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The Chicken or the Egg: PHEX, FGF23 & SIBLINGs unscrambled

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Abstract

The eggshell is an ancient innovation that helped the vertebrates' transition from the oceans and gain dominion over the land. Coincident with this conquest several new eggshell and noncollagenous bone-matrix proteins (NCPs) emerged. The protein ovocleidin-116 is one of these proteins with an ancestry stretching back to the Triassic. Ovocleidin-116 is an avian homolog of Matrix Extracellular Phosphoglycoprotein (MEPE) and belongs to a group of proteins called Small Integrin-Binding Ligand Interacting Glycoproteins (SIBLINGs). The genes for these NCPs are all clustered on chromosome 5q in mice and chromosome 4q in humans. A unifying feature of the SIBLING proteins is an Acidic Serine Aspartate Rich MEPE associated motif (ASARM). The ASARM motif and the released ASARM peptide play roles in mineralization, bone turnover, mechanotransduction, phosphate regulation and energy metabolism. ASARM peptides and motifs are physiological substrates for PHEX, a Zn metalloendopeptidase. Defects in PHEX are responsible for X-linked hypophosphatemic rickets. PHEX interacts with another ASARM motif containing SIBLING protein, Dentin Matrix Protein-1 (DMP1). DMP1 mutations cause bone-renal defects that are identical with the defects caused by loss of PHEX function. This results in autosomal recessive hypophosphatemic rickets (ARHR). In both XLH and ARHR increased FGF23 expression occurs and activating mutations in FGF23 cause autosomal dominant hypophosphatemic rickets (ADHR). ASARM peptide administration *in vitro* and *in vivo* also induces increased FGF23 expression. This review will discuss the evidence for a new integrative pathway involved in bone formation, bone-renal mineralization, renal phosphate homeostasis and energy metabolism in disease and health.

I. INTRODUCTION: Back to the Future

Our planet is covered by over 139 million square miles of water that encompasses more than 71% of the earth's surface. The deepest part of this aquatic realm, the Mariana Trench plunges over 6.8 miles, a distance equivalent to the cruising height of commercial aircraft. In this vastness, the nascent beginnings of life began over 2 billion years ago. Life that was and is nurtured by geothermal fulminations and tectonic forces still active today. Approximately 530 million years ago a quite remarkable event occurred that resulted in the rapid, unexplained and unprecedented birth of a cornucopia of new phyla, the Cambrian explosion. Amongst the new phyla the vertebrates emerged and evolved into the boney fish or teleosts. Approximately 300 million years ago the vertebrates began their conquest of gravity and the dry land. This new environment required ingenious changes in reproduction, waste secretion and bone physiology. In particular, the dry land was unable to sustain the reproductive process that was previously nurtured by the aqueous marine environment. Evolution came up with a solution and the egg was born. The sea was effectively transported to the land by the fashioning of this new and quite ingenious vessel. The shell surrounding the egg contained the minerals present in abundance in the oceans plus a new ancestral protein (ovocleidin-116)^{1–7}. This protein likely first appeared with the dinosaurs and was preserved through the theropod lineage in modern birds and reptiles^{5–7}.

Ovocleidin-116 is an avian homolog of Matrix Extracellular Phosphoglycoprotein (MEPE) and belongs to a group of proteins called Small Integrin-binding Ligand N-linked Glycoproteins (SIBLINGs) that includes Dentin Matrix Protein 1 (DMP1), osteopontin (OPN), dentin sialo-phosphoprotein (DSPP), bone sialoprotein (BSP), MEPE and statherin^{4, 5, 7–10}. SIBLING proteins also comprise a subgroup of the Secretory Calcium-Binding Phosphoprotein family (SCPP) that share a common evolutionary heritage^{11, 12}. The broader SCPP family includes enamel, milk and distinct salivary proteins (amelogenin, enamelin, ameloblastin, caseins, histatins, proline rich proteins and mucins)^{11, 12}. The appearance of these noncollagenous bone matrix proteins (DMP1 and MEPE) coincided with an internuncial sequestration and regulation of two older proteins FGF23 and PHEX. The regulatory link between the SIBLINGs and FGF23 is orchestrated through a common SIBLING-motif, the ASARM motif and the corresponding free protease-resistant peptide (ASARM peptide). The ASARM-sequence previous role was likely to orchestrate the mineralization of eggshell and bone. This role was retained but nature parsimoniously extended the properties of this peptide and motif to both transduce and suppress FGF23 signaling. The FGF23 signaling is primarily rendered by the competitive displacement of a DMP1-ASARM-motif and PHEX interaction by free ASARM peptide as discussed in more detail in the review. FGF23 is a member of the fibroblast growth factor (FGF) family of cytokines and surfaced 500 million years ago with the boney fish (teleosts) that do not contain SIBLING proteins (MEPE or DMP1), (Figure 1). In terrestrial vertebrates, FGF23 like the SIBLINGs is expressed in the osteocyte. The boney fish however are “an-osteocytic” and so a physiological bone-renal link with FGF23 and the SIBLINGs was likely cemented when life ventured from the oceans to the land in the Triassic, approximately 300 million years ago. This link is exquisitely revealed by recent research that indicates a competitive displacement of a PHEX-DMP1 interaction by ASARM peptide that leads to increased FGF23 expression.

In this review we will discuss the roles of several molecules and hormones that link back to the tentative transition and adaptations of life from marine to terrestrial environments. These include FGF23, MEPE, DMP1, KLOTHO, PHEX and ASARM peptides. These new molecules have direct impact in disease and health and have relevance in inherited bone-teeth mineral disorders, tumor induced osteomalacia, chronic kidney disease, end stage renal disease, renal osteodystrophy, ectopic arterial calcification, renal calcification, cardiovascular disease, diabetes and obesity.

II. PHEX, its physiological substrate (ASARM peptide), osteomalacia & hypophosphatemic rickets

In 1995, the HYP consortium discovered a new gene PHEX (previously known as PEX) and identified its primary role in X-linked hypophosphatemic rickets (HYP also known as XLH)^{9, 13–24}. Pictures of the “*first*” human XLH patient shown to have a mutation in the PHEX gene and the human murine-homolog of the disease are shown in Figure 2. Following this MEPE was cloned as a phosphaturic factor expressed from the tumor of a patient suffering from tumor-induced osteomalacia TIO^{9, 25}. Clinically, TIO is similar to XLH with an overlapping pathophysiology. Loss of PHEX function causes defective mineralization, hypophosphatemia, abnormal vitamin-D metabolism and gross skeletal abnormalities as illustrated in Figure 2¹⁰. PHEX is a Zn metalloendopeptidase of the M13 family and MA clan that includes endothelin converting enzyme-1 (ECE-1 α , ECE-1 β and ECE-II), ECE-like enzyme/distress-induced neuronal endopeptidase (ECEL1/DINE), soluble endopeptidase/NEP-like enzyme-1/neprilysin 2 (SEP/NL1/NEP2), membrane metallo-endopeptidase-like 2 (MMEL2), and Kell blood group protein antigen (KELL)^{10, 26–29}, Figure 3. Extensive studies confirm PHEX binds with high specificity to MEPE a bone-renal extracellular matrix-protein and ASARM peptide^{10, 30–32}. Figure 4 depicts the PHEX ASARM binding

region as first deduced from mutation analyses on PHEX and related M13 Zn metalloendopeptidases²². A virtual three dimensional (3D) x-ray crystallographic scheme for PHEX and the ASARM substrate binding region is presented in Figure 5. The 3D structure was rendered for this review using known M13 Zn metalloendopeptidase X-ray crystallographic data and the Protein Homology/analogy Recognition Engine V.20 or Phyre2 server to model domains³³. MEPE has a motif (ASARM motif) located at the tip of the COOH terminus consisting of 23 residues enriched for aspartate, serine and glutamate. The motif also occurs at the DMP1 COOH terminus (a related SIBLING protein) but is capped by an extra 33 residues^{9, 10, 31}. PHEX also binds to and hydrolyzes with high affinity and specificity phosphorylated and nonphosphorylated small ASARM peptides from MEPE and the related SIBLING-protein osteopontin^{10, 30–32, 34–37}. The SIBLING-motifs (ASARM) and their potential role or roles were first described in the paper that reported the original cloning of MEPE from a tumor resected from a patient with tumor induced osteomalacia (TIO)⁹. These ASARM peptides (~2.2 kDa) are essentially the released MEPE or SIBLING ASARM motif (DMP1, osteopontin, DSPP) and are the only known physiological *substrate/ligand* for PHEX^{10, 31, 32, 34, 36–39}. The ASARM peptide is otherwise resistant to proteolysis^{9, 10, 16, 30–32, 38, 40}. This motif (ASARM), when released as a phosphorylated peptide (ASARM peptide), behaves like a biological bisphosphonate and inhibits mineralization and renal/intestinal phosphate uptake^{10, 32, 34, 37–39, 41–47}. Indeed, like bisphosphonates and salivary-statherin^{48–50}, the ASARM peptide binds strongly to hydroxyapatite^{10, 31, 34, 37}. Compelling evidence for the role of DMP1 (the closest relative to MEPE) in mineralization and phosphate regulation is the finding that DMP1 null-mutations result in a phenotype identical with XLH. This newly characterized disease is called autosomal recessive hypophosphatemic rickets (ARHR)^{51, 52}. Null mutations in MEPE result in an opposite bone phenotype with age dependent increased mineralization apposition rate (MAR), hastened mineralization *in vitro*, increased bone mass, increased trabecular volume, increased trabecular thickness/number, and increased cortical bone volume that is age dependent⁵³. These findings are in agreement with several genome-wide studies in humans (males and females) that show strong correlations with volumetric bone mineral density (vBMD)^{54–57} and confirm a major role for MEPE in osteocyte mechanotransductive response to load^{58–63}. Also, recent studies using MEPE transgenic mice show MEPE and MEPE-PHEX interactions are component to age-diet dependent pathways that regulate bone turnover, suppress mineralization and renal calcification³⁵. This novel pathway also modulates bone-renal vascularization and bone turnover. In a separate study, ASARM peptides were shown to be responsible for the *in vitro* mineralization defect in XLH-mice bone-marrow-stromal-cells (BMSCs)³¹. In these studies a bioengineered, 4.2 kDa **S**mall-synthetic **P**HEX **R**elated-**P**eptide (SPR4) that specifically binds and neutralizes ASARM peptides was described. This peptide (SPR4) also corrects the mineralization defect *in vitro* and has marked effects on osteogenic and bone resorption markers^{31, 39}. These discoveries (ASARM and SPR4-peptides) may help provide new strategies to treat select hypophosphatemic bone-mineralization disorders (XLH, ADHR, ARHR and TIO) and manage hyperphosphatemia in chronic kidney disease (CKD) and end stage renal disease (ESRD).

