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Role of phosphate sensing in bone and mineral metabolism

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Abstract

Inorganic phosphate (P_i) is essential for signal transduction and cell metabolism, and is also an essential structural component of the extracellular matrix of the skeleton. P_i is sensed in bacteria and yeast at the plasma membrane, which activates intracellular signal transduction to control the expression of P_i transporters and other genes that control intracellular P_i levels. In multicellular organisms, P_i homeostasis must be maintained in the organism and at the cellular level, requiring an endocrine and metabolic P_i-sensing mechanism, about which little is currently known. This Review will discuss the metabolic effects of P_i, which are mediated by P_i transporters, inositol pyrophosphates and SYG1–Pho81–XPR1 (SPX)-domain proteins to maintain cellular phosphate homeostasis in the musculoskeletal system. In addition, we will discuss how P_i is sensed by the human body to regulate the production of fibroblast growth factor 23 (FGF23), parathyroid hormone and calcitriol to maintain serum levels of P_i in a narrow range. New findings on the crosstalk between iron and P_i homeostasis in the regulation of FGF23 expression will also be outlined. Mutations in components of these metabolic and endocrine phosphate sensors result in genetic disorders of phosphate homeostasis, cardiomyopathy and familial basal ganglial calcifications, highlighting the importance of this newly emerging area of research.

Phosphorus found in living organisms is referred to as inorganic phosphate (P_i) when present as phosphoric acid ($H_2PO_4^-$ and HPO_4^{2-}), in its monovalent or divalent soluble sodium or potassium salts or its less soluble calcium salt (such as hydroxyapatite). Phosphate can also form dimers (such as pyrophosphate) and polymers (polyphosphate) or might be covalently bound in organic molecules (such as inositol pyrophosphates, membrane phospholipids, phosphoproteins and ribonucleic acids)[1–3]. In mammalian systems, P_i is essential for metabolic functions, such as intracellular signal transduction and energy production in most tissues. In addition, P_i is an important structural component as it is needed to form hydroxyapatite in the extracellular matrix of the skeleton. The intracellular concentration of free (soluble) P_i is approximately equal to the extracellular P_i concentration (3–5 mg/dl in humans), but levels of insoluble salts, multimers or organically bound phosphate are

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approximately tenfold higher. In total, intracellular phosphate makes up ~14% of total body P_i, whereas 85% of P_i is stored as hydroxyapatite in the extracellular matrix of bone and teeth[4]. Only 1% of overall phosphate is present in extracellular fluids, where it serves an important additional role as a buffer to maintain total body pH. Dysregulation of the intracellular P_i concentration affects cell metabolism and muscle function, whereas dysregulation of the P_i concentration in the extracellular fluid is implicated in skeletal disorders and in the development of vascular calcification as complications in chronic kidney disease (CKD) and cardiovascular disease (reviewed by us[5] and others[6–16]). Owing to its important function in many cellular processes, bacteria and yeast have developed membrane-anchored and intracellular signalling pathways to sense extracellular P [17]. This P_i sensing mechanism is well understood in bacteria and yeast; however, the mechanism of P_i sensing in mammalian systems is still unclear [6,16,18].

This Review will summarize the role of P_i sensing in bacteria, yeast and higher organisms, focusing on the role of P_i sensing in mineral metabolism and the consequences of P_i imbalance in human diseases. We will also discuss new findings about the crosstalk between iron and P_i homeostasis for regulation of fibroblast growth factor 23 (FGF23) expression, human mutations in P_i transporters resulting in genetic disorders of phosphate homeostasis, cardiomyopathy and familial basal ganglial calcifications and functions of inositol pyrophosphates and SYG1–Pho81–XPR1 (SPX)-domain proteins in cellular phosphate homeostasis.

Metabolic P_i sensing and function

Metabolic P_i sensing functions to maintain levels of P_i in the intracellular compartment to support cellular metabolism. P_i uptake in unicellular organisms (such as bacteria and yeast) is regulated at the plasma membrane, where metabolic P_i sensing activates signal transduction pathways that control the expression of phosphatases and the number of P_i transporters. In multicellular organisms, uptake of P_i into the intracellular compartment in muscle, bone and other tissues is regulated by P_i itself like in unicellular organisms[19,20], and additionally by hormones such as adrenaline[21], platelet-derived growth factor (PDGF)[22], insulin, insulin-like growth factor 1 (IGF1)[23], FGF2 (REF.[24]) and transforming growth factor-β (TGFβ)[25] (FIG. 1). Some of these genes are highly conserved, as shown in TABLE 1. Therefore, metabolic P_i sensing is distinct from endocrine P_i sensing, which regulates hormones produced by the parathyroid, kidneys and bones (as discussed further in the Review), which in turn regulate P_i in the extracellular compartment.

Bacteria

Unicellular organisms sense P_i through plasma membrane protein complexes to regulate intracellular levels of P_i (REFs[26,27]) (FIG. 1a). In bacteria, the transporters that make up the membrane protein complex are similar to the ATP-binding cassette (ABC) transporters in higher species and comprise P_i-binding protein PstS (PstS), P_i transport system (Pst) permease protein A (PstA), P_i import ATP-binding protein B (PstB) and Pst permease protein C (PstC). During high P_i conditions (as described in detail in FIG. 1a), P_i binding to the membrane protein complex results in dephosphorylation and deactivation

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of P_i regulon transcriptional regulatory protein (PhoB)[27]. PhoB deactivation reduces the expression of high-affinity P_i transporters and phosphatases, which are upregulated in P_i-limiting conditions and permit bacteria to scavenge P_i from the environment to increase intracellular levels of P_i[27]. Low-affinity P_i transporters 1 and 2 (PitA and PitB) mediate bacterial uptake of P_i during high P_i conditions[17]. When present in excess, P_i is stored by a specialized polyphosphate kinase (PPK) as a linear polymer (polyphosphate)[28,29]. This mechanism is conserved from bacterial to human cells; however, the role of polyphosphate as a metabolic regulator is only beginning to be understood in higher species[28,29].

Yeast

Whereas P_i is sensed in the inner bacterial membrane, P_i uptake by the P_i transporter Pho84 is required for P_i sensing in yeast[30,31] (FIG. 1b). Pho84 is a high-affinity P_i transporter related to the solute carrier family 17 (SLC17) protein family in higher species, and they share 7.6–12.9% amino acid identity[32]. Pho89, a second high-affinity transporter, has the PIT family signature sequences at the amino terminus and carboxyl terminus, which is shared with bacterial PitA and PitB, plant P_i transporter 2–1 (PHT2–1) and human sodium-dependent P_i transporter 1 and 2 (PIT1 and PIT2; which are encoded by *SLC20A1* and *SLC20A2*, respectively). Both high-affinity P_i transporters Pho84 (587 amino acid residues) and Pho89 (574 amino acid residues) are composed of 12 transmembrane domains. In Pho84, these 12 transmembrane domains are arranged as 2 homologous sequence segments, each containing 6 transmembrane domain regions that are separated by a large cytoplasmic loop[33,34], whereas in Pho89, a large intracellular hydrophilic loop is positioned between domains 7 and 8 (REF.[35]). The amino acid sequence identity in transmembrane domains 4, 8 and 10 for these transporters is 8–11%[36]. Conversely, the low-affinity transporters Pho87 and Pho90 and the vacuolar transporter Pho91 are related to metazoan sodium–sulfate transporters (solute carrier family 13 member 1–4 (SLC13A1–4))[37].

Under high P_i conditions, low-affinity P_i transporters Pho87 and Pho90 are responsible for P_i uptake, whereas under low P_i conditions, Pho84 and Pho89 are required[27]. Similar to bacteria, high environmental P_i reduces expression of the high-affinity transporters in yeast. This process involves cAMP-dependent protein kinase A (PKA)-dependent internalization and degradation of the Pho84 transporter in the vacuole[30]. A second mechanism requires the uptake of P_i by Pho84, which activates a signalling cascade (described in detail in FIG. 1b) that results in the repression of the yeast Pho regulon, which encodes yeast P_i starvation-induced genes[30,31]. Conditions of low P_i induce genes that encode for the high-affinity P_i transporters Pho84 and Pho89, secreted phosphatase Pho5 and putative cyclin-dependent kinase inhibitor SPL2, which in turn suppresses the low-affinity transporter (REF.[38]).

5-Diphosphoinositol pentakisphosphate (Ip7) is an important intermediary to signal P_i starvation in yeast[39]. In response to P_i starvation, Ip7 is synthesized from inositol hexakisphosphate (Ip6) by the yeast Ip6 kinase 1 (Kcs1) and diphosphoinositol-pentakisphosphate kinase (Vip1)[40]. Ip7 binds to the SPX domain, which is named after the plasma membrane protein that suppresses lethality of G_α protein deficiency (Syg1), the cyclin-dependent kinase inhibitor phosphate system positive regulatory protein (Pho81), and the mammalian xenotropic and polytropic retrovirus receptor 1 (XPR1; which is encoded

by *SLC53A1* and might function as a P_i exporter)[41]. Ip7 induces the expression of genes with protein products that increase P_i uptake, such as Pho84 and other P_i transporters[27,40]. In addition, Ip7 can activate another yeast SPX-domain protein, the vacuolar transporter chaperone 4 (Vtc4), which, together with Vtc1, Vtc2 and Vtc3, forms a yeast polyphosphate polymerase that stimulates polyphosphate synthesis from ATP under low P_i conditions in the vacuole[42]. This polyphosphate is converted to P_i by endopolyphosphatase (Phm5; also known as Ppn1) and transported from the vacuole to the cytosol by the vacuolar P_i transporter (Pho91). Therefore, by synthesizing polyphosphate, ATP can provide P_i for cytosolic processes when the exogenous supply of P_i is low[27,43,44]. Ip7 finally inhibits transcription of genes important for glycolysis and class 1 histone deacetylase in yeast[45], thereby reducing consumption of P_i. In summary, Ip7 might function as a second messenger in yeast to signal reduced cytosolic P_i levels by binding to several SPX-domain proteins, to stimulate P_i uptake from the environment and release of P_i from vacuolar polyphosphate stores and to reduce P_i consumption.

Mammalian

Unlike bacterial and yeast P_i sensing, mammalian P_i sensing is only partially understood. Multicellular organisms require homeostatic regulation in the extracellular and intracellular compartments. Although there are no orthologous proteins of the P_i-sensing histidine kinase P_i regulon sensor protein PhoR in higher species, a human plasma phosphate binding protein has been identified with 25% sequence identity to bacterial P_i binding protein PstS[46]. Evidence furthermore suggests that the type 3 sodium-dependent P_i transporters PIT1 and PIT2 (encoded by *SLC20A1* and *SLC20A2*, respectively) have an important role in mammalian P_i sensing. Interestingly, PIT1 and PIT2 might sense extracellular P_i without requiring translocation of P_i, which is also referred to as P_i transport-independent sensing[47,48]; therefore, it is possible that these transporters serve as sensors for extracellular P_i in addition to regulating intracellular levels of P_i. This process might involve a co-receptor (or co-receptors) that switches between extracellular sensing and transport functions (FIG. 1c; one sensor hypothesis). However, it is also possible that there are separate sensors for extracellular and intracellular P_i levels (FIG. 1c; multiple sensor hypothesis). In this hypothesis, extracellular P_i levels are detected by a sensor located only in the cell membrane of endocrine cells, whereas the intracellular P_i level is sensed in most tissues to maintain cell metabolism.

