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Editorial Review

Dietary phosphate modifies lifespan in *Drosophila*

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Abstract
Phosphate is required for many important cellular processes and having too little phosphate (hypophosphatemia) or too much (hyperphosphatemia) can cause disease and reduce lifespan in humans. *Drosophila melanogaster* has been a powerful tool to discover evolutionarily well-conserved nutrient-sensing pathways that are important for the lifespan extension. We have established *Drosophila* as a model system for studying the effects of dietary phosphate during development and adult life. When absorption of phosphate is blocked by sevelamer or cellular uptake is inhibited by phosphonoformic acid (PFA), larval development is delayed in a phosphate-dependent fashion. Conversely, restriction of phosphate absorption with sevelamer or reduced cellular uptake after treatment with PFA is able to extend the adult lifespan of otherwise normal flies. Gaining an understanding of the specific pathways and mediators that regulate cellular and organismic phosphate levels might ultimately lead to the development of improved dietary and therapeutic approaches to the treatment of human disorders of hypo- and hyperphosphatemia.

Keywords: CKD; drosophila; hyperphosphatemia; lifespan; phosphate metabolism

Introduction

Disturbances of phosphate homeostasis can result in serious human disorders [1]. The clinical consequences of severe hypophosphatemia, which for example are seen under conditions of malnutrition or tumor-induced hypophosphatemia include hemolysis, skeletal muscle myopathy, cardiomyopathy, neuropathy and osteomalacia and, in some cases, contribute to death [2]. On the other hand, hyperphosphatemia leads to soft tissue calcifications and metabolic changes, which to date are poorly understood. Hyperphosphatemia is encountered most frequently in patients with chronic kidney disease (CKD), which currently affects 20 million Americans, and the serum phosphate level is an important predictor of mortality [3–5]. Also non-dialysis-dependent CKD stages 2–3 may already go along with 'compensated hyperphosphatemia'. This, in turn, may cause maladaptive endocrine changes such as elevation of fibroblast growth factor 23 (*FGF23*), suppression of 1-α-hydroxylation in the kidney and development of secondary hyperparathyroidism [6]. The outcomes in the at-risk population can be improved by dietary phosphate restriction or treatment with phosphate binders such as sevelamer (Renagel™), which reduce the level of phosphate absorption from the diet. Hyperphosphatemia is also seen in familial hyperphosphatemic tumoral calcinosis, a human disorder that was recently attributed to loss-of-function mutations in the genes encoding *FGF23*, *UDP-GalNAc transferase* 3 (*GALNT3)* and *Klotho* (*KL*) [7]. Furthermore, mouse models of hyperphosphatemia due to loss-of-function mutations in *Fgf23*, *Kl* or *Galnt3* die prematurely on a standard diet [8–10]. Similar to the benefit seen with dietary phosphate restriction in humans with CKD, this early lethality can be rescued and their lifespans extended when fed a phosphate-restricted chow [8, 9].

Cellular and endocrine effects of phosphate in multicellular organisms

How hyperphosphatemia reduces lifespan is largely unstudied. Inorganic phosphate is required for cellular functions such as DNA/RNA and membrane phospholipid synthesis, generation of high-energy phosphate esters and intracellular signaling [11]. Many enzymes of key metabolic pathways are regulated by phosphate; these pathways include those for anaerobic glycolysis, gluconeogenesis, mitochondrial metabolism, glutamine, purine and nucleic acid metabolism. Although most studies were performed with purified enzymes *in vitro*, some enzymes respond to phosphate concentrations that could reasonably be expected intracellularly in the organs/cells from which they were isolated (Kd = 1–10 mM), and thus these enzymes may also be regulated by intracellular phosphate *in vivo* [11], and hyperphosphatemia may lead to dysregulation of these processes. In addition to the metabolic changes, phosphate appears to activate distinct nutrient-sensing pathways. These are best understood in unicellular organisms like bacteria and yeast.