Major progress has been made in understanding the molecular processes regulating bone formation remodeling and mineralization in disease and health^{64, 65}. For example, the proteins of the PHEX, DMP1, FGF23, KLOTHO and the MEPE/ASARM peptide axis have emerged as novel and important regulators of phosphate homeostasis and bone-mineralization⁶⁵. More recent seminal and exciting discoveries have shifted the paradigm and shown the skeleton to be a dynamic endocrine organ that has major influence in regulating energy-metabolism and fat mass⁶⁶. In line with this, there is strong evidence that PHEX, DMP1, FGF23 and ASARM peptides are also involved in orchestrating cross talk between bone and energy metabolism^{35, 42, 67}.

III. SIBLING PROTEINS, MEPE, DMP1 and the ASARM/Dentonin motifs

Human MEPE was first cloned in 2000 from a tumor resected from a patient with TIO⁹. Later that year rat MEPE was cloned⁶⁸ and this was followed by the cloning of murine MEPE in 2001⁶⁹. The human-MEPE paper by Rowe et al 2000, also mapped the MEPE gene to chromosome 4q and first described the close similarity of MEPE to a group of bone dentin non-collagenous matrix proteins (NCP's) that all clustered on chromosome 5q in mice and 4q in humans (Figure 6)⁹. These proteins include MEPE, DMP1, Dentin Sialo-Phosphoprotein (DSPP), Osteopontin (SPP1), Bone Sialoprotein (BSP), enamel and statherin. Each protein was reported to contain a key COOH terminal MEPE motif that was named ASARM (Acidic Serine-Aspartate-Rich MEPE associated motif)⁹. Of note, DSPP is cleaved into three proteins, an N-terminal Dentin Sialoprotein (DSP)^{70, 71}, a COOH terminal portion Dentin Phosphoprotein (DPP)⁷²⁻⁷⁴, and Dentin Glycoprotein (Dgp)⁷⁵. The COOH terminal DPP portion of this protein (DSPP) contains an RGD integrin binding sequence and a long extended "repeat" of the SIBLING ASARM motif as illustrated in (Figure 7B)⁹. Cleavage and release of the DPP (ASARM containing) portion of DSPP is catalyzed by a group of astacin Zn metallopeptidases that include BMP1 (tolloid), MEPE1A and MEPE1B⁷⁶. These proteases are closely related to PHEX, the enzyme defective in X-linked hypophosphatemic rickets (XLH)²⁴, a disease with increased levels of ASARM peptides^{10, 32, 34, 37-39, 42, 77}. Specifically, BMP1, MEPE1A and MEPE1B belong to the M12 family and astacin subfamily of Zn metallopeptidases and as indicated earlier PHEX belongs to the M13 family and MA clan of Zn metallopeptidases that also include endothelin converting enzyme-1 (ECE-1 α , ECE-1 β and ECE-II), ECE-like enzyme/distress-induced neuronal endopeptidase (ECEL1/DINE), soluble endopeptidase/NEP-like enzyme-1/neprilysin 2 (SEP/NL1/NEP2), membrane metallo-endopeptidase-like 2 (MMEL2), and Kell blood group protein antigen (KELL)^{10, 26-29}, Figure 3. Other shared genetic and structural features of the SIBLINGs include: (1) a small non-translational first exon, (2) a start codon in the second exon (3) the last exon contains a large coding segment (the number of exons varies among the different genes), (4) common exon-intron features, (5) an integrin-binding tripeptide Arg-Gly-Asp (RGD) motif that mediates cell attachment/signaling via interaction with cell surface integrins (6) conserved phosphorylation and N-glycosylation sites and (7) a strong signal peptide for extracellular release (Figures 8 and 9)^{8-10, 78, 79}. A strong association to an ancestral mineralization-gene statherin that also contains an ASARM motif and maps to the same region of chromosome 5 was confirmed in subsequent papers^{10, 41}. Statherin, a small 63 residue salivary protein, maintains mineral solution dynamics of enamel by virtue of its ability to inhibit spontaneous precipitation and crystal growth from supersaturated solutions of calcium phosphate minerals⁵⁹⁻⁶¹. Statherin's role in preserving the calcium-phosphate supersaturated state of saliva is crucial for re-calcification and stabilization of tooth enamel and for the inhibition of formation of mineral accretions on tooth surfaces. In addition, statherin has been proposed to function in the transport of calcium and phosphate during secretion in the salivary glands⁵⁹. As with the MEPE ASARM peptide a single cathepsin-B site is present in statherin that would potentially release the highly charged and phosphorylated aspartate-serine rich statherin ASARM peptide. In recognition of the similarities Fisher et al coined the name SIBLING proteins (Small Integrin-Binding Ligand Interacting Glycoproteins) as a family name for this group of unique proteins⁷⁸.

Two key motifs of MEPE the ASARM motif and an RGD integrin binding motif (also known as dentonin or AC100) are highly conserved across species^{5, 9, 79}. The dentonin motif contains two cell-matrix adhesion sequences (RGD and SGDG) and the PFSSGDGQ is almost immutable (Figure 10). Dentonin or AC100 has potent anabolic activity on osteoblast and odontoblasts pulp precursor cells *in vitro* and these effects require the induction of COX-2⁸⁰⁻⁸⁷. The ASARM motif is not only conserved in MEPE but also in other

SIBLINGs and Figure 11 illustrates as an alignment both the MEPE and DMP1 ASARM regions for different species. Figure 7 shows the position of the ASARM motif across the human SIBLINGs as calculated using IANview software and alignment algorithms. Of note several conserved serines within the ASARM motif are substrates for casein kinase. As shown in Figure 11 a block of serines is invariant not only in MEPE but also in the DMP1 ASARM motif. This is further illustrated in Figure 12 that shows a comparison of DMP1 and MEPE ASARM motifs from two representative species (rabbit and human). The casein kinase serine residues that are substrates for phosphorylation are indicated in the figure. These phosphoserines are not only of key importance for interactions with hydroxyapatite but also for binding and hydrolysis with PHEX in both DMP1 and MEPE^{9, 31, 32, 34, 37, 47, 88–90}. Free ASARM peptide is a potent mineralization inhibitor in bone and teeth and suppresses renal calcification (*in vivo* and *in vitro*)^{31, 32, 34, 35, 37, 39, 41, 47, 89, 91, 92}. The GD domains as illustrated in both Figure 11 and Figure 12 are “*invariant*” for DMP1 and MEPE across all species and play a key role in the kinetics of PHEX binding and hydrolysis. The ASARM motif in DMP1 is capped by 35 residues and is also highly conserved in DMP1 across species. This region is labeled as the minfostin motif since a frame shift mutation that alters this region (DMP1 minfostin-motif) results in Autosomal Recessive Hypophosphatemic Rickets (ARHR)^{51, 52, 93}. The DMP1 minfostin region is thus required to foster or promote mineralization^{31, 42}. Also, this frame shift mutation results in the loss of the GD residues that are conserved in both MEPE and DMP1 and play a key role in the binding kinetics and hydrolysis of PHEX (Figure 11 & 12)^{31, 32}.

Another feature of the ASARM region in MEPE is the high degree of conservation of cathepsin K and cathepsin B protease sites that are N-terminal to and juxtaposed to the ASARM motif (Figure 13). Release of the ASARM motif by cysteine proteases as a protease resistant ASARM peptide likely plays a major role in mineralization and likely energy metabolism^{31, 32, 34, 36, 37, 39, 40, 42}. The biological relevance of these cleavage sites is discussed in more detail in the following sections.