Extracellular P_i sensing.

The mitogen-activated protein kinases (MAPKs) extracellular-signal-regulated kinase 1 (ERK1) and ERK2 (also known as MAPK3 and MAPK1, respectively) are activated by extracellular P_i in most cell types, which is an evolutionarily conserved process between *Drosophila melanogaster* and humans[32]. This activation is blocked by pharmacological inhibition or genetic ablation of type 3 sodium-dependent P_i transporters[49]. Because both PIT1 and PIT2 can fulfil this role, it might be intracellular P_i that is sensed to activate ERK1 and ERK2. However, PIT transporters might also bind and signal to the cell independent of P_i transport, as shown in HeLa cells and vascular smooth muscle cells (VSMCs) expressing the transport-deficient PIT1 mutant Glu70Lys[47,48]. Activation of PIT1, ERK1 and ERK2 is required for P_i-dependent stimulation of the expression of genes involved

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in bone mineralization in osteoblasts[50] and for the apoptosis of hypertrophic chondrocytes through the mitochondrial caspase 3 pathway[51]. In addition, P_i regulation of mitochondrial respiration and ATP flux in skeletal muscle requires the activation of ERK1 and ERK2 (REFs[52,53]). Finally, FGF receptor substrate 2 (FRS2) is phosphorylated following treatment with P_i in human embryonic kidney cells (HEK293)[54] and mouse osteoblastic cells (MC3T3)[53,55], which is blocked by short interfering RNA silencing of FGF receptor 1 (FGFR1). This result suggests that FGFR1 not only mediates FGF23 signalling but also P_i sensing, as discussed later in the Review.

Intracellular P_i sensing.

Subcellular compartments might further sequester intracellular P_i. The mitochondrial P_i carrier protein (PIC; which is encoded by *SLC25A3*) is part of the mitochondrial permeability transition pore (mPTP), which is a multiprotein complex. The mPTP regulates mitochondrial membrane potential and mitochondrial apoptosis[56] and is important for skeletal and cardiac muscle function[57]. PIT1 localizes to the endoplasmic reticulum (ER), where it seems to be involved in regulating the ER stress of growth plate chondrocytes[58]. In addition, large and small conductance chloride channels transport P_i into the sarcoplasmic reticulum of rabbit skeletal muscle[59]. The advent of novel imaging techniques to visualize subcellular P_i (such as fluorescence-lifetime imaging, fluorescence resonance energy transfer microscopy technologies, synchrotron-based X-ray fluorimetry and nanoscale secondary ion mass spectrometry) might provide insights into this poorly understood area of research in the future[60–63].

IP7.

In a genome-wide association study for genetic determinants of serum P_i concentration, two orthologues of yeast Ip6 kinase, inositol hexakisphosphate kinases 2 and 3 (IP6K2 and IP6K3, respectively), were identified as well as PIT1, extracellular calcium-sensing receptor (CaSR) and FGF23 (REF.[64]). IP6K2 seems to catalyse the synthesis of IP7 in various human cell lines, including HCT116 (human colon colorectal carcinoma) and U2OS (human bone osteosarcoma epithelial) cells[65]. Similar to yeast, IP7 regulates P_i consumption in mammalian cells. For example, IP7 inhibits insulin signalling by potently inhibiting the phosphorylation of AKT by 3-phosphoinositide-dependent protein kinase 1 (PDPK1), thereby preventing its activation in the human hepatocellular carcinoma cell line HePG2 (REF.[66]). In addition, IP7 enhances casein kinase 2 (CK2; which is encoded by *CSNK2*)-mediated phosphorylation of cellular tumour antigen p53 and thereby activates cell death pathways in human U20S bone osteosarcoma cells[67]. Furthermore, the highly conserved IP7 binding domain, SPX, is present in mammalian XPR1, which may function as a phosphate exporter[68]. Interestingly, mutations in the SPX domain of XPR1 stimulate the formation of calcium–P_i deposits in the basal ganglia of individuals with primary familial brain calcification, which might be caused by abnormal glial cell P_i export[69]. XPR1 expression is in turn stimulated by the receptor activator of nuclear factor (NF)-κB (RANK; which is encoded by *TNFRSF11A*)–RANK ligand (RANKL) pathway in osteoclasts[70]. Taken together, these findings suggest that IP7 has a role in mammalian metabolic P_i sensing, although the underlying mechanism remains unknown[39,69].

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Polyphosphate.

Similar to IP7, polyphosphate is found in mammalian cells, although its function is incompletely understood[71]. Mammalian cells lack orthologues of both yeast polyphosphate kinases (Vtc and Ppk1) (see FIG. 1b), and mammalian polyphosphate synthesis pathways are yet to be discovered. As mammalian polyphosphate synthesis is blocked by oligomycin, a mitochondrial ATP synthase (complex V of the respiratory chain) inhibitor, but does not require ATP as a substrate[72], it is suggested that polyphosphate is a by-product of mitochondrial ATP synthesis. Consistent with its role as a P_i store, polyphosphate seems to stimulate P_i consumption by activating the mammalian target of rapamycin (mTOR) pathway[73], the mitogenic activities of FGF1 and FGF2 by physically and functionally stabilizing the two, similar to heparin sulfate, or by potentially preventing their degradation in human fibroblasts[74]. However, polyphosphate also stimulates apoptosis by activating caspase 3 in human plasma cells[75] and neurons[76]. Further research is needed to better understand the role of polyphosphate in intracellular signalling and metabolism of mammalian cells.

Metabolic P_i in musculoskeletal biology

Here, we describe how P_i regulates differentiation and production of cartilage matrix by chondrocytes in joint surfaces and growth plates, how P_i regulates bone remodelling by controlling the differentiation and function of the three types of bone cells (bone-forming osteoblasts, mechano-sensing osteocytes and bone-resorbing osteoclasts) and how P_i regulates vascular, skeletal and cardiac muscle function (FIG. 2). Identification of the phosphaturic hormone FGF23 (REFs[77,78]), which is secreted by late-stage osteoblasts and osteocytes, also established the skeleton as an important endocrine regulator of P_i homeostasis[79], which will be discussed later in the Review.

Chondrocytes

Chondrocytes produce and maintain the extracellular matrix of joint cartilage and permit longitudinal growth of long bones by a process called endochondral ossification. Abnormal proliferation and differentiation of chondrocytes lead to degenerative diseases such as rickets and osteoarthritis[20].

The observation of very low levels of P_i in pre-mineralized cartilage, which increase during mineralization, led to the speculation that P_i might be required for bone growth[80,81]. A study in which P_i was added to the chondrogenic cell line ATDC5 showed reduced levels of type II collagen and parathyroid hormone receptor–parathyroid hormone-related peptide receptor (PTH–PTHr receptor (PPR1) which is encoded by *PTH1R*) gene expression and increased type X collagen expression[82], suggesting that P_i induces terminal differentiation and apoptosis of chondrocytes. The ability of P_i to rescue delayed differentiation of cultured murine metatarsals, prepared from heterozygous or homozygous knockout of parathyroid hormone-related protein (*Pthrp*$^{-/+}$ or *Pthrp*$^{-/-}$; which is encoded by *Pthlh*) in mice, further supported the role P_i has in bone growth[83]. Additionally, P_i induces hypertrophic differentiation[20] and apoptosis[84] in the chondrocytic cell line CFK2. P_i also stimulates hypertrophy in primary chondrocytes from human osteoarthritic joints[85]. PIT1 and PIT2

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facilitate P_i uptake into bovine articular chondrocytes[86]. Ablation or pharmacological inhibition of PIT1 or the inhibition of dual specificity MAPK kinase 1 (MEK1, which is encoded by MAP2K1) blocks the phosphorylation of ERK1 and ERK2 and mitochondrial apoptosis induced by P_i in primary chondrocytes[51] and CFK2 cells[84]. Chondrocytes might also regulate systemic P_i homeostasis by secreting FGF23, independently of ERK1 and ERK2 (REF.[85]). In addition to the MAPK pathway, P_i increases the nitrate:nitrite (NO_3^-:NO_3^-) ratio by stimulating nitric oxide synthase (NOS), which in turn induces chondrocyte apoptosis[84].

Studies in genetically modified mice suggest that PIT1 is important for liver development and haematopoesis[87,88]. Mice carrying two hypomorphic alleles of the *Pit1* gene, which results in an 85% reduction of PIT1 expression, show reduced femur length[89] but have surprisingly normal bone and mineral metabolism. Therefore, cell-type-specific ablation of PIT1 might be required to fully understand its skeletal functions. For example, mice lacking phosphoethanolamine–phosphocholine phosphatase (PHOSPHO1), an enzyme that hydrolyses phosphoethanolamine to generate P_i for matrix vesicle mineralization, have defective matrix vesicle mineralization in the growth plates[90]. Mice with double knockout of *Pit1* (specifically in the chondrocyte) and *Phospho1* (full-body deletion) show worse matrix vesicle mineralization than *Phospho1*-null mice. Furthermore, acute chondrocyte-specific deletion of *Pit1* in mice results in pronounced cell death in the first two postnatal days, possibly owing to P_i transport-independent ER stress[58]. Finally, universal overexpression of *Pit1* in rats caused an incisor enamel defect and decreased bone mineral volume[91]. Despite normal skeletal development, the mutant animals display biochemical abnormalities, including increased serum levels of P_i, FGF23 and parathyroid hormone (PTH), develop proteinuria and body weight loss and experience premature death.

In summary, P_i stimulates hypertrophic differentiation and apoptosis in chondrocytes via PIT1, ERK1 and ERK2 and possibly via NOS, which is necessary for normal bone growth and possibly articular cartilage function.

Osteoblasts and osteocytes

Osteoblasts and osteocytes synthesize bone matrix[92], which is composed of type I collagen, non-collagenous proteins (such as osteocalcin) and small integrin-binding ligand, *N*-linked glycoprotein (SIBLING) proteins (including dentin matrix acidic phosphoprotein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE), osteopontin (OPN; also known as SPP1) and hydroxyapatite)[93]. When osteoblasts become buried in bone matrix, they undergo terminal differentiation into osteocytes, which serve as mechanosensors and secrete endocrine and paracrine factors to maintain skeletal homeostasis[94].