Yeast uses high affinity phosphate transporters [*Pho84* (yeast H^+-coupled Pi transporter) and *Pho89* (yeast Na^+-coupled Pi transporter)] to take up phosphate from the environment, which then represses an intracellular signaling cascade, composed of *Pho81* (yeast cyclin-dependent kinase (CDK) inhibitor), *Pho80* (yeast cyclin)/*85* (yeast CDK) and *Pho4* (yeast basic helix-loop-helix transcription factor). When yeast cells are starved of phosphate, the phosphate-dependent multiple ankyrin-repeat-containing CDK inhibitor *Pho81* inactivates *Pho80/Pho85*. As a consequence, unphosphorylated *Pho4* enters the nucleus and binds to a phosphate response element [12] in genes belonging to the yeast Pho-regulon [13]. As a result, *Pho4* activates the transcription of *Pho84, Pho89* and secreted acid phosphatases, which permit yeast cells to better assimilate phosphate from the surroundings [14]. Although it is possible that a similar signaling cascade mediates phosphate effects in multicellular organisms, database searches based on amino acid sequence homology alone failed to identify metazoan orthologs. First progress was made over the past decade, when several investigators discovered activation of MAPK by inorganic phosphate at physiological concentrations between 5–10 mM, which was subsequently demonstrated in multiple mammalian cell lines including MC3T3 mouse fibroblast cells [15], chondrogenic ATDC5 cells, MC3T3-E1 osteoblasts and ST2 murine bone marrow stromal cells [16], HEK293 human proximal tubular cells [17] and lung alveolar cells [18]. Although some cell lines, for example C2C12 or L929 cells, are less responsive than others [16], activation of MAPK by phosphate appears to be quite universal and evolutionarily conserved in invertebrates including in *Drosophila* [19]. Recent evidence suggests that the type III sodium–phosphate co-transporter, *SLC20A1*/*Pit1*, mediates cellular effects of phosphate in mammalian cells. Furthermore, addition of phosphonoformic acid (PFA), a competitive antagonist of phosphate transporters and cellular phosphate uptake [20], as well as siRNA-mediated knockdown of the type III transporter *Pit1,* blocks the activation of MAPK by phosphate in HEK293 cells [17]. *SLC20A1/Pit1* is related to yeast *Pho89* and thus this entry point in the phosphate-signaling pathway appears to be conserved from yeast to mammals. Targeted deletion, hypomorphic and over-expression murine mutants of *Slc20a1*/*Pit1* support a role for this transporter in liver growth and phosphate homeostasis [21–23]; however, surprisingly, *Pit1*-null mice showed normal embryonic and fetal development. Haploinsufficiency of the related phosphate transporter *SLC20A2/PIT2* leads to basal ganglial calcifications in humans without affecting mineral homeostasis [24]. Using a comparative genomics approach, we recently showed that orthologs of yeast *Pho84* mediate activation of ERK/MAPK in *Drosophila* cell lines [19]. Thus, multiple membrane transporters and transporter families may be involved in cellular phosphate uptake and intracellular phosphate may be what is sensed in metazoan species. However, it is also possible that phosphate binds and signals extracellularly. Using cell lines expressing a Pi-transport-deficient *Pit1* transporter, Salaun *et al*. for example reported that *Pit1* may have transport-independent effects on cell proliferation and tumor growth *in vitro* and *in vivo*, although it remains to be shown whether these effects depend on phosphate binding to *Pit1* [25].

Furthermore, it is currently unknown, whether endocrine regulation by phosphate depends on the MAPK-pathway, since humans with cancer syndromes caused by activating mutations in this pathway, and mutant mice with similar genetic changes, generally have normal serum parathyroid hormone (*PTH*), *FGF23*, 1,25-dihydroxy vitamin D ($1,25(OH)_2D$) and phosphate levels [26]. Furthermore, when assayed using a luciferase reporter, treatment with $1,25(OH)_2D$, lithium, an activator of wnt-signaling, or *FGF2* can stimulate *FGF23* promotor activity in ROS17/2.8 rat osteosarcoma cells, whereas phosphate or pyrophosphate cannot [27]. Stimulation by *FGF2* is blocked by PD173074, an inhibitor of FGFR tyrosine kinase activity [27]. *In vivo* administration of the MAPK inhibitor PD0325901 to *Phex*-null mice blocks *FGF23* signaling in the kidney and reverses the biochemical consequences of *FGF23* excess in these mice, but was unable to decrease circulating *FGF23* levels [28]. At least at the level of gene-expression activation of MAPK by phosphate, therefore, it does not appear to have a role in the endocrine regulation of *FGF23*. Likewise, stimulation of *PTH* secretion by chronic hyperphosphatemia is thought to occur mostly indirectly by lowering extracellular calcium [29], and *FGF23*-effects on the parathyroids [30, 31], while hypophosphatemia suppresses *PTH* secretion indirectly by up-regulation of $1,25(OH)_2D$. In addition to these indirect effects, phosphate may directly act on the parathyroids to stimulate *PTH* synthesis through post-transcriptional mechanisms [32, 33], which have been elucidated in recent years, involving a balanced interaction of the *PTH* mRNA with the stabilizing proteins *AUF1* (AU-rich element binding protein, isoforms p37, p40, p42 and p45) and *CSDE1* (cold-shock domain containing 1, the fly ortholog of *Unr* (upstream of N-ras)) and the destabilizing protein *KSRP* (K-homology splicing regulator protein) *in vivo* [29]. The upstream sensing mechanism and how phosphate suppresses generation of 1,25-dihydroxycholecalciferol ($1,25(OH)_2D$) from inactive precursors in the proximal tubules of the kidney by 1-α-hydroxylation are completely unknown [34].