IV. ASARM peptides and renal calcification

Since MEPE ASARM peptides are mineralization inhibitors^{30–32, 35, 37, 41, 89} their presence in urine^{35, 42, 94} may well help suppress renal calcification. Indeed, transgenic mice over expressing MEPE (MEPE tgn) are resistant to diet induced renal calcification³⁵, Figure 14. In these mice, urinary Ca X PO₄ product correlates positively with urinary ASARM peptides and this is accompanied by a suppressed dietary renal-calcification. Also, in normal mice, urinary ASARM peptides are significantly higher in mice fed high phosphate diets⁴². Intriguingly, null-mutant Na-dependent phosphate co-transporter (NPT2a^{-/-}) mice^{95–97} are hypophosphatemic with increased 1,25(OH)₂-Vit-D₃ and massive renal-stones⁹⁶. Secondary ablation of the 1- α -hydroxylase gene resulting in a double null-mutant (NPT2a^{-/-}/1- α -^{-/-}) corrects the renal-stones defect⁹⁸. Since MEPE expression (protein and mRNA) is suppressed by 1,25(OH)₂-Vit-D₃^{35, 41, 42, 69} the increased renal-stones in the NPT2a^{-/-} mice may well have been precipitated by an increased urinary CaPO₄ product and exacerbated by low urinary MEPE ASARM peptides. Further studies are required to confirm this.

MEPE also has a significant effect on neovascularization and likely mediates this by interacting with α v β 3 integrin via the RGD-motif³⁵. Specifically, renal-bone angiogenesis and VEGF secretion is increased in transgenic mice over expressing MEPE (MEPE trg)³⁵. Coincident with the increased vascularization, urinary aldosterone excretion is increased with hypokalemia and normal urinary output. Aldosterone exerts powerful effects on blood vessels, independent of blood pressure rise mediated via regulation of salt and water

balance⁹⁹. Specifically, aldosterone increases neovascularization through an angiotensin II dependent pathway with commensurate increase in VEGF expression¹⁰⁰. Also, aldosterone inhibits vascular reactivity and tone as calculated by intravital video-microscopic measurement of vessel diameter¹⁰¹. Of further relevance, activation of the Renin-Angiotensin-Aldosterone System (RAAS) in mice markedly influences osteoclastogenesis also independent of hypertension¹⁰². Indeed, angiotensinogen II (AngII), a component of the RAAS system increases aldosterone expression that stimulates RANKL expression in osteoblasts¹⁰³. The changes in MEPE tgn renal vascularization may also in combination with the factors discussed earlier contribute to the reduced susceptibility to renal calcification. Of relevance, recent studies confirm that in chronic kidney disease and mineralization bone disorders (CKD-MBD) there is cross talk between the renin-angiotensin-aldosterone system (RAAS) and the Vitamin-D, FGF23 klotho pathway¹⁰⁴.

V. The ASARM Model, bone-renal mineralization and phosphate homeostasis

It is clear PHEX regulates (directly or indirectly) FGF23 expression and or stability since loss of PHEX activity leads to increased FGF23 expression⁶⁵. Loss of DMP1 (SIBLING protein) has the same effect as loss of PHEX function; notably, increased FGF23 expression and an autosomal recessive form of hypophosphatemic rickets (ARHR)^{51, 52}. Both XLH (PHEX defect)²⁴ and ARHR (DMP1 defect)^{51, 52} have increased ASARM peptides in circulation, bone and teeth where they inhibit mineralization and play a component part in the hypophosphatemia^{31, 32, 38, 39, 42, 77, 105}. Thus, inactivation of PHEX or DMP1 could “logically” either inactivate or activate a “mineralization inhibitor and Pi-regulator”. In this regard, the experimental evidence suggests a PHEX-DMP1 interaction is responsible for locally orchestrating mineralization and phosphate homeostasis. Figure 15A, B & C provides schemes illustrating component parts of the ASARM model that incorporates this fact. The following text is labeled in alphabetic sequence and contains a detailed and evidence based description for each of the corresponding labels in the diagrams:

1. Fig 15A: ASARM displacement of the PHEX-DMP1-integrin complex regulates FGF23 expression.

As illustrated in Figure 15A, PHEX, DMP1 and cell surface $\alpha v \beta 3$ integrin when bound are proposed to “co-activate” a pathway that leads to suppression of FGF23 expression and or decreased FGF23 stability^{10, 31, 42, 106}. Also, competitive displacement of DMP1 by ASARM peptide modulates the PHEX-DMP1 mediated FGF23 expression and this may play an important role in energy metabolism and vascularization and contribute to the cross talk between bone and energy metabolism⁴². In overview, the experimental data supports the following global hypothesis: (1). PHEX binding to DMP1 via the DMP1-ASARM motif leads to decreased active FGF23. (2). ASARM peptide competitive displacement of DMP1-PHEX increases FGF23 activity. (3). ASARM-peptide-PHEX interactions further modulate fat mass and bone-renal vascularization. (4) ASARM peptides influence glucose & insulin-metabolism (Pi & vitamin D dependent). The bold and highlighted letters (**A** to **I**) in Figure 15A sequentially highlight the key points of the pathway as discussed in the following text: (**A**); PHEX (a Zn metalloendopeptidase) is proposed to interact with DMP1 by binding to the DMP1-ASARM motif adjacent to and *N-terminal* to the DMP1 ‘*minfostin*’ motif⁴². The DMP1 minfostin-motif³¹ refers to a region that when mutated results in Autosomal Recessive Hypophosphatemic Rickets (ARHR)^{51, 52, 93} and is thus required to foster or promote mineralization (Figure 11 & 12)^{31, 42}. Note that a DMP1 frame shift mutation (Figure 11) results in the loss of the GD residues that are conserved

in both MEPE and DMP1 and play a key role in the binding kinetics and hydrolysis of PHEX to the ASARM region^{31, 32}. Recent experiments further support the notion that FGF23 is regulated through a PHEX-DMP1 common pathway involving FGF receptor (FGFR) signaling¹⁰⁶. This was done by comparing phenotypes of compound and single mutant DMP1 and PHEX mice. **(B)** DMP1 also contains an RGD motif that interacts with $\alpha v \beta 3$ integrin and stimulates phosphorylation of focal adhesion kinase (FAK) leading to downstream activation of the MAPK pathway^{107, 108}. **(C)**; The PHEX-DMP1-Integrin cell-surface complex may be involved in suppressing FGF23 expression and possibly increases FGF23 protein degradation through 7B2 co activation of SPC2 proprotein-convertase as proposed by DreZner et al^{109, 110} (discussed further in Figure 15C). Thus, in XLH and ARHR, mutations in PHEX and DMP1 respectively result in hypophosphatemia through increased FGF23 expression and stability. There is precedent for this since PHEX binds with high affinity and specificity to ASARM peptides (MEPE & osteopontin derived) and MEPE protein^{31, 32, 34, 37, 39}. PHEX also cleaves ASARM peptides the only known physiological substrate for PHEX^{31, 32, 34, 36, 37} and SIBLINGs (like DMP1) activate PHEX related Zn matrix metalloproteinases (MMP's) by direct binding interactions that also involve cell-surface integrins^{107, 111–115}. DMP1 for example binds and activates a PHEX related Zn Matrix metalloproteinase 9 (MMP9)^{112, 114} and signals through cell surface interactions with $\alpha v \beta 3$ integrin in human mesenchymal cells and osteoblast like cells^{107, 108}. Also, as discussed earlier, DSPP (a SIBLING protein) is cleaved into three proteins, an N-terminal Dentin Sialoprotein (DSP)^{70, 71}, a COOH terminal portion Dentin Phosphoprotein (DPP)^{72–74}, and Dentin Glycoprotein (Dgp)⁷⁵. Like DMP1, The DPP protein fragment of DSPP contains a SIBLING RGD-motif and a COOH terminal ASARM motif. In DPP, the ASARM motif is repeated multiple times (Figure 7). Of relevance to this motif-sequence and alignment-similarity, recent elegant experiments confirm that DPP (like DMP1) binds to cell surface integrins via the RGD-motif and activates integrin-mediated anchorage dependent signals in undifferentiated mesenchymal cells and dental cells^{107, 108, 116}. The cell surface binding generates intracellular signals that are channeled along cytoskeletal filaments and activates the non-receptor tyrosine kinase FAK, which plays a key role in signaling at sites of cellular adhesion¹¹⁶. This is the same signaling pathway activated by DMP1 cell surface integrin binding in human mesenchymal cells and osteoblast like cells^{107, 108}. Since DMP1 and DSPP both contain RGD and ASARM motifs and PHEX is expressed in the cell lines investigated in these studies^{31, 117–119}, this also supports a cell surface PHEX-DMP1-Integrin axis for signal transduction through a Focal Adhesion Kinase (FAK) and downstream MAPK pathways, namely ERK and JNK^{107, 108, 116}. Moreover, the *in vitro* addition of recombinant DMP1 to UMR-106 cells causes a dose-dependent decrease in FGF23 expression (mRNA and protein)¹²⁰ and FGF23 upregulates DMP1 expression in MLO-Y4 cells (a well established murine osteocyte cell line) in the presence of Klotho.