Microarray, next-generation sequencing and proteomics studies show that P_i induces expression of genes important for cell proliferation, energy metabolism and mineralization in osteoblast-like cells[53,95,96], suggesting that P_i stimulates osteoblast proliferation and differentiation. P_i, in the form of β-glycerophosphate, is commonly added to cell culture media to stimulate proliferation and mineralization of primary human and mouse mesenchymal stromal cells. This process requires the presence of PIT1 and FGFR1 and activation of the GTPase NRAS[53]. In MC3T3 cells and primary murine calvaria-derived

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osteoblasts, P_i induces the expression of Fos-related antigen 1 (*Fra1*; also known as *Fosl1*), *Opn* and matrix Gla protein (*Mgp*; which are genes required for mineralization), which is dependent on ERK1 and ERK2 (REFs[97,98]). P_i might also stimulate IGF1 expression in the mouse-derived osteoblast cell line MC3T3-E1, which enhances osteoblast proliferation in an autocrine fashion[98,99]. The function of polyphosphate is less clear. Similar to pyrophosphate, polyphosphate can inhibit mineralization by preventing apatite crystal growth and by blocking alkaline phosphatase, tissue-nonspecific isozyme (TNSALP; which is encoded by *ALPL*), which in turn prevents degradation of the mineralization inhibitor pyrophosphate[100].

As previously discussed, the *Phospho1–Pit1* double-knockout mouse showed reduced matrix vesicle levels of minerals, suggesting that PIT1 is required for P_i uptake into matrix vesicles and initiation of skeletal mineralization through hydroxyapatite formation[90]. In addition to PIT1, osteoblasts also express PIT2 and the type 2 sodium-dependent phosphate transport protein 2A and 2B (NPT2A and NPT2B; which are encoded by *SLC34A1* and *SLC34A2*, respectively)[101–103]. In pre-osteoblastic bone marrow stromal cells, nano-hydroxyapatite stimulation of gene expression (which can influence osteoblast lineage commitment and cell function) requires FGFR signalling, phosphate transporters (*PIT1* and *PIT2*) and ERK1 and ERK2 signalling[104]. Blood levels of calciprotein particles (a complex of calcium, P_i and other proteins that transports hydroxyapatite to bone without crystallization in other tissues) elevate with excess P_i and calcium, contributing to CKD just before the rise of FGF23 (REF.[105]). Understanding hydroxyapatite formation and signalling might therefore improve clinical outcomes for CKD.

Although ablation of *Pit1* does not seem to affect osteoblast or osteocyte functions[89], global *Pit2*-knockout mice show placental calcification, growth retardation, reduced cortical and trabecular BMD[106,107], reduced dentin mineralization[107], cornea and brain calcification, increased levels of P_i in the cerebrospinal fluid (CSF) and decreased levels of P_i in peripheral blood[108]. Furthermore, these mice seem to be unable to suppress serum levels of intact FGF23 (iFGF23) in response to a low-P_i diet or to increase levels of iFGF23 in response to high-P_i diet unlike wild-type mice, whereas relative FGF23 gene expression did not show any change[109]. Global *Pit2* haploinsufficiency also causes decreased BMD in response to 5/6 nephrectomy but has no effect on blood levels of calcium and P_i and the mutant mice had normal fractional excretion of P_i (REF.[106]). Thus, PIT2 is required for normal bone function in mice; however, it remains to be confirmed whether PIT2 is required for the regulation of serum iFGF23.

Unexpectedly, *Npt2a*$^{-/-}$ mice have increased bone mass, which might be due to a reduction in osteoclast number[110]. In addition, the difference in osteoclast number is less pronounced at 115 days of age; therefore, there is an increase in mineralizing and osteoblast surfaces later in life in these mice[111]. *Npt2b*$^{-/-}$ mice show embryonic lethality at embryonic day 8 (REF.[112]), and adult *Npt2b–/+* mice have pulmonary alveolar microlithiasis but no skeletal abnormalities[113]. Therefore, the function of NPT2B in bone biology remains poorly understood[113].

Osteocytes, similar to osteoblasts, express PIT1 and PIT2 transporters[114]. P_i stimulates osteocyte maturation and matrix formation in the osteocyte lacuna. Hypophosphataemic

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Dmp1-null mice have impaired osteocyte maturation and decreased mineralization[115]. When 10 mM P_i and 10 nM calcitriol (the active form of vitamin D, also known as 1,25-dihydroxyvitamin D_3) are added to murine IDG-SW3 osteocyte-like cells, the gene expression of polypeptide *N*-acetylgalactosaminyltransferase 3 (*Galnt3*), *Dmp1*, phosphate regulating endopeptidase homologue, X-linked (*Phex*), ectonucleotide pyrophosphatase/phosphodiesterase 1 (*Enpp1*) and *Mepe* is induced and results in matrix mineralization[116]. In response to PTH, osteocytes stimulate osteoclast recruitment and differentiation by secreting RANKL and inhibit osteoblast differentiation by secreting sclerostin, which blocks the wingless (WNT)–β-catenin pathway[117]. P_i and other factors (such as calcitriol and PTH) cause osteocytes to secrete FGF23 to regulate systemic phosphate homeostasis[118–120], and in response to IL-6 and myostatin, osteocytes secrete osteocalcin to regulate body energy metabolism[121].

In summary, P_i stimulates differentiation of osteoblasts and osteocytes, matrix maturation and bone formation, which involve the function of P_i transporters and ERK1 and ERK2 signalling in vitro. Surprisingly, mild bone and mineral metabolism phenotypes of the global *Pit1*-null and *Pit2*-null mice suggest a high degree of redundancy of these generally co-expressed transporters. Bone-specific ablation of *Pit1* and *Pit2* (individually and in combination) in mice might be required to shed light on their metabolic and endocrine functions.

Osteoclasts

Osteoclasts are large multinucleate cells derived from the monocyte lineage and are responsible for bone resorption[122], which is necessary for the remodelling and repair of the skeleton. The formation and function of osteoclasts are stimulated by macrophage colony-stimulating factor (MCSF) and RANKL produced by osteoblasts. In turn, osteoclasts stimulate osteoblastic bone formation through two (and possibly more) signalling pathways (ephrins–ephrin receptors and semaphorins–plexins)[123,124].

Osteoclasts express NPT2A, PIT1 and PIT2 (REF.[125]). High levels of extracellular P_i (4 mM) inhibit osteoclast-like cell formation in mouse bone marrow cells[126] and decrease the number and area of resorption pits formed by mature rat osteoclasts on sperm whale dentine slices, which is a common assay for osteoclast function[103]. This observation presumably reflects a feedback mechanism to limit the degradation of hydroxyapatite and might involve NPT2A-dependent inhibition of RANK–RANKL signalling, inhibition of osteoclast growth by P_i (REF.[127]) and the suppression of microRNA-223 expression, which was reported in the pre-osteoclast RAW264.7 cell line[128]. Similar to P_i, polyphosphate might inhibit the maturation of RAW264.7 cells into functional osteoclasts by blocking RANK–RANKL signalling[129]. However, some P_i is required for normal osteoclast function, as phosphonoformic acid (an inhibitor of sodium-phosphate cotransporters) reduces bone resorption in cultured osteoclasts, possibly by inhibiting ATP production, for which uptake of extracellular P_i is required[130]. Extracellular P_i also stimulates reactive oxygen species production, which is required for osteoclastogenesis and resorptive function of RAW264.7 cells[131]. Wild-type mice fed a low-P_i diet and Hyp mice (a mouse model of human X-linked hypophosphataemic rickets) showed similar results. Both wild-type mice fed a

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low-P_i diet and Hyp mice exhibited decreased osteoclast numbers in osteoclast-like cells derived from bone marrow cells compared with wild-type mice fed normal-P_i diets, and this defect was reversed by a high-P_i diet[132]. Finally, the high bone mass phenotype observed in 21-day *Npt2a*-null mice was attributed to impaired osteoclast function[133]. In summary, osteoclasts express NPT2A, PIT1 and PIT2 transporters. Release of P_i during bone resorption might provide a mechanism of feedback inhibition, limiting survival and differentiation of osteoclasts at high P_i levels. However, some P_i seems to be required for normal osteoclast function.

Myocytes

Myocytes are present in vascular, skeletal and cardiac muscle tissue and contribute to the control of blood pressure, locomotion and cardiac functions.

Hypophosphataemia causes myopathy and heart failure. Although steady-state muscle P_i and ATP concentrations in Hyp mice seem to be preserved[134], phosphocreatine recovery time in humans with hypophosphataemia is delayed[135], which is similar to findings in individuals with vitamin D deficiency[136]. After 12 weeks of vitamin D supplementation, there was an increase in serum levels of P_i and restoration of phosphocreatine recovery time in individuals with vitamin D deficiency, suggesting a connection between vitamin D status, serum P_i and mitochondrial muscle ATP synthesis[136]. By adapting 31P-magnetic resonance spectroscopy techniques[137] to noninvasively examine mitochondrial energy production (by measuring basal and insulin-stimulated ATP flux (V_{ATP})), we studied two hypophosphataemic mouse models in vivo (wild-type mice fed a low-P_i diet and *Npt2a*$^{-/-}$ mice)[133]. Both mouse models had ~50% reduction in V_{ATP}, and this reduction was rapidly restored by intravenous P_i supplementation[52]. V_{ATP} was likewise reduced by ~50% in a patient with untreated hereditary hypophosphataemic rickets with hypercalciuria, which normalized upon treatment with oral P_i supplements[52].

At the molecular level, P_i stimulates mitochondrial energy production (measured by V_{ATP}) by serving as a substrate for ATP synthesis during oxidative phosphorylation at complex V[138]. Furthermore, P_i maintains cytochrome *b* oxidation and cytochrome *c* reduction[139] and stimulates the activity of several Krebs cycle dehydrogenases (2-oxoglutarate dehydrogenase, isocitrate dehydrogenase and malate dehydrogenase)[140–143]. This action increases the concentrations of mitochondrial electron donors (FADH, NADH and NADPH) to fuel the electron transport chain. In addition, P_i binds to glyceraldehyde 3-phosphate dehydrogenase and is thereby an important cofactor for a rate-limiting glycolytic enzyme that converts triose phosphates (dihydroxyacetone and glyceraldehyde 3-phosphate) to 1,3-bisphosphoglycerate[144,145].