Inorganic phosphate is stored as a linear polymer in intracellular vacuoles comprising ten to many hundred phosphate residues (polyphosphate, poly P); these are linked by the same phosphoanhydride bonds found in ATP. Poly P was originally discovered in bacteria and yeast, in which it can serve both as a phosphate reservoir and as an energy source in different biological processes, where it substitutes for ATP [35]. The bacterial poly P kinase gene, *PPK*, which is involved in poly P synthesis, appears to be under the control of the *PhoR* (*E. coli* sensory histidine kinase)/*PhoB* (*E. coli* DNA-binding response regulator) two-component bacterial phosphate-signaling complex [36]. Conversely, poly P is able to regulate the expression of bacterial phosphate transporters such as *PitA* expression, and furthermore, *PPK, PstS,A,B,C and PitA* are able to compensate for each other to regulate cellular phosphate uptake [37]. Similarly in yeast, Poly P is synthesized and accumulated in the vacuole and represents a phosphate reserve used during periods of phosphate starvation [38], but it also strongly

influences the expression of *Pho5*, the main secreted acid phosphatase [39] by which yeast mobilizes phosphate from extracellular sources. Little is known about poly P in animal cells beyond its widespread abundance in tissues and subcellular compartments [40]. Poly P, at the concentrations of 0.15–1.5 μM found in mammalian cells [41] and with poly P chain lengths ranging from 15 to 750 phosphate residues, appears to stimulate *mTOR* (mammalian target of rapamycin), a ser–thr protein kinase that is at the center of signaling pathways in cell growth and proliferation and is activated by autophosphorylation [42]. *mTOR* function is controlled by nutrient availability to ensure that protein synthesis is repressed when the supply of precursors, for example amino acids, is insufficient. The key downstream targets of *mTOR* are *eukaryotic initiation factor 4E-binding protein* (*eIF4E-BP*), and the kinase for the 40S ribosomal S6 protein (*S6K*) [43]. *eIF4E-BP* is inhibited by *mTOR*, while *S6K* is activated [44]. Via activation of *mTOR* poly P thus stimulates global protein translation. Extracellular poly P also appears to enhance the mitogenic activities of acidic *FGF1* and basic *FGF2* in human fibroblasts by functioning as co-activator similar to heparin [45].

In mammals, circulating inorganic phosphate, in addition to serving to maintain intracellular phosphate levels for cell metabolism and growth, serves to support extracellular mineralization (complexes of phosphate with calcium), which appears to furthermore depend on the concerted expression of collagen type 1 and tissue-nonspecific alkaline phosphatase [46]. To control mineralization and cellular delivery, extracellular phosphate levels and total body phosphate content are tightly regulated by a number of hormones, including *PTH*, 1,25-dihydroxy vitamin D, the active form of vitamin D ($1,25(OH)_2D$), and *FGF23* and serum phosphate feeds back to regulate these factors in an endocrine fashion [1]: high phosphate increases the secretion of *PTH* and *FGF23*, while low phosphate stimulates the synthesis of $1,25(OH)_2D$. Hypophosphatemia leads to demineralization of the skeleton (osteomalacia), whereas hyperphosphatemia is an important risk factor for the development of vascular calcifications [47]. Sodium–phosphate bowel preps (e.g. Fleet enema™), presumably as a result of increased renal phosphate excretion, can lead to nephrocalcinosis and irreversible renal failure [48]. Maladaptive endocrine responses to hyperphosphatemia in CKD can cause secondary hyperparathyroidism and metabolic bone disease [49]. Furthermore, increased circulating *FGF23* levels may lead to left ventricular cardiac hypertrophy in a calcineurin-pathway-dependent, *KL*-independent fashion [50]. Finally, recent studies showed that down-regulation of vascular *KL* and the resulting *FGF23*-resistance may contribute to vascular calcification [51].