Of note, one group attributes “dual” functionality to DMP1. Specifically, they propose DMP1 is both a “nuclear” transcriptional co-activator and also acts as an extracellular matrix orchestrator of mineralization¹²¹. However, as indicated earlier, recent research suggests DMP1 and DPP both signal on the cell surface through integrin interactions^{107, 108, 116}. Also, a more recent report that used DMP null mice (DMP1-KO) found no rescue of DMP1-KO mice by the targeted re-expression of artificially localized nuclear DMP1 (nlsDMP1) in osteoblast-osteocytes¹²². The authors in agreement with previous studies^{107, 108, 116} concluded that DMP1 is not a “nuclear co-transcription factor” but an extracellular matrix

protein¹²². Clearly, DMP1 may have dual functions (nuclear and extracellular) and the extracellular signaling appears well established (consistent with the ASARM model).

(D); Specific cleavage of MEPE and/or other bone SIBLING-proteins (DSPP, OPN, DMP1 etc.,) generate free protease resistant ASARM peptides^{31, 38, 39, 42, 77, 105}. In XLH, MEPE, and several bone-proteases including cathepsins, ECEL1/DINE and NEP are markedly increased^{31, 38, 123–125} resulting in excess ASARM peptide production and inhibition of mineralization^{31, 37, 38, 77, 105}. **(E)**; Increased ASARM peptides are proposed to competitively displace the DMP1-PHEX complex in “normal mice” by forming a high affinity/specificity PHEX-ASARM complex^{31, 32, 36} that is slowly hydrolyzed (low K_{cat}/K_m) by PHEX^{34, 36, 37}. Loss of PHEX and DMP1 in XLH and ARHR respectively results in “ASARM-independent” constitutive over expression and increased stability of FGF23. **(F)**; Competitive displacement of DMP1 by ASARM peptide(s) in normal mice results in increased FGF23 expression. This is supported by *in vivo* and *in vitro* murine experiments using ASARM peptides with bolus, osmotic-pump infusion, perfusion, renal/intestinal micropuncture, *ex vivo* cell culture experiments with transgenic FGF23 Green-Fluorescence-Protein promoter reporters, and transgenic mice models^{30, 31, 41–45}. Additional compelling support for this hypothesis comes from *in vitro* observations that show MEPE/PHEX mRNA and protein ratios are excellent indicators of mineralization progression¹²⁶. Specifically, the MEPE/PHEX ratio is low when osteoblasts are actively differentiating to the mineralization stage and high when the mineralization stage is reached. At the late mineralization stage the osteocyte is thus presumed to release ASARM peptide in order to maintain a hypomineralized space around the osteocytic lacuna via a “MEPE-ASARM-peptide-BMP2” pathway^{59, 126–129}. Of relevance, the SIBLING protein osteopontin is expressed at high levels along osteocyte lacunae and canaliculi within a structure known as the lamina limitans¹³⁰. This suggests the osteopontin ASARM motif may also play a role. There is compelling evidence that ASARM peptides from MEPE and DMP1 play key roles in regulating the osteocyte-mediated mechanotransductive response to load, bone formation and osteoclastogenesis^{31, 35, 58–61, 63, 80, 126, 129, 131–133}. **(G)**; Increased FGF23 results in decreased serum 1,25(OH)₂-Vit-D₃ by the well documented alteration of renal 1- α -hydroxylase and 24-hydroxylase expression and activities¹³⁴. **(H)**; 1,25(OH)₂-Vit-D₃ regulates several proteases and protease inhibitors in different cell types including bone^{135–138}. Cystatins for example are strong inhibitors of the cathepsin protease family (cathepsin B, D and K for example) and 1,25(OH)₂-Vit-D₃ is a potent stimulator of cystatin expression¹³⁵. Also, cystatin C stimulates differentiation of mouse osteoblastic cells, bone formation and mineralization *in vitro* and *ex vivo*, consistent with a suppression of cathepsin mediated release of ASARM peptide¹³⁸. Of note, cathepsin D activity is increased in XLH^{38, 123–125} and cathepsin D inactivates cystatins¹³⁹ and activates cathepsin B¹⁴⁰. This in turn also contributes to increased proteolytic release of protease resistant ASARM peptides^{10, 35, 38, 42, 77}. As depicted schematically in Figure 13 the cathepsin B and K regions N-terminal to and adjacent to the ASARM motif are highly conserved^{10, 31, 32, 38, 41}. Recent phylogenetic analyses of MEPE confirm that this region is also under positive selection^{5, 79}. **(I)**; Thus, FGF23 suppression of 1,25(OH)₂-Vit-D₃ in diseases with increased FGF23 levels may be partly responsible for the markedly increased levels of osteoblastic proteases in XLH and ADHR^{31, 35, 38, 123, 124, 141}. **(D)**; In turn, the increased osteoblastic-protease activity is likely responsible for the increased proteolytic release of protease-resistant ASARM peptides from SIBLING proteins including MEPE and

DMP1^{10, 31, 32, 38, 39, 42, 77, 105}. Of note, classic experiments show treatment of XLH and ARHR with phosphate supplements does not correct the endosteal mineralization defect but partially corrects the growth defect^{142–144}. Co-supplementation with 1,25(OH)₂-Vit-D₃ is required to impact the mineralization defect but is still not completely satisfactory since this then results in increased FGF23 production (vicious cycle)¹⁴⁴. The partial correction of the mineralization defect by 1,25(OH)₂-Vit-D₃ supplementation is thus consistent with the ASARM model. This is because the 1,25(OH)₂-Vit-D₃ dietary supplements may help reduce free ASARM peptide production by inhibiting extracellular matrix proteases (cathepsins etc) and suppressing MEPE expression. Also, *in vivo* administration of cathepsin protease inhibitors pepstatin and CAO74 partially corrects the mineralization defect in XLH mice³⁸. This sequence of events leads to a coordinated feedback loop involving 1,25(OH)₂-Vit-D₃, PHEX, DMP1, FGF23 and ASARM peptides as illustrated in further detail in Figure 15B.

2. Fig 15B: ASARM & bone mineral renal phosphate regulation through FGF23 and 1,25(OH)₂-Vit-D₃.

The physiological loop described in Figure 15A also impacts bone mineralization and renal phosphate regulation as illustrated in Figure 15B. (A); specifically, accumulation of ASARM peptides inhibits mineralization and (B); coordinately inhibits Na⁺ dependent phosphate uptake in the kidney as shown *in vitro* and *in vivo*^{32, 35, 41–45, 145}. The pathophysiological ASARM-pathway inhibits renal phosphate uptake independent of FGF23 and exacerbates the FGF23 induced hypophosphatemia found in familial XLH, ARHR and ADHR^{10, 42}. ASARM peptides are acidic, phosphorylated, highly-charged, with low pI's and are extraordinarily resistant to a wide range of proteases (see references^{10, 32, 38}; also unpublished observations) and have physicochemical similarities to bisphosphonates, phosphonoformic acid (PFA) and phosphonoacetic acid (PAA). Also, they share biological properties *in-vivo* and *in-vitro* with bisphosphonates, PFA and PAA in that they all inhibit mineralization and interfere with renal phosphate handling and vitamin D metabolism^{31, 35, 41–45, 146–158}. Thus in this abnormal context, ASARM peptides likely play a direct but component part in the hypophosphatemic pathology^{31, 32, 35, 37–39, 41–46, 77}. Specifically, in XLH and ARHR, FGF23 is the chief hypophosphatemic stimulus and ASARM peptides exacerbate the renal phosphate leak by virtue of their over-abundance and intrinsic, hypophosphatemic, physicochemical properties^{32, 41–46}. In further support of the secondary ASARM inhibition of phosphate uptake in the renal proximal tubules of the kidney, Baum et al 2004 observed phosphatonin washout in XLH mice renal proximal tubules¹⁵⁹. In these elegant experiments evidence for a secondary posttranscriptional defect in XLH mice that complemented the down regulation Na dependent phosphate co-transporters (NPT2a) mRNA expression was provided. The authors concluded that a secondary posttranscriptional mechanism regulates renal phosphate uptake but were unable to define the factor(s) involved. Specifically, these workers observed a temporal normalization of the phosphate transport defect in XLH proximal tubule cells perfused *in vitro* (independent of protein synthesis) that was consistent with phosphatonin washout. Similar findings were reported independently by researchers using immortalized XLH renal proximal cell lines^{160, 161}. Thus, ASARM peptides may also inhibit phosphate transport by directly binding to the phosphate transporter as demonstrated with PFA and etidronate^{147, 148, 152–155, 158, 162, 163} and in turn exacerbating the effects of FGF23 on NPT2 transcription⁴². (C,D,E) as shown, free ASARM peptides competitively displace the DMP1, PHEX and integrin complex. It should be noted

that inorganic pyrophosphate (PP_i) also plays a coordinate physiological role in skeletal mineralization. An excellent review of this pathway in disease and health is presented by White 2010¹⁶⁴. **(F,G,H,I)**; This results in up regulation of FGF23 expression down regulation of 1,25(OH)₂-Vit-D₃ and inhibition of renal phosphate uptake or hypophosphatemia^{30–32, 41, 42, 69}. **(J)** The decrease in 1,25(OH)₂-Vit-D₃ also increases expression of a 100 kDa transcription factor (100kDa-TRP) that is required for PHEX expression^{165, 166}. **(K)**; This results in increased PHEX expression that fulfills a classic feedback loop involving FGF23, DMP1, PHEX and 1,25(OH)₂-Vit-D₃. Specifically increased PHEX favors increased hydrolysis of ASARM peptides and increased PHEX-DMP1 binding. This leads to reduced FGF23 expression and increased 1,25(OH)₂-Vit-D₃. Increased 1,25(OH)₂-Vit-D₃ results in reduced PHEX expression and decreased PHEX-DMP1 binding that leads to increased FGF23 and suppression of 1,25(OH)₂-Vit-D₃ synthesis. The reduced 1,25(OH)₂-Vit-D₃ results in increased protease and MEPE expression and thus increased ASARM peptide production and this feeds back to reduce FGF23 expression by modulating DMP1 processing, FGF23 stability and possibly directly suppressing FGF23 expression via 1,25(OH)₂-Vit-D₃ as illustrated in Figure 15C.