By contrast, hyperphosphataemia stimulates the expression of OPN, increases cell proliferation and mineralization and downregulates myocardin and smooth muscle α-actin (SMAαA) in a PIT1 and PIT2 transport-independent manner[147–149]. This process is dependent on ERK1 and ERK2 and results in osteogenic transdifferentiation[147–149], which causes vascular calcification in patients with CKD[146]. This VSMC transdifferentiation might depend on WNT–β-catenin–runt-related transcription factor 2 (RUNX2) signalling, which is an important anabolic signalling pathway for osteoblast and osteocyte function[150–153] and is

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inhibited by secreted frizzled-related protein 5 (SFRP5)[154]. Furthermore, research in skinned muscle fibres showed that P_i released during ATP hydrolysis raises cytosolic P_i from 3 mM to 15 mM, which reduces peak force by decreasing force per actin–myosin bridge and/or by increasing the number of low-force bridges in skeletal muscle, by decreasing cytosolic ionized calcium and by causing Ca–P_i precipitations in the sarcoplasmic reticulum. Thus, high intracellular P_i appears to contribute to muscle fatigue[155], arguing against the simple depletion of intracellular P_i as a substrate to explain the observed reduction in V_{ATP} and hypophosphataemic myopathy.

In summary, P_i is required for maintaining muscle function, but excess P_i leads to calcification, which is best documented in vascular smooth musculature, and fatigue of skeletal muscle. This process might involve the functions of PIT1 and PIT2 transporters and ERK1 and ERK2 signalling.

Phosphate homeostasis and endocrine actions

Different from metabolic P_i sensing, which maintains intracellular P_i levels to support cell metabolism, endocrine P_i sensing maintains extracellular blood levels of P_i. The blood concentration of P_i is regulated within a narrow range (2.5–4.5 mg/dl in humans) through the control of intestinal absorption of P_i from the diet, P_i release from stores by bone modelling and renal excretion (reviewed by REFs[5,9,10,156]). In turn, P_i feeds back to regulate its intestinal absorption, release from bone mineral and renal excretion by inducing the secretion of PTH and FGF23 and by inhibiting the synthesis of calcitriol.

After its discovery, now almost two decades ago, it rapidly became clear that FGF23 is a key endocrine regulator of renal P_i excretion[157–160]. Meanwhile, the list of factors regulating FGF23 and renal P_i excretion has grown (FIG. 3; TABLE 2). FGF23 requires the co-receptor Klotho for binding to the receptor FGFR1 to activate ERK1–ERK2 signalling[161], whereas non-canonical signalling via FGFR4–nuclear factor of activated T cells (NFAT)–calcineurin seems to be independent of this co-receptor[162]. Interestingly, there is some evidence that FGFR1 might also contribute to P_i sensing[53,55]; however, as levels of FGF23 are high, rather than low, in tumoural calcinosis type 3 (REF.[163]) and *Klotho*$^{-/-}$ mice[164], Klotho is probably not involved in endocrine P_i sensing.

FGF23

Primarily secreted by osteoblasts, osteocytes and possibly other cell types, FGF23 is a 227-amino-acid intact peptide. Dietary P_i stimulates the increase in serum FGF23 in healthy men, which is inversely related to renal P_i absorption and calcitriol levels[165]. This inverse relationship is because FGF23 downregulates NPT2A and NPT2C expression in the proximal tubules of the kidneys, which resembles the action of PTH, and results in renal phosphate excretion. Conversely, neutralizing FGF23 antibodies increase serum levels of P_i (REF.[166]). Different from PTH, FGF23 suppresses 25-hydroxyvitamin D-1 α-hydroxylase (which is encoded by *CYP27B1*) and stimulates 1,25-hydroxy-vitamin D-24-hydroxylase, mitochondrial (which is encoded by *CYP24A1*) in the proximal tubules, thereby reducing serum levels of calcitriol[167]. However, one other study failed to show suppression of CYP27B1 and stimulation of CYP24A1 in response to recombinant human FGF23 in

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12-week-old mice, which triggered a phosphaturic effect, possibly owing to suppression of endogenous iFGF23 in these mice[168]. Both actions of FGF23 at the proximal tubule might be indirect and mediated by WNT–β-catenin signalling as FGFR1 and Klotho are predominantly expressed in the distal tubules[156,169]. Whole-nephron and global deletion of Klotho cause a similar severe phenotype, characterized by accelerated ageing, disturbed mineral metabolism, growth retardation, organ dysfunction and vascular calcification[170,171]. Although Klotho ablation in the proximal tubules is not as severe as whole-nephron and global ablation, these mice show impaired P_i excretion and increased NPT2A abundance when fed a high-P_i diet, suggesting that FGF23 also acts directly at the proximal tubules[172].

Osteocytes of *Dmp1*-null mice and Hyp mice that have loss-of-function (LOF) mutations in the *Dmp1* and *Phex* genes, respectively, have elevated FGF23 expression, which at least in part is mediated by the activation of canonical FGF–FGFR signalling[173]. In addition, mice expressing a transgenic variant of FGF23 that is resistant to proteolytic cleavage within a highly conserved subtilisin-like proprotein convertase site (176RHTR179/S180AE182) overexpress FGF23 when made iron deficient. As a result, intact and carboxy-terminal FGF23 levels are increased, leading to hypophosphataemia. Similarly, iron deficiency stimulates FGF23 expression in wild-type mice; however, only the carboxy-terminal FGF23 levels are increased and hypophosphataemia is absent, suggesting that P_i controls the secretory checkpoint[174]. Degradation of iFGF23 is enhanced by post-translational phosphorylation of FGF23 by the Golgi-associated secretory pathway kinase FAM20C at Ser180 and reduced by *O*-glycosylation by polypeptide *N*-acetylgalactosaminyltransferase 3 (GALNT3) at Thr178 (REF.[175]).

The effects of iron deficiency on FGF23 gene transcription might be indirect and mediated via erythropoietin. Von Hippel–Lindau disease tumour suppressor (VHL) ablation-mediated overexpression of hypoxia-inducible factor 1α (HIF1α) in the osteoblastic linage induces erythropoietin expression in these cells[176]. This effect might be relevant in light of emerging data that erythropoietin stimulates FGF23 synthesis and secretion by myeloid lineage LSK cells in the haematopoietic bone marrow[177]. In turn, FGF23 might feed back to regulate haematopoiesis, as suggested by the low erythrocyte counts found in FGF23-null mice[178].

The osteocyte-like mouse cell lines IDG-SW3 (REF.[116]) and MLO-Y4 (REF.[179]) reportedly express substantial levels of FGF23 mRNA. Both cell lines differentiate into osteocytes after growing in cell culture on a collagen-coated surface in osteogenic media[180]. P_i and calcitriol increase FGF23 mRNA expression along with that of other osteocyte markers in IDG-SW3 cells, but these cells do not secrete the FGF23 protein[116]. In MLO-Y4 cells, in the absence of FGF2, extracellular P_i alone induces DMP1 expression. However, increased extracellular levels of P_i partially inhibited FGF2-induced DMP1, suggesting a coordinated regulation of DMP1 expression by FGF signalling and extracellular P_i (REF.[179]). Additionally, FGF23 mRNA was detected in UMR-106 rat osteosarcoma cells and MC3T3-E1 osteoblast-like cells[181]. P_i induces FGF23 mRNA expression in UMR-106 by an ERK-dependent mechanism, which might require the production of NADPH and reactive oxygen species[182]. In addition, in UMR-106 cells, advanced glycation end products (sugarmodified proteins, nucleic acid and lipids that contribute to FGF23 disorders) induce the transcription of *FGF23*, partially owing to upregulation of NF-κB[183]. P_i also induces

DMP1 mRNA expression in MC3T3-E1 cells, but not *FGF23* mRNA, and this induction is blocked by MEK inhibitor UO126 (REF.[55]).

In summary, P_i seems to regulate a post-translational checkpoint for secretion of bioactive FGF23 in vivo, and suitable in vitro models are needed to further study this mechanism.

Parathyroid

Intact PTH is a peptide of 84 amino acids that is secreted by the parathyroid glands and signals via its receptor PPR1, which is expressed in osteoblasts, osteocytes, chondrocytes and proximal tubular cells. Its net effect is the reduction of blood levels of P_i. P_i in turn stimulates PTH release from sections of bovine parathyroid glands, an effect not observed in dispersed cells[184].

In the parathyroids, FGF23 decreases *PTH* mRNA levels through the activation of Klotho–FGFR1 and MAPK[185]. In addition to a Klotho-dependent mechanism, a Klotho-independent calcineurin–NFAT signalling mechanism has been suggested on the basis of the observation that *Klotho*$^{-/-}$ mice show a preserved PTH response when treated with FGF23, which could be blocked by the calcineurin inhibitor cyclosporine A[162]. PTH in turn increases serum levels of FGF23 via PPR1 and activation of PKA[186], WNT pathways[187] and nuclear receptor family 4 group A member 2 (NR4A2)[188]. Circulating levels of FGF23 are more substantially elevated in patients with humoral hypercalcaemia of malignancy than in patients with primary hyperparathyroidism[189,190], suggesting that PTHrP stimulates FGF23 secretion more effectively than PTH (possibly by PPR1-independent pathways)[191,192].

P_i stabilizes *PTH* mRNA levels via A+U-rich element binding factor 1, heterogeneous nuclear ribonucleoprotein K (HNRNPK) and cold shock domain-containing protein E1 (also known as N-ras upstream gene protein (UNR), which is encoded by *CSDE1*), which might have a role in parathyroid cell proliferation and the pathogenesis of secondary hyperparathyroidism that develops in patients with CKD[185]. An alternative mechanism for the development of secondary hyperparathyroidism caused by hyperphosphataemia is binding of P_i to the seven-transmembrane-spanning G protein-coupled CaSR[193], which might inhibit calcium signalling and thereby stimulate PTH secretion.

Iron deficiency

Iron deficiency might induce FGF23 gene transcription via the transcription factor HIF1α[174]. HIF1α binding to hypoxia-responsive elements in the FGF23 promoter stimulates *FGF23* mRNA expression in bone marrow stromal cells and MC3T3 cultures treated with deferoxamine (an iron-chelating agent)[174]. However, bone cell-specific ablation of *Hif1α* does not block the effect of iron deficiency on production of iFGF23; thus, this mechanism might not be relevant in vivo[194]. Rather, iron deficiency might stimulate erythropoietinexpression in the juxtaglomerular macula densa in the kidneys, which acts on osteoblasts and erythropoietic cells to induce FGF23 expression[177,195].

Although co-regulation of P_i and iron homeostasis to support energy metabolism is plausible, it remains unclear how both systems stay sufficiently independent, as hypophosphataemia rarely develops with iron deficiency in otherwise healthy individuals.