Nutrient-sensing and lifespan

The observation that dietary phosphate is critically important for cellular processes, while it in excess appears to reduce lifespan, bears resemblance to the effects of dietary composition on lifespan. There furthermore appears to be cross-talk between the phosphate and nutrient-sensing pathways for protein and glucose. For example, vascular calcifications are worsened in a uremic rat model by reducing the dietary content of protein [52] and in diabetic individuals with CKD [53]. Furthermore, deletion of one copy of 'insulin response substrate *1* (*IRS1*)' in mice deficient in *KL* is able to rescue survival despite persistent hyperphosphatemia [54]. On a molecular level, studies in yeast show that phosphate via *Pho81* may regulate the activity of the protein kinase *Rim15*, which plays a central role in the integration of phosphate, glucose, nitrogen and amino acid availability during the yeast cell cycle [55]. In higher species, *TOR/S6K* may furthermore be regulated by poly P and integrate phosphate with glucose and nitrogen-sensing pathways as discussed above. Finally, phosphate stimulates apoptosis, which requires changes of the mitochondrial membrane potential and activation of caspase-9 [56, 57]. Conversely, inhibition of phosphate uptake or phosphate restriction prevents apoptosis [58].

Dietary restriction (DR) reduces the activity of various signal transduction pathways which are highly conserved from yeast to mammals [59–61] with some exceptions: the role of *TOR* and *S6K* in promoting aging appears to be conserved in yeast, worms, flies and mice. By contrast, the 'adenylate cyclase (AC)–protein kinase A (PKA)' pathways promote aging in yeast and mammals, whereas an 'insulin/IGF-1-like receptor' or the upstream 'growth hormone (GH)' (mammals) accelerates aging in worms, flies and mice. Similar transcription factors (*Gis1, Msn2/4*, *Daf-16* and *FOXO*) activated by these pathways affect cellular protection and/or aging in all the major model organisms. Notably, in the multicellular worms, flies and mice, these genes may promote aging within the cells in which they are expressed but also in other cells through the regulation of circulating factors. The mechanisms proposed for the longevity extension caused by inhibition of these nutrient signaling pathways include a decrease in the free radical superoxide (mediated in part by superoxide dismutases) and of its damage to macromolecules, protection of proteins by chaperones (heat-shock protein 70, *Hsp70*), decreased translation, the activation of autophagy and the switch to hypoxia-associated gene-expression patterns (in yeast and mice) [59–61]. Modulation of these nutrient-signaling pathways is able to regulate adult physiology and lifespan independent of DR, and pharmacological interventions that alter the flow of information through these pathways could, in principle, be used to mimic the effects of DR and extend a healthy lifespan in humans [60]. In yeast, for example, reduced activities of the *Tor-Sch9* and *Ras-AC-PKA* pathways, the serine–threonine kinase *Rim15* and of the transcription factors *Gis1, Msn2/4* can extend lifespan independent of nutrient availability. Although in both worms and flies, deletion of *Daf-2/FOXO* shortens lifespan as would be expected, the animal continues to respond to DR. In flies, activation of the transsulfuration pathway (TSP), a phylogentically ancient metabolic process that involves a network of enzymes responsible for the metabolism of sulfur-containing amino acids, was recently shown to be necessary and sufficient to mediate effects of DR, although it remains unclear, whether the rate-limiting enzyme 'cystathionine β-synthase (dCBS)' is downstream of and controlled by

other nutrient-sensing pathways. Flux through the TSP is known to affect overall cellular metabolism by directly influencing cysteine and methionine levels. Methionine availability affects protein synthesis and methylation, and it has been implicated in murine aging [62]. In mice, the longevity effects of DR appear to furthermore involve endocrine signals, for example reduced activity of the growth hormone receptor (GHR)/Insulin-like growth factor 1 (*IGF-1*) pathways, because DR does not extend further the lifespan of GHR-deficient mice. A better understanding of the phosphate-sensing pathway(s) and how it cross-talks with known nutrient-sensing pathways may based on these considerations have important implications for healthy lifespan in human individuals with impaired or normal renal function.

Using *Drosophila* to study dietary phosphate toxicity

Drosophila melanogaster has been a tool of discovery for entire signal transduction pathways based on similar phenotypes of genes targeted by genome-wide mutagenesis. Advances in RNAi technology have eliminated the need for subsequent mutation-mapping to identify genes of interest. Endocrine regulation may be simpler, for example flies only possess three FGF-like peptides, two FGF receptors and one *KL* in it's genome. However, virtually all signaling pathways identified in the fly were found to be highly conserved in humans and thus they were of immediate relevance to advance the understanding of human physiology. Finally, many human disease genes have fly orthologs and mutations in these genes cause similar phenotypes in flies and humans.