3. Fig 15C: The ASARM pathway and processing of BMP1 and DMP1 by SPC2 convertase and 7B2.

The ASARM pathway is also regulated by the processing of DMP1 by BMP1 and BMP1 is in turn processed by 7B2 activated SPC2 proprotein-convertase^{167, 168}. FGF23 is also proposed to be processed by 7B2 activated SPC2 as illustrated in Figure 15C^{109, 110}. **(A,B,C,D)**; As discussed earlier competitive displacement of the DMP1-PHEX-Integrin complex results in increased expression of FGF23. **(E)**; recent exciting developments indicate that the PHEX DMP1 axis may also coordinately regulate expression of 7B2^{109, 110}. **(F)** 7B2 protein is a co activator of SPC2 proprotein-convertase (SPC2) and in XLH mice there is constitutively decreased 7B2 expression, presumably due to loss of PHEX. Cell surface PHEX is required for normal 7B2 expression and this is likely co-activated by the PHEX-DMP1-integrin complex. In XLH, absence of cell surface PHEX results in constitutive over expression of FGF23 since the PHEX-DMP1-integrin complex is required to suppress FGF23 and activate 7B2. Thus in XLH and ADHR, loss or suppression of 7B2 leads to increased stability of FGF23 (reduced 7B2 and thus SPC2 proprotein-convertase activation)^{109, 110}. In XLH mice there is therefore increased FGF23 expression and stability and consequential bone-renal pathology. In normal mice the 7B2-PHEX-DMP1-FGF23 axis coordinates a feedback loop to exquisitely regulate bone-renal metabolism. **(E)**; Specifically, in normal mice the PHEX-DMP1-Integrin complex results in increased expression of 7B2 and decreased expression of FGF23. **(F)** Protein 7B2 co-activates SPC2 that in turn cleaves and inactivates FGF23. SPC2 activation also cleaves a protease “pro-BMP1” (also called tolloid and a member of the astacin Zn metalloendopeptidase family) that in turn cleaves pro-DMP1 into an active (57 kDa) and inactive form (37 kDa)^{167, 168}. Of relevance, elegant experiments by Feng et al show conclusively the 57 kDa BMP1 cleaved form of DMP1 is indeed the active form. Specifically, transgenic overexpression and reintroduction of 57 kDa DMP1 in DMP1 null mice almost completely corrects the bone renal phenotype¹³³. **(K)** The activated 57 kDa DMP1 then binds to PHEX and integrin further suppressing FGF23 expression. This is coordinated in turn by an increase in 1,25(OH)₂-Vit-D₃ consequential to the reduced FGF23. The regulatory loop is closed by the recent finding that BMP2 strongly stimulates MEPE expression¹²⁶. Thus, the competitive

displacement of DMP1 by ASARM peptides provides an additional “bone” fine tuning of FGF23 and bone renal phosphate mineralization.

VI. PHEX, DMP1, ASARM regulates FGF23 and modulates energy metabolism

Recent seminal discoveries have revealed an integrated relationship between bone and energy metabolism^{66, 169}. Leptin, an adipocyte hormone regulating appetite influences osteoblast function and bone formation peripherally and centrally^{169, 170}. In turn, osteocalcin a bone hormone produced by osteoblasts influences energy metabolism^{169–171}. Recent studies show serotonin plays a major role in regulating bone remodeling through contrasting pathways^{169, 170}. These pathways originate from the duodenum (via LRP5) the brain (via Leptin) and bone (LRP5)¹⁶⁹. Of note, the duodenal serotonin pathway is currently controversial^{172–176}. Osteoblasts and osteocytes play key roles in glucose homeostasis and mediate insulin signaling in pancreatic β -cells, hepatocytes and adipocytes^{177, 178}. The surface area of the lacuno-canalicular Haversian complex is vast, containing large numbers of osteocytes. Relevant to this, osteocytic expression of FGF23, DMP1 and MEPE is high (mRNA and protein)^{35, 59, 61, 126, 179}. Thus, this new microcosmic endocrine-system undoubtedly plays a major role in disease and health. Several studies confirm over expression or infusion of ASARM peptides or MEPE results in major changes in fat mass, energy metabolism, vascularization and soft tissue metastatic calcification (renal)^{35, 42, 180}. Also, these changes are PHEX, phosphate and vitamin D dependent. These findings are consistent with several independent studies that show XLH mice are hypoinsulinemic, hyperglycemic with increased gluconeogenesis in bone, liver and kidney^{160, 161, 181–188}. The reverse is the case for FGF23 null mice (hypoglycemic)¹⁸⁹. Klotho, an FGF23 co-activator or vitamin-D likely play a direct indirect role^{189–193}. Indeed, earlier seminal studies by Dr. Louis Avioli’s laboratory showed the XLH osteoblast has a markedly higher rate of gluconeogenesis¹⁸⁶. These elegant studies not only confirmed osteoblasts are capable of glucose production but also clearly showed the XLH osteoblast has decreased intracellular pH. The acidic intracellular milieu was proposed to be the chief reason for the increased gluconeogenesis but the cause for the decreased pH was unknown. Our studies show increased acidic ASARM peptide production (pH 3 in solution), is chiefly responsible for the altered pH and increased gluconeogenesis. Also, acidic ASARM peptides impact osteocalcin expression^{35, 42, 180} and may activate osteocalcin by acidic γ -de-carboxylation (Figure 16). Osteocalcin is a key osteoblast derived protein that links bone and energy metabolism by regulating insulin secretion, insulin sensitivity and energy expenditure (Figure 16)⁶⁶. The changes in gluconeogenesis in XLH, Vitamin D receptor null-mice (VDR $^{-/-}$) and FGF23 null mice occur in bone^{184–186}, liver¹⁸⁷ and kidney^{160, 161, 181–184, 188}. The kidney is almost of equal importance to the liver in glucose homeostasis supplying >40% of glucose output^{194, 195}. Of relevance, specific expression of MEPE and acidic ASARM peptides occurs in the proximal convoluted tubules and acidosis also increases renal and bone gluconeogenesis^{35, 42, 77, 94, 186, 195}. Extraosseous infusion, bolus or micropuncture administration of MEPE or MEPE derived ASARM peptides *in vivo* and *in vitro* induces hypophosphatemia and inhibits intestinal and renal Na-dependent phosphate co-transport^{41–46}. Also, hypophosphatemic rodents or humans develop hyperglycemia with reduced insulin secretion and sensitivity^{196–198}. Moreover, our data confirms this accompanies an increase in circulating ASARM peptides in mice^{35, 42}. Consistent with this, increased Glucose-6-Phosphatase activity (a gluconeogenic enzyme) occurs in rats fed with Pi-deficient diets^{199, 200}. These Pi diet-restricted animals are also hypophosphatemic, hypoinsulinemic, and hyperglycemic (35% increase)^{199, 200}. Furthermore, clinical hypophosphatemia is associated with impaired glucose tolerance and insulin resistance^{197, 198}. Also, in type 2 diabetes patients subjected to a 4 hour euglycemic-

hyperinsulinemic clamp show major increases in FGF23 that correlated positively with insulin infusion²⁰¹.

Mice over expressing MEPE, ASARM peptides or infused ASARM peptides using osmotic pumps provide further support for a SIBLING, FGF23, PHEX energy metabolism link^{21, 35, 42}. Intriguingly, as well as changes in mineralization, bone formation and phosphate regulation, these mice develop increased fat mass, leptin, adiponectin, osteocalcin and sympathetic tone. The mice are also hyperglycemic with changes in circulating insulin, serotonin, VEGF and urinary aldosterone. These changes are more profound in mutant MEPE and ASARM over expressing transgenic mice (MEPE-trg and ASARM-trg) or ASARM infused mice fed a low Pi and vitamin D3 diet. Figure 16 highlights the areas impacted by changes in DMP1, PHEX, ASARM and FGF23. Notably the key hormones from adipocytes, osteoblasts, kidney (adrenal glands) and liver are altered as indicated in the scheme. We previously reported an increase in urinary aldosterone and VEGF for our MEPE transgenic mice and showed this accompanied increased vascularization in bone and kidney³⁵. Of note, aldosterone suppresses vascular glucose-6-phosphate-dehydrogenase activity (glycolysis) and this leads to reduced vascular reactivity¹⁰¹. Also, through the renin-aldosterone-angiotensin pathway (RAAS), aldosterone increases osteoclastogenesis by increasing RANKL production^{102, 103}. Consistent with this, we have shown aldosterone is increased in MEPE tgn mice with increased osteoclast progenitor cells³⁵. Of relevance, recent studies confirm that in chronic kidney disease there is cross talk between the renin-angiotensin-aldosterone system (RAAS) and the Vitamin-D, FGF23 klotho pathway¹⁰⁴.