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One possible feedback mechanism is the degradation of iFGF23 during iron deficiency. Hypophosphataemia develops only if FAM20C and GALNT3 are abnormally regulated, which differentially phosphorylate and *O*-glycosylate FGF23, postmarking the protein for degradation or secretion, respectively, as described previously[175]. Of interest in this context is that treatment with saccharated ferric oxide[196] and iron polymaltose complex[197] worsened FGF23-dependent osteomalacia despite correcting the iron deficiency, whereas iron dextran, as expected, corrected it[198]. The results suggest that saccharated ferric oxide and iron polymaltose complex block the cleavage of iFGF23, resulting in excess FGF23 bioactivity. FGF23 cleavage might also be inhibited by the chronic inflammatory state observed in CKD[195,199]. This process may be regulated by IL-1β[200] and explain why germ-free mice have low FGF23 levels[201]. In addition, insulin or IGF1 suppresses FGF23 expression in a phosphatidylinositol 3-kinase (PI3K)–AKT–forkhead box protein O1 (FOXO1)-dependent fashion in vitro, in mice and in humans[202]. Lastly, FGF23 expression is stimulated by the mineralocorticoid hormone aldosterone[203]. This in vitro observation was corroborated by murine knockout models of the renal thiazide-sensitive NaCl cotransporter; however, renal resistance to FGF23 might be an additional factor as these mice remain normophosphataemic despite increased circulating levels of iFGF23 (REF.[204]).

Calcitriol

In the proximal tubules of the kidneys, calcitriol is synthesized from its precursor calcifediol (also known as 25-hydroxy vitamin D) by CYP27B1 (REF.[205]). P_i decreases circulating levels of calcitriol by stimulating gene expression of *CYP24A1*, which degrades calcitriol, while simultaneously suppressing its synthesis by CYP27B1 (REF.[205]). This process is predominantly mediated by the actions of FGF23 (REFs[206–210]).

Cyp24a1-null mice or individuals with LOF mutations in this gene develop hyperphosphataemia, along with severe hypercalcaemia[211]. Mice lacking *Cyp27b1* expression or individuals with LOF mutations in this gene develop hypophosphataemia as well as hypocalcaemia, secondary hyperparathyroidism and rickets[212]. Calcitriol increases FGF23 levels and FGF23 decreases levels of calcitriol, forming a regulatory feedback loop to regulate plasma P_i (REF.[213]). Conversely, PTH increases calcitriol by stimulating CYP27B1 and inhibiting CYP24A1 and calcitriol suppresses PTH to regulate plasma calcium levels[214]. Owing to the opposite actions of both hormones on calcium metabolism, PTH is unable to compensate for the loss or excess of FGF23 in FGF23-dependent disorders of P_i homeostasis.

P_i might exert direct effects as shown in rabbit[215] and mouse[216] proximal tubule kidney cells in vitro and in vivo. Furthermore, regulation of CYP24A1 by P_i might require expression of NPT2A, as a high-phosphate diet fails to stimulate the gene expression of *Cyp24a1* in *Npt2a*$^{-/-}$ mice[217]. This observation could explain why severe hypercalcaemia develops in children with NPT2A LOF mutations, which resembles the phenotype of idiopathic infantile hypercalcaemia caused by LOF of CYP24A1 (REF.[218]).

Calcitriol binds to its nuclear hormone receptor vitamin D3 receptor (VDR) to promote intestinal absorption of P_i and calcium[205] by inducing NPT2B expression in the gut[219]. Elevated circulating levels of calcitriol are an important diagnostic feature in FGF23-

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independent disorders of renal phosphate wasting. However, this homeostatic response is not sufficiently robust to completely correct the hypophosphataemia owing to the development of hypercalcaemia and because elevated calcitriol also stimulates production of FGF23 in the skeleton, which might further worsen phosphaturia[220]. Conversely, suppression of calcitriol might contribute to the hypophosphataemia seen in FGF23-dependent disorders of renal phosphate wasting. Because secondary hyperparathyroidism often develops as a consequence of calcitriol deficiency (which should reduce FGF23 levels), PTH is unable to fully suppress and compensate for the phosphaturia caused by FGF23 excess.

Intestinal P_i sensing

Intestinal P_i absorption is stimulated by calcitriol. Both paracellular transport by passive diffusion and active transcellular transport have been described[221,222]. As suggested by findings in intestine-targeted *Npt2b*-knockout mice[223], active transcellular transport of dietary P_i raises P_i levels in the circulation and results in increased renal phosphate excretion to eliminate excess P_i. Decreased FGF23 levels in these knockout mice suggest that this renal response to P_i is regulated by the stimulation of FGF23. Additionally, it was observed that dietary P_i is a more potent stimulant of renal P_i excretion, when compared with intravenous P_i. Furthermore, homogenate from duodenal mucosa can stimulate renal P_i excretion, suggesting the existence of an intestinal–renal axis (where P_i is sensed in the intestinal mucosa to induce the expression of unknown intestinal phosphatonins that stimulate renal P_i excretion)[224]. However, an acute intravenous and intestinal P_i load caused nearly identical phosphaturic responses in humans; thus, definitive proof for the presence of these intestinal phosphatonins is still pending[225]. Paracellular P_i transport has not been well characterized, and it is unknown whether it contributes to the regulation of FGF23 and renal P_i excretion. Targeting dietary P_i absorption and intestinal P_i sensing could reduce the severity of hyperphosphataemia and cardiovascular complications in CKD[221].

Disorders of P_i homeostasis

In light of the essential role of P_i in energy metabolism, cell signalling, protein function and bone matrix mineralization, disorders of P_i homeostasis are expected to impair the function of many organ systems. The majority of disorders of P_i homeostasis primarily result in changes of extracellular P_i. However, disorders that primarily change intracellular P_i have been described. Because extracellular and intracellular P_i are intimately connected, symptoms of these disorders might overlap. However, some disorders of excess P_i uptake into cells result in hypophosphataemia and rhabdomyolysis, whereas disorders of reduced P_i uptake into cells result in hyperphosphataemia and matrix calcifications. In addition, some transporters have cell autonomous and systemic functions. For example, LOF mutations in *NPT2A* reduce intracellular levels of P_i and stimulate the synthesis of calcitriol in the proximal tubules of the kidneys, resulting in hyperabsorption of phosphate from the diet in the gut. However, because of its essential role in reclaiming P_i from the urine, the net effect of *NPT2A* LOF mutations is reduced extracellular levels of P_i, which results in hypophosphataemic rickets or osteomalacia. Although the mechanisms by which disorders of P_i homeostasis result in extracellular effects, such as loss of bone mineral or vascular

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calcification, are well defined, how intracellular effects such as myopathy, tumour formation and changes associated with accelerated ageing are mediated is less well understood[226].

Extracellular P_i homeostasis

Disorders of extracellular P_i homeostasis can be divided into acquired and familial forms, which might be FGF23-dependent, PTH-dependent or FGF23-independent and PTH-independent. Supplementary Table 1, FIG. 4 and several excellent reviews[8,220] provide an in-depth discussion of these disorders. In this section, we focus on the latest discoveries in each category.

Tumour-induced osteomalacia is caused by small benign or low-grade malignant phosphaturic mesenchymal tumours of a mixed connective tissue variant[227]. This disorder is characterized by the secretion of FGF23, and less commonly by FGF7, MEPE or SFRP4, which cause renal P_i wasting, hypophosphataemia and osteomalacia[228]. An important discovery is that >60% of phosphaturic mesenchymal tumours of mixed connective tissue variant bear a somatic rearrangement between the fibronectin promoter and *FGFR1* (REF.[229]) or *FGF1* genes[230]. Together with reports that inhibitors of FGFR1 reverse the phenotype of a mouse model of X-linked hypophosphataemia[231,232] and reduced FGF23 levels in a patient with malignant tumour-induced osteomalacia after treatment with the FGFR1 inhibitor BGJ398[233,234], these findings suggest that FGF1 and/or FGFR1 stimulate tumour growth and FGF23 synthesis and/or secretion. As initial observations with neutralizing FGF23 antibodies did not replicate[235] the impressive tumour regression observed with BGJ398 (REFs[233,234]), excess FGF23 itself does not seem to stimulate tumour-induced osteomalacia tumour growth in an autocrine fashion.

Germline mutations in several genes cause familial forms of FGF23-dependent hypophosphataemia. The most common X-linked hypophosphataemia is caused by mutations in *PHEX*[236], autosomal dominant hypophosphataemic rickets is caused by gain-of-function mutations in *FGF23* (REF.[237]) and autosomal recessive hypophosphataemic rickets is caused by LOF mutations in *DMP1*. Two additional autosomal recessive forms (autosomal recessive hypophosphataemic rickets types 2 and 3) were described. Autosomal recessive hypophosphataemic rickets type 2 is caused by LOF mutations in *ENPP1* (REF.[238]), and autosomal recessive hypophosphataemic rickets type 3, a variant of Raine syndrome, is caused by LOF mutations in *FAM20C*[239,240].

ENPP1 is a cell membrane protein that is essential for pyrophosphate synthesis. Because LOF mutations in *ENPP1* cause hypophosphataemia owing to increased bioactive FGF23, it was speculated that the P_i:pyrophosphate ratio might participate in the post-translational regulation of iFGF23 (REF.[241]). The mechanism of FAM20C LOF mutations was discussed above and results in underphosphorylation of FGF23 at Ser180 (REF.[175]), thereby increasing secretion of bioactive iFGF23 in humans[239,240] and mice[242], whereas LOF mutations in *GALNT3* reduce levels of bioactive iFGF23 and lead to hyperphosphataemic tumoural calcinosis[243].

Observations made in patients with autosomal dominant hypophosphataemic rickets led to the discovery that the condition often improves after puberty in men, but not women. This

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discovery raised the possibility that iron deficiency, often a result of menstrual bleeding in women[244], might sustain FGF23 excess in women, but not men, which uncovered an important link between iron and phosphate homeostasis[244,245]. Subsequently, it was found that iron deficiency stimulates FGF23 expression in healthy individuals and those who are affected, but only patients with autosomal dominant hypophosphataemic rickets, who express the autosomal dominant hypophosphataemic rickets variant of *FGF23*, become hypophosphataemic as discussed previously[246].

McCune–Albright syndrome is a condition that causes benign tumours in bones (also known as fibrous dysplasia) and in multiple endocrine glands. Individuals with this condition sometimes develop renal phosphate wasting owing to high circulating levels of FGF23, suggesting that guanine nucleotide-binding protein G(s), subunit α (GNAS1) likewise stimulates secretion of bioactive iFGF23 owing to inhibition of subtilisin or furin-type proteases[247]. Furthermore, work in mice expressing GNAS-XL, a large splice variant of GNAS1, suggests that GNAS-XL stimulates the secretion of FGF23 by upregulating FGFR1 (REFs[248,249]).

Current therapies for FGF23-dependent disorders of P_i homeostasis aim to correct blood levels of P_i and calcitriol, which is discussed in several excellent reviews[250–252]. Additionally, Crysvita (burosumab, an FGF23-inactivating antibody[253]) was approved by the FDA for FGF23-dependent hypophosphataemic disorders, such as X-linked hypophosphataemia. Also, modified carboxy-terminal FGF23 peptides with increased half-lives are able to improve blood levels of P_i and bone quality in Hyp mice by blocking the action of bioactive FGF23[254]. As mentioned above, two individuals with tumour-induced osteomalacia showed marked reduction of FGF23 levels upon treatment with the FGFR1 inhibitor BGJ398 (REF.[233]). Furthermore, FGF23 hormone replacement and blocking excessive degradation of iFGF23 by supplementing GALNT3 enzyme[255] were proposed for the treatment of FGF23-deficient hyperphosphataemic disorders.