Summary

The skeletal Haversian system or osteon is the functional and architectural unit of bone and an exquisitely designed micro-endochondral organ. This complex system is maintained by specialized bone cells (osteocytes) that communicate through dendrites occupying intricately designed channels that compenetrates the hard calcium-phosphate hydroxyapatite matrix. In terrestrial vertebrates this living lacuno-canalicular complex is vast and is the repository for signaling molecules that orchestrate bone formation, mineralization, bone-renal phosphate homeostasis and energy metabolism. The teleosts or boney fish do not have osteocytes or a Haversian system and so the emergence of this elaborate system coincided with a colonization of the land by the terrestrial invertebrates approximately 300 million years ago. The primary cell of this system the “osteocyte” expresses key bone-matrix proteins that communicate with other cells from organs such as the gut, brain, kidney, liver, bone and pancreas. The past decade has revealed a new group of important *osteocyte-expressed* proteins (FGF23, DMP1, PHEX, MEPE) and processed forms (ASARM peptides for example) involved in the internuncial integration and regulation of bone turnover, bone-renal mineralization, bone-teeth mechanical loading, renal mineral-homeostasis, mineralization and vascularization. Ovocleidin-116, an avian protein homolog of the osteocyte expressed protein MEPE occurs in the eggshell and bones of the chicken. This protein contains an ASARM motif and is ancestral to the SIBLING family of proteins (DMP1, MEPE etc). The properties of the ASARM motif and free peptide likely played a key role in adapting the new terrestrial phyla to gravity and the dry land. Recent research indicates the ASARM peptide regulates FGF23 expression by competitive displacement of a cell surface PHEX-DMP1-integrin interaction. This has important implications for the study of bone-renal, mineral and energy metabolism in disease and health.

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Abbreviations

PHEX	PHosphate-regulating gene with homologies to Endopeptidases on the X chromosome
MEPE	Matrix Extracellular Phosphoglycoprotein
DMP1	dentin matrix protein 1
ASARM	Acidic Serine Aspartate Rich MEPE associated motif
XLH	X-linked hypophosphatemic rickets (also abbreviated as HYP in some publications)
ARHR	Autosomal Recessive Hypophosphatemic Rickets
ADHR	Autosomal Dominant Hypophosphatemic Rickets
FGF-23	fibroblast growth factor 23
OPG	osteoprotegerin
RANKL	Receptor Activator for Nuclear Factor κ B Ligand
OPN	osteopontin
BSP	bone sialoprotein
DSPP	Dentinsialophosphoprotein
DPP	Dentin Phosphoprotein
DSP	Dentin Sialo Protein
Dgp	Dentin Glyco Protein
FEP	fractional excretion of phosphate
WT	wild type mice
NPT2a, b,c	sodium dependent phosphate co-transporters
1,25(OH)₂-Vit-D3	1,25-dihydroxycholecalciferol
PTH	parathyroid hormone 1–34
OCN	osteocalcin
CKD-MBD	Chronic Kidney Disease and Mineralization Bone Disorders
ESRD	End Stage Renal Disease
BMSC	Bone Marrow Stromal Cells
TIO	Tumor Induced Osteomalacia, also known as OHO
SIBLING	Small Integrin-Binding Ligand Interacting Glycoproteins
RAAS	Renin-Angiotensin-Aldosterone System
VEGF	Vascular Endothelial Growth Factor
BMD	Bone Mineral Density
BMC	Bone Mineral Content
SPR4	Small-synthetic PHEX Related Peptide 4
NCPs	Noncollagenous Proteins
Ovocleidin-116	Avian eggshell and bone matrix SIBLING-protein

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Evolution

Phyla/Class	Timeline million years	PHEX	KLOTHO	FGF23	SIBLINGs
Unicellular eukaryotes	2000				
Round worms	600	PHEX	Klotho		
Marine chordates	550				
Insects	400				
Boney Fish	500			FGF23	
Amphibia	360				
Terrestrial vertebrates	300				SIBLINGs

Figure 1.

Scheme showing the temporal emergence and evolution of the key players involved in regulating vertebrate bone-mineral homeostasis. PHEX and KLOTHO appeared approximately 600 million years ago and were followed by the appearance of FGF23 in teleosts or boney fish during the Triassic 500 million years ago. KLOTHO is a co-activator that is required to convert FGF receptor (FGFr) specificity to FGF23 signaling in the kidney. FGF23 is predominantly expressed in bone osteocytes in terrestrial vertebrates but teleost fish do not have osteocytes. It is likely that KLOTHO first diversified functionally in teleost fish. Specifically, FGF23 was co-opted with KLOTHO to regulate mineral phosphate homeostasis in the marine-kidney approximately 500 million years ago. SIBLINGs appeared later and may well have played a role in fashioning the eggshell as exemplified by the modern avian eggshell and bone protein ovocleidin-166. Ovocleidin-116 is an avian homolog of MEPE. The SIBLING proteins (in particular DMP1 and MEPE) may have in turn sequestered FGF23 300 million years ago to extend signaling across a bone-renal axis. This was an adaptive response to the conquest of the dry terrestrial environment and the increased gravitational burden as discussed in the text.

X-linked Hypophosphatemic Rickets (HYP)

Figure 2.

Pictures showing the first human XLH patient (TK11)²⁴ discovered with a PHEX mutation and for comparison a murine homolog of XLH. This patient contained a deletion of approximately 55 kb that helped narrow the search down to a specific and defined chromosomal region in Xp22.1²⁴. The young boy has the classic skeletal deformities of XLH that are caused by softening of the bones (osteomalacia) and induced by hyperosteoridosis or severe under mineralization. In children, the large amount of uncalcified osteoid at the epiphyses of growing bone results in a weight bearing induced dramatic bowing of the long bones (genu varum) that is accompanied by curvatures of the spine (kyphosis or scoliosis). Other deformities include a protruding square forehead (caput quadratum) with frontal bossing and a flattening of the posterior skull. A pigeon chest is also common and XLH patients have severe dental problems. The loss of both phosphate and calcium in the skeleton eventually results in a destruction of the supportive matrix and in adults the bones become soft and wax-like with tremendous flexibility. There is also hypophosphatemia due to defective renal phosphate handling and a passive loss of phosphate into the renal glomerular filtrate. This is quantified as a severely lowered transfer maximum of phosphate per unit volume of glomerular filtrate (TMPO₄/GFR). Circulating 1,25(OH)₂-Vit-D₃ is inappropriately normal with increased FGF23, MEPE, ASARM peptides and serum alkaline phosphatase.

Figure 3.

Co-alignment of the key protein features of the M13 and MA clan of Zn metalloendopeptidase that includes PHEX, the enzyme defective in XLH. The prototypic member of this group is neprilysin (also known as CD10, gp100, NEP and chronic acute lymphoblastic leukaemia antigen [CALLA])^{10, 28}. PHEX and the M13 family are type II glycoproteins with short transmembrane domains, distinctive Zn binding motifs that form a pentacoordinated complex with the zinc atom, multiple conserved cysteines that play a major tertiary structure role and a large number of exons (22 for PHEX)¹⁰.

Figure 4.

Scheme showing the key PHEX amino acid residues involved in the binding and hydrolysis of ASARM-substrate with corresponding locations²². Also shown are the key ASARM residues including the scissile bond and their topographic alignments with the PHEX substrate site as deduced from mutation analyses for PHEX and related Zn metalloproteinases plus studies with ASARM peptide substrate^{10, 22, 31, 32, 34, 36, 37}. Hydrolysis of the substrate occurs through formation of a penta-coordinated complex of the metal that includes the three amino acids of the peptidase, the oxygen of the scissile bond and a water molecule bound to the Zn²⁺ atom²⁸. The three zinc-coordinating ligands characteristic of the Zn²⁺ metalloproteinase groups occur in PHEX at positions His580, His584 and Glu642 as highlighted in the figure. An additional Glu residue at position 581 has a role in catalysis by polarizing a water molecule. An important residue shown to be essential for stabilization of the transition state in thermolysin, and confirmed by site-directed mutagenesis is His711²⁰². This residue is conserved in the M13 family and MA clan zinc metalloproteinases including PHEX, NEP, KELL, ECE1 and NL1. Arginine at 747 of PHEX corresponding to Arg747 of NEP is important for correct 'lock and key' alignment. Of note, in PHEX a serine (Ser100) is substituted for an arginine (Arg102) at position 100 that is present in NEP and ECE1²². This residue is located at the edge of the active site and is responsible for the dipeptidyl-carboxypeptidase activity of NEP as observed in the release of Phe-Leu from Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu)^{203, 204}. This would indicate a lack of PHEX terminal dipeptidyl-carboxypeptidase activity and is consistent with the specific PHEX ASARM cleavage patterns reported^{34, 36, 37}.