Different from the already-discussed disorders, hereditary hypophosphataemic rickets with hypercalciuria and autosomal recessive Fanconi syndrome are FGF23-independent and are caused by LOF mutations in *NPT2C* and *NPT2A*, respectively[218,256,257]. Treatment of hereditary hypophosphataemic rickets with hypercalciuria and idiopathic infantile hypercalcaemia 2 is founded on oral P_i supplementation, although there is concern in mouse models lacking the *Npt2a* gene that P_i supplementation can worsen renal calcifications and Fanconi syndrome, despite normalization of hypercalciuria[258–260]. *NPT2A* LOF mutations can also cause idiopathic infantile hypercalcaemia type 2, which clinically resembles idiopathic infantile hypercalcaemia type 1 (caused by LOF mutations in *CYP24A1*). Both disorders present with hypercalcaemia and elevated calcitriol levels, whereas renal phosphate wasting and hypophosphataemia are mild, which suggests a role of NPT2A in the regulation of CYP24A1, as previously discussed.

Intracellular P_i homeostasis

Mutations in several P_i transporters that cause a new group of disorders of intracellular P_i homeostasis have been reported in the past 5 years. Individuals with hypertrophic cardiomyopathy, muscular dystrophy and lactic acidosis[261,262] were found to carry LOF

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mutations in the mitochondrial phosphate carrier (PIC; which is encoded by *SLC25A3*), which mediates uptake of P_i by the mitochondria. These findings suggest that P_i is important for the function of the mitochondrial respiratory chain and ATP synthesis, which has been reported by us[52] and others[134–136,263,264]. PIC is also a component of the mPTP, which requires P_i to permit the influx of calcium into the mitochondrial matrix[57]. Detailed evaluation of hearts in a mouse model with an inducible cardiac-specific deletion of *Slc25a3* (REF.[265]) showed reduced mPTP opening in response to calcium challenge. PIC therefore also has P_i transport-independent roles in mitochondrial function.

LOF mutations in *PIT2* (REF.[266]) and *XPR1* (REF.[267]) were reported in individuals with primary familial brain calcification, or Fahr syndrome. These individuals develop vascular calcifications in the basal ganglia of their brains, leading to seizures and in some cases disturbance of sustained phonation and orofacial apraxia[268]. Inhibition of P_i uptake into the microglia because of LOF in *PIT2* or the inhibition of P_i export from VSMCs due to LOF in XPR1 might stimulate formation of calcium– P_i deposits inside these cells[267,269]. A similar phenotype was observed in human individuals and mouse models with LOF mutations in PDGFB receptor (*PDGFBR*) and *PDGFB*[270]. Together with reports of a physical interaction between XPR1 and PDGFRB in mice[271], it was suggested that PDGFB, PDGFBR and phosphate transporters functionally interact. Similar to humans with *PIT2* LOF mutations, *Pit2*-knockout mice develop brain calcifications[272]. Interestingly, *Pit2*-knockout mice have increased CSF levels of P_i and glymphatic pathway-associated arteriolar calcification[273], which suggests a role of this transporter in CSF P_i homeostasis[274].

Pseudoxanthoma elasticum is caused by LOF mutations in the multidrug resistance-associated protein 6 (encoded by *ABCC6* (REF.[275])), which, similar to mutations in *ENPP1*, result in abnormal pyrophosphate metabolism, mineralization and fragmentation of the elastin-containing fibres in connective tissue, which may lead to vascular disease in humans. Mice with LOF mutations of *ABCC6* also exhibit renal calcifications that are different from autosomal recessive hypophosphataemic rickets type 2 as hypophosphataemic rickets is absent in pseudoxanthoma elasticum[276]. As the substrate of ABCC6 is unknown, it remains unclear whether pseudoxanthoma elasticum can be considered a disorder of intracellular P_i homeostasis.

In summary, although the majority of disorders of P_i homeostasis primarily result in changes in extra-cellular P_i, new disorders that primarily change intracellular levels of P_i have been reported. As mammalian P_i sensing is only partially understood, it remains unclear whether these disorders are caused by toxicities due to the lack or excess of P_i in the extracellular or intracellular compartment.

Conclusion

Disorders of P_i homeostasis are important in human health and disease. Major advances in P_i homeostasis in humans have been made with the discovery of FGF23 and its regulation of body P_i levels. However, it is unknown how P_i feeds back to regulate FGF23, PTH and calcitriol. Although the P_i-sensing mechanism has been extensively studied in bacteria and yeast, no P_i sensor has been identified in humans. One possible P_i signalling

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pathway involves the binding of P_i and/or transport of P_i by the type 3 sodium-phosphate cotransporters PIT1 and PIT2, followed by the activation of ERK1 and ERK2, which modulate gene expression in bone and VSMCs. However, it remains to be shown whether metabolic and endocrine actions of P_i involve the same or different signalling pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

Phosphaturic hormone

Hormone causing excretion of phosphate in the urine

Hypomorphic alleles

Genes that have a mutation that causes a partial loss of gene function

Haploinsufficiency

Refers to complete loss of function of one copy of a gene when the remaining functional copy of the gene is not adequate to produce the needed gene product to preserve normal function

5/6 nephrectomy

Model of progressive renal failure with reduced nephron number achieved by either infarction or surgical excision of both poles and removal of the contralateral kidney

Pulmonary alveolar microlithiasis

A rare autosomal recessive disease of widespread intra-alveolar accumulation of minute calcium phosphate calculi called microliths caused by homozygous loss-of-function mutations in *SLC34A2* (which encodes NPT2b)

Tumoural calcinosis

Group of rare autosomal recessive metabolic disorders characterized by the development of severe ectopic calcifications in soft tissues due to homozygous loss-of-function mutations in the *GALNT3, FGF23* or *KL* genes

Osteomalacia

Osteomalacia is a rare disorder of bone metabolism leading to reduced bone matrix mineralization

Phosphatonins

Phosphatonins is the collective term used for major regulators of P_i homeostasis, which generally function as phosphaturic hormones and lower blood levels of P_i

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Key points

- Endocrine regulation of gastrointestinal absorption, storage in the mineral deposits of the skeleton and renal excretion of inorganic phosphate (P_i) maintains the serum concentration of P_i within a narrow range.
- P_i activates extracellular-signal-regulated kinases 1 and 2 in mammalian cells, which are required for stimulation of mitochondrial respiration and transcription of bone matrix proteins.
- P_i stimulates the synthesis and secretion of parathyroid hormone and fibroblast growth factor 23 and blocks the synthesis of calcitriol; however, the endocrine sensor for P_i remains unknown.
- Mutations in the endocrine regulators of P_i lead to genetic disorders characterized by abnormal bone and mineral metabolism and ectopic calcifications.
- Identification of loss-of-function mutations in several P_i transporters highlights the importance of intracellular P_i for muscle function and vascular calcifications.
- How intracellular P_i causes myopathy, tumour formation and changes associated with acclerated ageing is less well understood.

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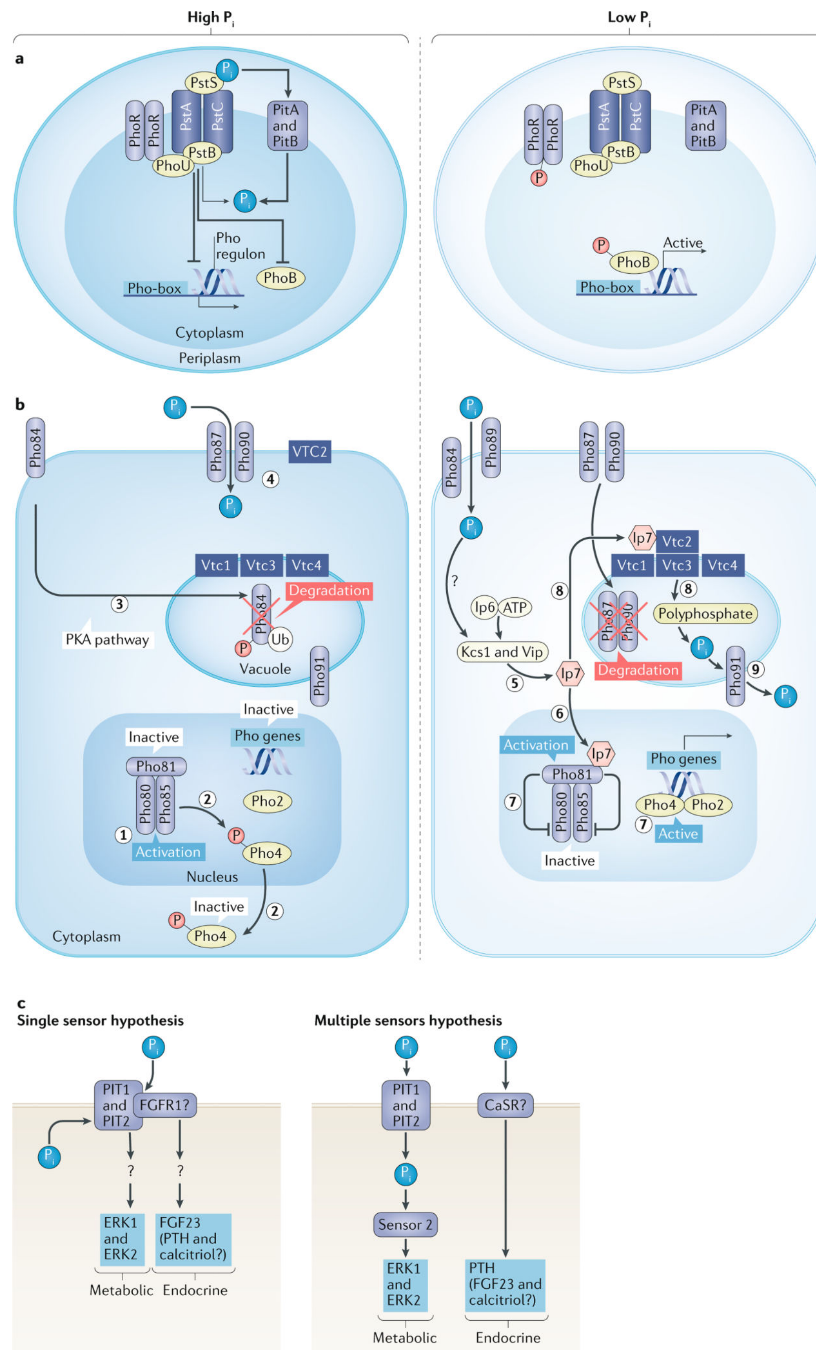

Fig. 1 |. P_i sensing pathways.

a | P_i sensing in bacteria. High P_i levels are sensed by PstS. Then, together with the Pst–ABC complex (PstA, PstB and PstC), it forms a plasma membrane protein complex in the bacterial inner membrane that stimulates the binding of PhoU to PhoB and PhoR. This process inactivates the transcription factor PhoB and the Pho regulon. The low-affinity P_i transporters 1 and 2 (PitA and PitB) facilitate uptake of P_i for cellular metabolism. Under low P_i conditions, PhoR is autophosphorylated and phosphorylates PhoB and activates the Pho box, allowing downstream activation of the Pho regulon. **b** | In yeast, high P_i levels

activate the Pho80 and Pho85 cyclin–cyclin-dependent kinase complex (1), which results in the phosphorylation and export of Pho4 into the cytosol (2) and inactivation of the yeast Pho regulon. The high-affinity P_i transporter and sensor Pho84 is internalized and degraded (3), whereas the low-affinity P_i transporters Pho87 and Pho90 are responsible for P_i uptake in high P_i conditions (4). Low P_i levels stimulate synthesis of Ip7 by the yeast inositol hexakisphosphate (Ip6) kinase 1 (Kcs1) and Vip (5), which activates Pho81 (6). Pho81 inhibits Pho80 and Pho85, preventing phosphorylation of Pho4, resulting in the association of Pho4 with Pho2 (7) in the nucleus to activate the Pho regulon. Ip7 also stimulates Vtc proteins 1–4, which stimulate polyphosphate synthesis from ATP (8) and the conversion of polyphosphate into P_i by endopolyphosphatase (Phm5). P_i is transported by Pho91 from the vacuole to the cytosol (9), thereby indirectly using ATP to supply P_i for metabolic processes. Pho84 and Pho89 are responsible for P_i uptake in low P_i condition, whereas Pho87 and Pho90 are internalized and degraded. **c** | Metabolic P_i sensing in mammalian cells is mediated by PIT1 and/or PIT2, resulting in activation of the ERK1 and ERK2 pathway, which might also have a role in endocrine P_i sensing but could require co-receptors (single sensor hypothesis), possibly FGFR1, which was shown to be activated by P_i and might regulate FGF23 secretion by osteocytes. Alternatively, endocrine P_i sensing might involve the calcium-sensing receptor (CaSR) or other molecules as a second sensor (multiple sensor hypothesis), which might mediate secondary hyperparathyroidism in the parathyroids. Question marks indicate unknown mechanisms or sensors. P, phosphate; PTH, parathyroid hormone; Ub, ubiquitin.

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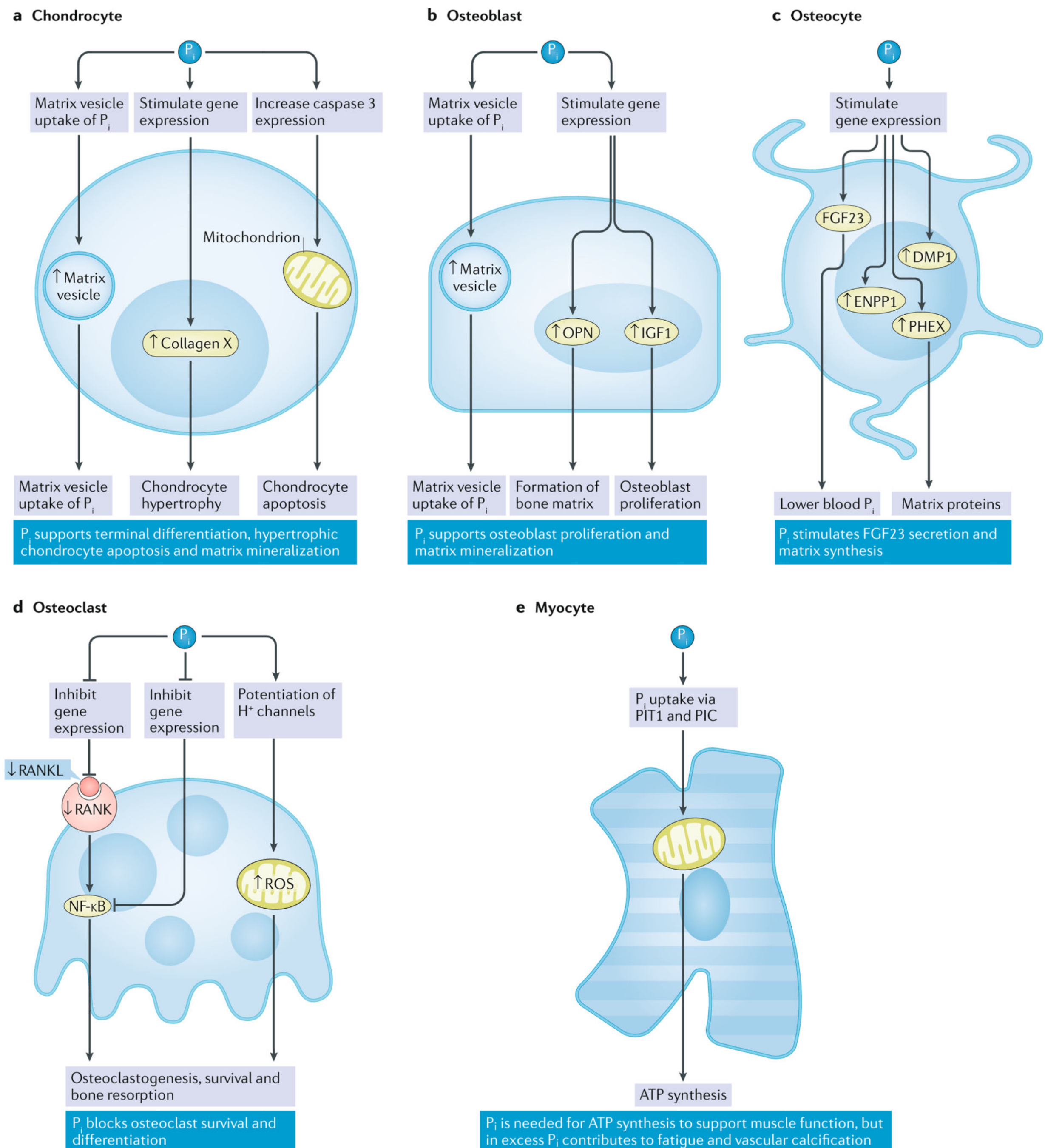

Fig. 2 |. Regulation of bone cell function and matrix mineralization by P_i.

a | In chondrocytes, P_i stimulates the expression of hypertrophic chondrocyte markers (that is, collagen X) and induces apoptosis via the mitochondrial caspase 3 pathway in an ERK1-dependent and ERK2-dependent fashion[83]. P_i also stimulates PIT1-dependent matrix vesicle mineralization[90]. **b** | In osteoblasts, P_i induces the expression of osteopontin (OPN) through an ERK1-dependent and ERK2-dependent mechanism to support the formation of bone matrix[98]. P_i also stimulates IGF1 secretion, which increases osteoblast proliferation in an autocrine fashion[99]. **c** | In osteocytes, DMP1, ENPP1 and PHEX expression are

stimulated by P_i (REF.[116]), which also induces the secretion of bioactive intact FGF23 (REF.[103]). **d** | In osteoclasts, P_i reduces gene expression of RANKL and thereby suppresses RANK[126], which results in the inhibition of osteoclastogenesis and bone resorption. P_i also induces the production of ROS, possibly through proton (H+) channels, which increases osteoclast function and survival[131]. **e** | In myocytes, P_i is important for the function of the mitochondrial respiratory chain and ATP synthesis[52]. This process is possibly due to the function of the muscle-specific isoform of PIC, which mediates mitochondrial uptake of P_i (REF.[57]), and PIT1 (REF.[277]). ↑, upregulation; ↓, downregulation.

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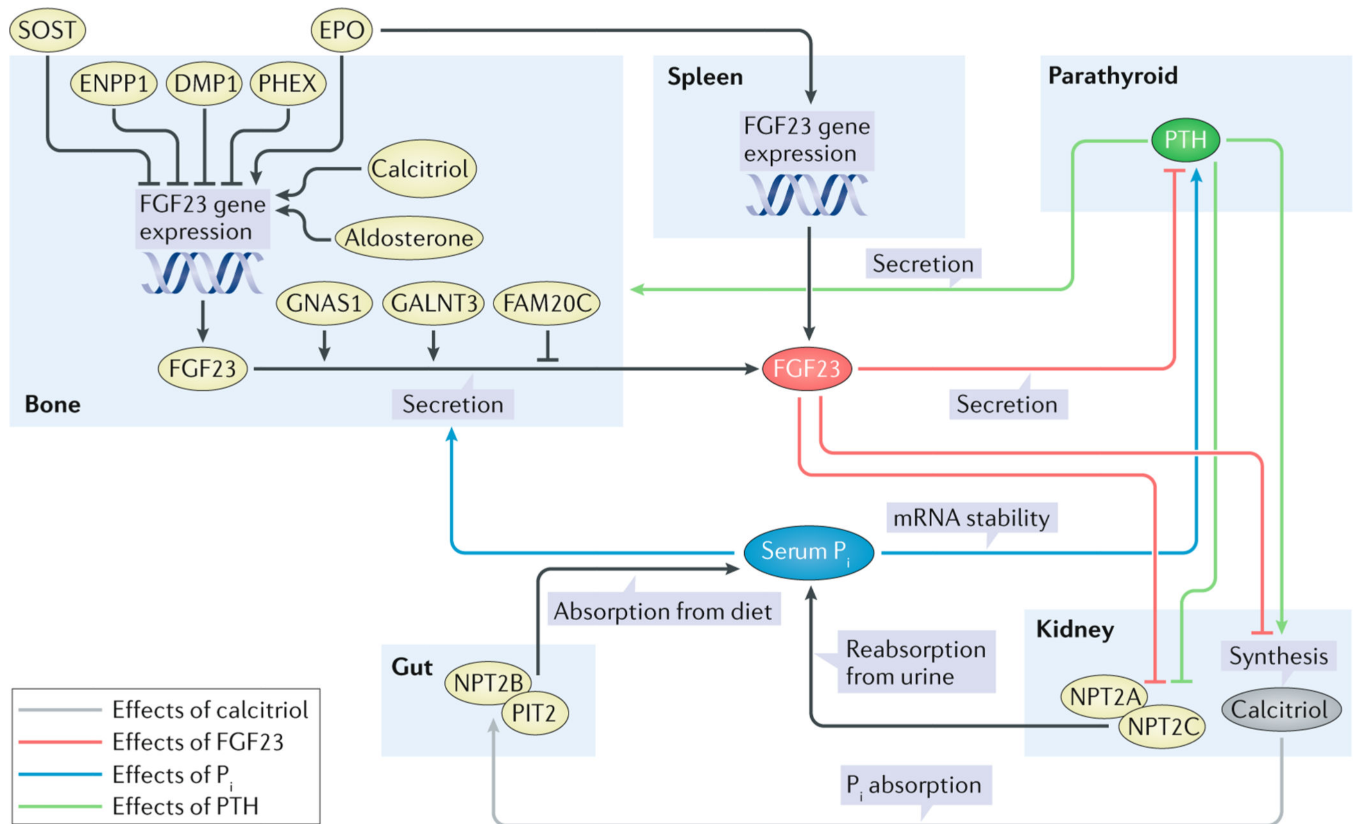

Fig. 3 |. Endocrine regulation of P_i homeostasis.