Figure 5.

Scheme that shows a virtual 3D X-ray crystallographic structural model of PHEX and the PHEX ASARM-substrate/ligand binding site. The 3D structure was rendered (for this review) using known M13 Zn metalloendopeptidase X-ray crystallographic data and the Protein Homology/analogy Recognition Engine V.20 or Phyre2 server to model domains³³. A total of three M13 family Zn metalloendopeptidase templates (neprilysin [NEP], endothelin converting enzyme 1 [ECE-1] and mycobacterium tuberculosis zinc metalloprotease 1 [Zmp1]) were selected to model human PHEX based on heuristics to maximize confidence, percentage identity and alignment coverage. More than 91% of the residues were modeled at >90% confidence. All the key PHEX residue positions shown in figure 4 and previously reported as important for PHEX ligand and substrate specificity are consistent with the 3D model and the topographic substrate/ligand representation shown in the figure. Note, PHEX contains a short intracellular domain, a transmembrane domain and a large extracellular domain. Thus, PHEX is anchored to the plasma membrane and the active binding site is extracellular. Also, there is a flexible region that attaches the membrane bound PHEX to the globular extracellular domain. This region may introduce flexibility and allow PHEX to bind and conform topographically to a DMP1 and $\alpha v \beta 3$ integrin signaling complex on the cell surface. All reported PHEX mutations result in protein trafficking defects or loss of endopeptidase activity^{22, 205–207}. Further support for the extracellular cell surface function of PHEX comes from the finding that PHEX mutations that do not affect catalytic activity result in intracellular retention of the protein²⁰⁵.

Figure 6.

Scheme showing the chromosomal locations of MEPE, SIBLING proteins and other genes for reference (Rowe et al 2000)⁹. All the SIBLINGs including MEPE map to the long arm of chromosome 4 (4q12) between markers D4S1534 and D4S3381 in humans and chromosome 5 in mice. Codes are translated as follows: (1) BMP3, bone morphogenetic protein 3; (2) DSPP, dentin sialo phospho protein; (3) OPN, osteopontin; (4) DMP-1, dentin matrix protein 1; (5) ANX, annexin; (6) BSP, integrin binding sialo protein/bone sialoprotein II; (7) BMPR1B, bone morphogenetic receptor protein 1B; 8, MEPE, matrix extracellular phosphoglycoprotein; (9) Statherin, salivary statherin; (10) ENAM, Enamelin. Human ASARM sequence is shown above the scheme with conserved casein kinase serine-phosphorylation sites. See also Figures 6 and 7 for illustrations of the casein kinase ASARM-conservation across species for DMP1 and MEPE.

Figure 7.

Scheme illustrating the sequence alignments and positions of the ASARM peptide in MEPE, DMP1, DSPP, Osteopontin (OPN) and Statherin⁹. The sequence similarity analysis was carried out using “sim” and “lalview” mathematical and software tools^{208–210}. In each computation the gap open penalty was set to 12, and the gap extension penalty was 4. Comparison matrix was set to BLOSUM62 with similarity a configured score of 70%. The highlighted and colored blocks shown on each protein scheme represent sequence percentage homologies that are color indexed on the similarity scale at the top of the figure. Note that in MEPE versus DSPP (B), there are several repeated ASARM homology blocks that extend across the COOH terminal Dentin phosphoprotein (DPP) region of DSPP. This region also contains a single integrin binding RGD motif. The MEPE and DMP1 ASARM motif sequence (DDSESSDSGSSSES DGD) is shown in Figures 6, 11, 12 and 13. For other SIBLING ASARM sequences see Rowe et al 2000⁹ and also recent publications^{34, 37, 47}.

Figure 8.

Signal Peptide analysis prediction using Signal peptide-NN software confirming that all SIBLING proteins have strong signal-peptide motifs and are therefore secreted extracellular matrix proteins. Analysis was conducted using the SignalP 4.0 software²¹¹ and server at <http://www.cbs.dtu.dk/services/SignalP/>. Three scores (C, S and Y) are provided as shown in the scheme. The red line shows the C-score or “cleavage site” score. The C-score is calculated for each sequence position. In all Siblings (MEPE, DMP1, DSPP, OPN, BSP and Statherin) a highly significant cleavage score (>0.8) was reported for the same sequence position. Y-max is a derivative of the C-score combined with the S-score resulting in a better cleavage site prediction than the raw C-score alone (blue peak). This is due to the fact that multiple high-peaking C-scores can be found in one sequence, where only one is the true cleavage site. The cleavage site is assigned from the Y-score where the slope of the S-score is steep and a significant C-score is found. The S-mean is the average of the S-score, ranging from the N-terminal amino acid to the amino acid assigned with the highest Y-max score, thus the S-mean score is calculated for the length of the predicted signal peptide. The calculated S-mean score for all SIBLINGs indicates strongly that all the SIBLINGs are secretory proteins.

Secondary structure MEPE:

Figure 9.

Secondary structure prediction for MEPE as calculated using GCG peptide structure software^{9, 212} (see also Accelrys computer platform software details at <http://accelrys.com/products/>); The primary amino acid backbone is shown as a central line with curves indicating regions of predicted turn. Regions of hydrophilicity and hydrophobicity are represented as ellipsoids (red) and diamonds (blue) respectively. The RGD motif is highlighted with a pentagon. The N-glycosylation sites are represented as blue ellipsoids on stalks (C-terminus) and an alpha helix is indicated by undulating regions on the primary backbone. The signal peptide is indicated by a checkered box and coincides with a hydrophobic region at the N-terminus. The COOH-terminal ASARM motif is highlighted and the PHEX binding region is indicated.

Alignment Name: alignment shorte.aaa_bsm1
Length: 23

	10										20												
Human MEPE RGD	T	D	L	Q	E	R	G	D	N	D	I	S	P	F	S	G	D	G	Q	P	F	K	D
Chimpanzee MEPE RGD	T	D	L	Q	E	R	G	D	N	D	I	S	P	F	S	G	D	G	Q	P	F	K	D
Gorilla MEPE RGD	T	D	L	Q	E	R	G	D	N	D	I	S	P	F	S	G	D	G	Q	P	F	K	D
Orangutan MEPE RGD	T	D	L	Q	E	R	G	D	N	D	I	S	P	F	S	G	D	G	Q	P	F	K	D
Gibbon MEPE RGD	T	D	L	Q	E	R	G	D	N	D	M	S	P	F	S	G	D	G	Q	P	F	K	D
Macaque MEPE RGD	T	D	L	Q	E	R	G	D	N	D	M	S	P	F	S	G	D	G	Q	P	F	K	D
Baboon MEPE RGD	T	D	L	Q	E	R	G	D	N	D	M	S	P	F	S	G	D	G	Q	P	F	K	D
Marmoset MEPE RGD	T	D	V	Q	E	R	G	D	N	D	I	S	P	F	S	G	D	G	Q	P	F	K	D
Bush Baby MEPE RGD	S	D	L	Q	G	R	G	D	N	D	I	S	S	F	S	G	D	G	Q	P	F	Q	D
Mouse Lemur MEPE RGD	A	D	L	Q	E	R	G	D	N	D	I	S	S	F	S	G	D	G	Q	P	F	E	D
Tree Shrew MEPE RGD	T	D	L	Q	E	R	G	D	N	A	F	P	P	F	S	G	D	G	Q	P	F	K	D
Pika MEPE RGD	T	D	L	Q	G	R	G	D	N	D	I	I	A	F	S	G	D	G	Q	P	F	K	D
Rabbit MEPE RGD	-	-	-	T	G	R	G	D	N	D	I	T	P	F	S	G	D	G	Q	P	S	K	D
Squirrel MEPE RGD	T	D	L	Q	E	R	E	D	N	D	I	F	P	F	S	G	D	G	Q	P	S	K	G
Guinea Pig MEPE RGD	T	G	A	Q	G	R	G	D	N	D	V	S	P	F	S	G	D	G	Q	P	F	Q	D
Rat MEPE RGD	P	D	L	L	V	R	G	D	N	D	V	P	P	F	S	G	D	G	Q	H	F	M	H
Mouse MEPE RGD	P	D	L	L	V	R	G	D	N	D	V	P	P	F	S	G	D	G	Q	H	F	M	H
Hedgehog MEPE RGD	S	A	P	E	G	R	G	D	-	D	T	S	P	F	S	G	D	G	Q	L	G	E	-
Cat MEPE RGD	P	D	L	Q	G	R	G	D	N	D	I	S	P	F	S	G	D	G	Q	P	F	K	D
Dog MEPE RGD	P	D	L	Q	E	R	G	D	N	D	I	S	P	F	S	G	D	G	Q	P	F	K	D
Horse MEPE RGD	P	D	L	Q	E	R	G	D	N	D	I	S	P	F	S	G	D	G	Q	P	F	K	D
Vicugna MEPE RGD	P	D	L	Q	G	R	G	D	N	D	I	G	P	F	S	G	D	G	P	P	F	K	D
Cow MEPE RGD	P	D	L	Q	G	R	G	D	N	D	L	S	P	F	S	G	D	G	P	P	F	K	D
Pig MEPE RGD	P	D	L	Q	G	R	G	D	N	D	I	S	P	F	S	G	D	G	P	P	F	K	D
Armadillo MEPE RGD	T	D	L	Q	A	R	G	D	N	N	I	S	P	F	S	G	D	G	Q	P	F	E	D
Sloth MEPE RGD	T	E	L	Q	E	R	G	D	N	N	I	S	P	F	S	G	D	G	Q	P	F	E	D
Tenrec MEPE RGD	P	H	L	R	Q	R	G	D	D	D	V	G	P	F	S	G	D	G	Q	P	S	E	D
Elephant MEPE RGD	T	N	L	Q	E	R	G	D	N	D	I	S	P	F	S	G	D	G	Q	P	F	E	D
Hyrax MEPE RGD	T	D	L	E	E	R	G	D	N	G	I	F	P	F	S	G	D	G	Q	P	F	E	D
Dolphin MEPE RGD	P	D	L	Q	G	Q	E	D	S	-	S	S	P	F	S	G	D	G	P	P	F	K	D
Microbat MEPE RGD	P	D	L	Q	E	R	G	-	Y	S	M	S	P	F	S	G	D	G	Q	P	F	K	D
Megabat MEPE RGD	P	D	L	Q	E	R	G	S	N	N	M	S	P	F	S	G	D	G	Q	P	F	K	D
Kangaroo Rat MEPE RGD	I	D	P	Q	E	T	G	D	N	D	L	S	P	F	S	G	D	G	Q	L	F	K	D
	X	d	l	q	e	r	g	d	n	d	i	s	p	F	S	G	D	G	q	p	f	k	d