Serum P_i stimulates secretion of bioactive FGF23 in osteoblasts and osteocytes (blue arrow), which directly or indirectly acts at the proximal tubule of the kidneys to inhibit synthesis of calcitriol and the function of NPT2A and NPT2C (red arrows). Inhibition of calcitriol reduces absorption of P_i from the diet in the gut (grey arrow) and mobilization of P_i from bone mineral. Downregulation of NPT2A and NPT2C reduces renal phosphate reabsorption (black arrows). The net effect of FGF23 action is to lower blood levels of P_i. Similar to FGF23, parathyroid hormone (PTH) downregulates NPT2A and NPT2B and reduces renal phosphate reabsorption (green arrows). However, different from FGF23, PTH induces calcitriol and bone turnover, which increase blood P_i (green arrows). However, the net effect of PTH is to lower blood levels of P_i. Although not completely understood, FAM20C, DMP1, ENPP1 and PHEX reduce FGF23 expression or secretion whereas phosphate, iron deficiency, erythropoietin (EPO), GALNT3 and GNAS1 stimulate it (black arrows)[278,279]. In addition, sclerostin (SOST) seems to negatively regulate FGF23 (black arrows)[117]. Furthermore, EPO might directly upregulate FGF23 gene expression in myeloid lineage stem cells of the spleen, providing a link to iron homeostasis (black arrows)[195]. PTH is suppressed by FGF23 in rodents but not in humans (red arrow).

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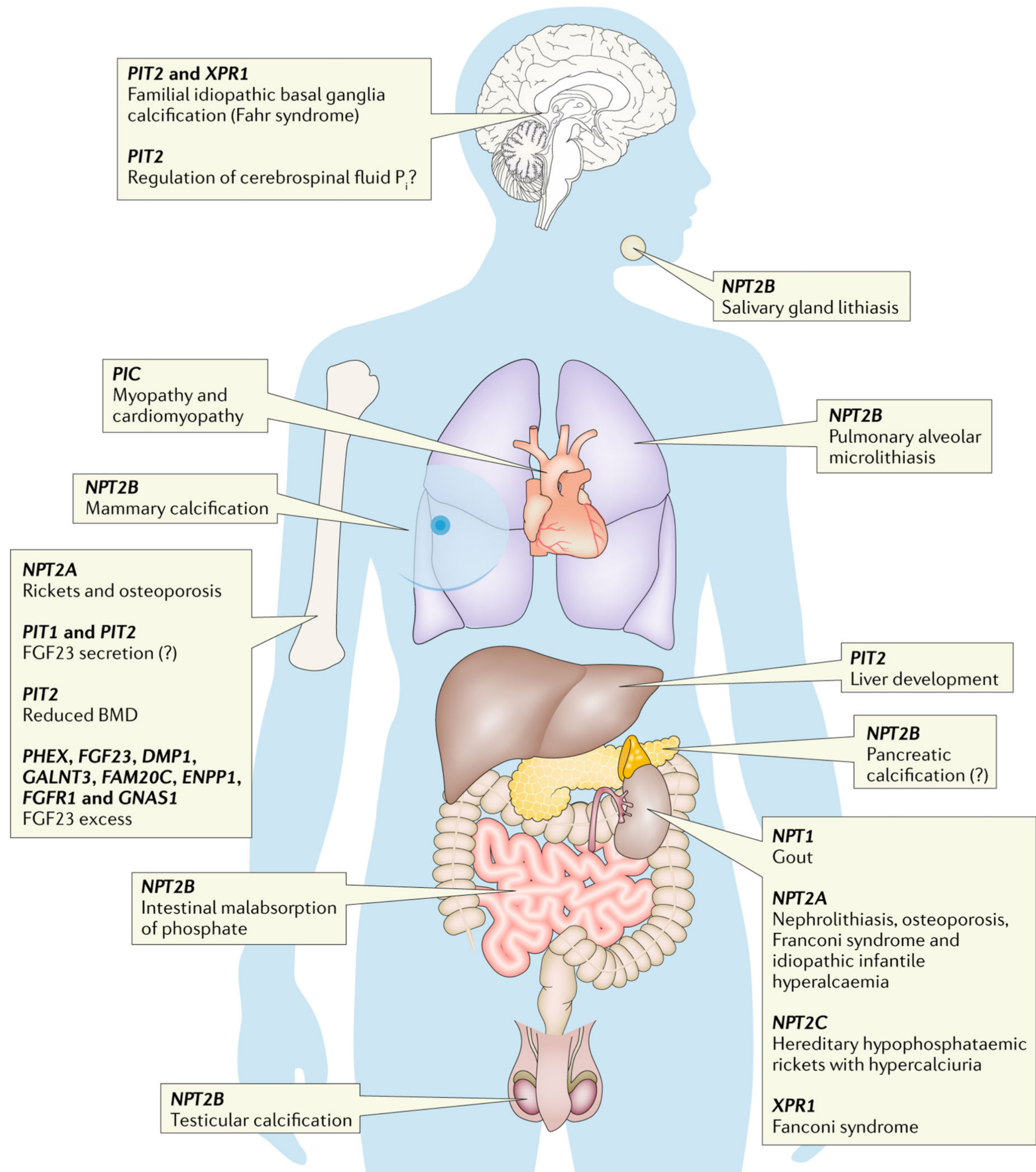

Fig. 4 |. Diseases of phosphate homeostasis organized by organ system.

Linkage analysis in human disorders of inorganic phosphate (P_i) homeostasis, cardiomyopathy and familial basal ganglial calcifications identified several novel genes important for the regulation of P_i homeostasis. *DMP1*, dentin matrix acidic phosphoprotein 1; *ENPP1*, ectonucleotide pyrophosphatase-phosphodiesterase family member 1; *FAM20C*, Golgi-associated secretory pathway kinase; *FGF23*, fibroblast growth factor 23; *FGFR1*, FGF receptor 1; *GALNT3*, polypeptide *N*-acetylgalactosaminyltransferase 3; *GNAS1*, guanine nucleotide-binding protein G(s), subunit α; *NPT1*, sodium-dependent phosphate

transport protein 1; *NPT2A*, sodium-dependent phosphate transport protein 2A; *PHEX*, phosphate-regulating endopeptidase homologue, X-linked; *PIT*, sodium-dependent P_i transporter; *SLC25A3*, solute carrier family 25 member 3; *XPR1*, xenotropic and polytropic retrovirus receptor 1. Question mark indicates unknown.

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Table 1 |

Genes involved in inorganic phosphate (P_i) sensing

Gene name	Organism	Orthologue	Function	Refs
PstS	Bacteria (*Escherichia coli*)	Human phosphate-binding protein	Phosphate periplasmic-binding component	46,280
PstA	Bacteria (*Escherichia coli*)	–	•Phosphate transporter subunit •Membrane component	281
PstB	Bacteria (*Escherichia coli*)	–	•Phosphate transporter subunit •ATP-binding component	282
PstC	Bacteria (*Escherichia coli*)	–	•Phosphate subunit •Membrane component	NA
PitA	Bacteria (*Escherichia coli*)	•*PIT1* and *PIT2* in *Homo sapiens* •*CG7628* in *Drosophila melanogaster* •*Pho89* in yeast	Low-affinity phosphate transport system	37
PitB	Bacteria (*Escherichia coli*)	•*PIT1* and *PIT2* in *Homo sapiens* •*CG7628* in *Drosophila melanogaster* •*Pho89* in yeast	Low-affinity phosphate transport system	37
Pho84	Yeast	•*SLC17A1–9* in *Homo sapiens* •*MFS10* and *MFS13* in *Drosophila melanogaster*	Major facilitator superfamily (MFS) P_i transporter (H^+-coupled)	32
Pho89	Yeast	•*PIT1* and *PIT2* in *Homo sapiens* •*CG7628* in *Drosophila melanogaster* •*PitA* and *PitB* in bacteria	P_i transporter (Na^+-coupled)	37
Pho87	Yeast	*SLC13A1–4* in *Homo sapiens*	Putative P_i transporter	18
Pho90	Yeast	*SLC13A1–4* in *Homo sapiens*	Putative P_i transporter	18
Pho91	Yeast	*SLC13A1–4* in *Homo sapiens*	Putative P_i transporter	18
SLC17A1	Mammalian	•*MFS10* and *MFS13* in *Drosophila melanogaster* •*Pho84* in yeast	Type 1 sodium-phosphate cotransporters (NPT1)	32
SLC13A1–4	Mammalian	*Pho87* in yeast	Sodium-dependent sulfate transporters	18
SLC34A1–A3	Mammalian	–	Type 2 sodium-phosphate cotransporters NPT2a, NPT2b and NPT2c	283
SLC20A1 and *SLC20A2*	Mammalian	•*CG7628* in *Drosophila melanogaster* •*Pho89* in yeast •*PitA* and *PitB* in bacteria	Type 3 sodium-phosphate cotransporters PIT1 and PIT2	37
SLC25A3	Mammalian	*MIR1* in yeast	Mitochondrial phosphate transporter PIC	37,284
SLC53A1	Mammalian	*Pho81* and *Syg1* in yeast	Phosphate exporter XPR1	285

H^+, proton; NA, not available.

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Table 2 |

Hormones and growth factors regulated by P_i

Factors	Regulation by P_i	Target organs	Effect	Blood P_i concentration	Refs
FGF23	Up	Kidney	Renal P_i wasting and decreased synthesis of calcitriol	Down	79
PTH	Up	Kidney and bone	Renal P_i wasting, increased synthesis of calcitriol and release of P_i by bone resorption	Down	286
Calcitriol	Down	Intestine and bone	Increased intestinal P_i absorption and release of P_i by bone resorption	Up	287
IGF1	Up	Bone and kidney	Increased P_i storage in bone mineral and increased renal P_i reabsorption	Up (down)	99,288
Osteopontin	Up	Bone	Increased P_i storage in bone mineral	(Down)	98

FGF23, fibroblast growth factor 23; IGF1, insulin-like growth factor 1; P_i, inorganic phosphate; PTH, parathyroid hormone.