Figure 10.

The MEPE RGD integrin binding region (dentonin or AC100) is highly conserved across species as illustrated in the clustalW alignment. There is complete conservation of a sequence (FSGDG) N-terminal to the RGD region and the consensus sequence RGDNDISPFSGDGQ is highly conserved. Species sequences for MEPE were searched for and downloaded from the “Ensembl” project resource at <http://www.ensembl.org>. The dentonin/AC100 peptide is a strong stimulator of bone/teeth formation *in vitro* and *in vivo*^{80, 81, 83–86, 213–215}. This contrasts with the ASARM peptide, an inhibitor of mineralization *in vitro* and *in vivo* (minhibin)^{31, 32, 34, 35, 37–39, 41, 47, 89, 91, 92}.

Figure 11.

DMP1 ASARM region (COOH residues 464 to 478) shows strong homology to MEPE ASARM peptide (across species) and the free ASARM peptide likely competes for PHEX binding⁴². Flanking this region is a sequence also conserved in DMP1 (minfostin motif) and mutations here result in ARHR^{51, 93}. Since MEPE and osteopontin ASARM peptides bind specifically to PHEX^{31, 32, 34, 36, 37, 42}, we would propose the DMP1 ASARM motif also interacts with PHEX protein. Thus, free ASARM peptides likely compete with PHEX for DMP1 binding as described in Figure 15. The ASARM region (DMP1 and MEPE) consists of serine residues interspersed with acidic aspartate (D) and glutamic acid (E) residues. The serine residues are phosphorylated and the SXSSSE(S/D) conserved sequence of DMP1 and ASARM contains identical consensus casein-kinase II phosphorylation sites (see also Figure 12). Of relevance, a highly conserved GD sequence occurs in both MEPE-ASARM peptide and DMP1-ASARM motif as highlighted in the alignment. The GD sequence resides in the DMP1 minfostin region and likely plays an important role in PHEX binding. A DMP1 frameshift mutation in this minfostin region that alters the COOH-terminal sequence at position 498 following residues LTVDA (with loss of the GD domain and an increase of 13 residues) results in ARHR^{11, 12}. The species alignments (top to bottom) for DMP1 are; Human, Chimpanzee, Gibbon, Macaque, Cat, Elephant, Tarsier, Cow, Dolphin, Tenrec, Kangaroo, Rabbit, Squirrel, Mouse, Rat, Hedgehog, and Vicugna. The species alignments for MEPE (top to bottom) are; Human, Chimpanzee, Orangutan, Gorilla, Gibbon, Macaque, Rhesus Monkey, Marmoset, Cow, Tree Shrew, Sloth, Alpaca, Rock Hyrax, Tarsier, Elephant, Rabbit, Squirrel, Ground Squirrel, Cat, Dog, Dolphin, Pig, Microbat, Hedgehog, Kangaroo, Rat and mouse. The above species sequences for MEPE and DMP1 were

searched for and downloaded from the “Ensembl” project resource at <http://www.ensembl.org>.

Figure 12.

DMP1 ASARM region (COOH residues 464 to 478) shows strong homology to MEPE ASARM peptide (across species) and the free ASARM peptide likely competes for PHEX binding⁴². The ASARM region in both DMP1 and MEPE also contain casein kinase II serine phosphorylation sites as depicted in the scheme. These phosphoserines sites are highly conserved between DMP1, MEPE and across species. The clustalW alignment compares DMP1 and MEPE and two representative species (human and rabbit).

Figure 13.

Cleavage sites for cysteine proteases (Cathepsin K and B) that are N-terminal and in close proximity to the MEPE ASARM motif are highly conserved as shown in the alignment. The ASARM motif and peptide sequence is resistant to proteases (except for PHEX). The ASARM peptide is the only known physiological substrate for PHEX. Species sequences for MEPE were searched for and downloaded from the “Ensembl” project resource at <http://www.ensembl.org>.

Figure 14.

Urinary ASARM peptides suppress diet induced renal calcification in transgenic mice over expressing MEPE protein³⁵. Three-dimensional microtomographs (μCT) comparing wild type (WT) and MEPE transgenic (MEPE-TGN) mice fed high phosphate diets. There is a significant reduction in renal calcification in MEPE-TGN mice compared to WT mice as shown graphically and visually.

Fig 15A

Fig 15B

Fig 15C

Figure 15.

Figure 15A. ASARM displacement of the PHEX-DMP1-integrin complex regulates FGF23 expression (see text for detailed discussion of the scheme). The highlighted and encircled letters in the diagram link to the discussion in section-1 titled “Fig 9A: ASARM displacement of the PHEX-DMP1-integrin complex regulates FGF23 expression”.

Figure 15B. ASARM peptide regulation of bone mineral and renal phosphate regulation through FGF23 and 1,25(OH)₂-Vit-D₃ (see text for detailed discussion of the scheme). The highlighted and encircled letters in the diagram link to the discussion in section-2 titled “Fig 9B: ASARM & bone mineral renal phosphate regulation through FGF23 and 1,25(OH)₂-Vit-D₃”.

Figure 15C. (9C); The ASARM pathway and the processing of BMP1 and DMP1 by SPC2 convertase and its co-activator 7B2 (see text for detailed discussion of the scheme). The highlighted and encircled letters in the diagram link to the discussion in section-3 titled “Fig 9C: The ASARM pathway and processing of BMP1 and DMP1 by SPC2 convertase and 7B2.”

Figure 16.

Competitive displacement of DMP1 by ASARM peptide modulates PHEX-DMP1 mediated FGF23 expression (see central osteoblast in green). Specifically, DMP1 and PHEX interact and signal a down regulation of FGF23 as discussed in the text. ASARM disrupts this binding (PHEX + DMP1) resulting in an up-regulation of FGF23 signaling as discussed in figure 9. This in turn leads to major changes in bone mineralization, bone turnover, vitamin D metabolism with hypophosphatemia. Recent evidence indicates this also impacts fat energy metabolism pathways as depicted in the scheme and discussed in the text. Profound changes in fat mass, weight, glucose metabolism, insulin sensitivity, leptin levels, serotonin levels, sympathetic tone, aldosterone levels, vascularization, and sympathetic tone, occur in mice with defects in PHEX, DMP1, FGF23, MEPE and ASARM expression^{35, 42, 183, 186–190, 192, 197, 199, 216–219}. **Index:** SNS, sympathetic nervous system; **ADRβ2**, Osteoblast β2 adrenergic receptors (responsive to epinephrine/norepinephrine); **Gla-OCN**, γ-carboxylated osteocalcin (inactive form); **Unc-OCN**, γ-de-carboxylated osteocalcin (active form); **Esp**, Gene for OST-PTP (tyrosine phosphatase) that phosphorylates and inactivates the insulin receptor. This directly/indirectly results in reduced active osteocalcin (reduced γ-de-carboxylation); **Tc1r1**, Osteoclast proton (H⁺) pump. Increases resorption lacuna acidity (acidic ASARM-peptides likely contribute) and thereby increases active osteocalcin (Unc-OCN) by acidic γ-de-carboxylation; **ASARM**, Acidic Serine Aspartate Rich MEPE associated peptide or motif.