Drosophila is reared, or 'cultured' in polystyrene or glass vials, which contain a small amount of medium composed of 17 g/L yeast, 9.8 g/L soy flour, 71 g/L corn meal, 5.6 g/L agar, 5.6 g/L malt and 75 mL/L corn syrup in the bottom and a cotton plug to permit air exchange, which closes off the vial. To prevent fungal growth, 4 mL/L propionic acid and 250 mg/L tegosept (Spectrum M1187) are generally added to this standard medium (SM). Flies lay eggs into the medium, developing, larvae climb up the glass wall where they pupate. After 10 days at 25°C adults eclose (i.e. emerge from the pupal casing as adults), to complete the fly's life cycle. We previously demonstrated that activation of MAPK by phosphate is evolutionarily conserved in *Drosophila* [19] and furthermore show here that likewise the effects of dietary phosphate on larval development and the adult fly lifespan are similar to those seen in higher species. Culture of three commonly used control strains, *yw*, Canton S (CS) and Oregon R (OR), on SM or SM supplemented with 30 mM sodium phosphate (P30) supported larval development and eclosion of adult flies after 10 days at 25°C. To further investigate the effects of phosphate on larval development, we added PFA to block sodium–phosphate co-transporters and cellular uptake of phosphate [63]. Supplementation of the SM with 0.1 mM PFA did not result in developmental delay or lethality of *yw* control flies. However, developmental delay was noticeable in *yw* animals fed SM with 1 mM PFA and led to pupal lethality. Larvae reared on the SM with 10 mM PFA died at the first instar stage. The effect of 1 mM and 10 mM PFA was reversible, or partially rescued, respectively, when the medium was further supplemented with 30 mM sodium phosphate (Figure 1A and B).

To confirm phosphate dependence of larval development, we used sevelamer, an anion exchange resin developed to reduce dietary uptake of phosphate in individuals with CKD [64]. When *yw* larvae were reared on the SM supplemented with 0.1% sevelamer, we did not observe any effects on larval development. However, increasing the dose to 0.5% delayed emergence of instar 3 larvae from the medium, pupation and eclosion of adults by at least one day (Figure 1C). Similar to what we found for PFA, the effect of sevelamer could be rescued by supplementation with 30 mM sodium phosphate. A dose of 1% sevelamer resulted in a more severe developmental delay, and partial reversal by the further addition of 30 mM sodium phosphate was observed. Similar results were obtained with the two other control strains, CS and OR.

Dietary phosphate modifies circulating phosphate levels and lifespan in genetically modified mice [8–10] and humans with CKD [3–5, 10]. To see, whether phosphate has similar effects in *Drosophila*, we next asked, if dietary phosphate could modify adult lifespan. The median adult lifespan of male *yw* flies at 25°C was 42 ± 0.8, 38 ± 2.4 and 44 ± 0.8 days when adults were cultured on the SM, Pi30 or Si30, respectively (Figure 2). The findings were similar for females and for CS and OR males (not shown). The effects of phosphate were dose-dependent and no lifespan reduction was seen when the SM was supplemented with iso-osmolar concentrations of sodium sulfate (data not shown). Food consumption was similar for each supplement when tested using a modified dye-feeding assay [65] (data not shown). If increasing phosphate levels through dietary availability results in shortened lifespan, then conversely, reducing phosphate intake might extend lifespan. Consistent with this idea, supplementation of the SM with 1% sevelamer (Sev1) increased the median lifespan to 49 ± 1.9 days, an effect that was lost when we additionally supplemented SM with 30 mM Pi. Similarly, the adult lifespan was extended to 47 ± 1.8 days when 1 mM PFA1 was added to the SM but not when adults were cultured on the SM with both 1 mM PFA and 30 mM sodium phosphate. These effects of high and low dietary phosphates were confirmed using flies that are reared on Roberts' defined medium [66] (DM, Figure 2), a synthetic medium to which defined amounts of phosphate can be added.

Based on the above, dietary phosphate levels appear to be critically important during larval development, whereas in adults, excess dietary phosphate reduces lifespan. Next we asked whether increasing dietary phosphate results in an increase of fly hemolymph ('blood') phosphate levels and whether hemolymph phosphate may be a determinant of adult lifespan. When *yw* females are cultured on the SM, Sev1%, and Pi30 for 5 days, excretions directly reflect intake of phosphate: 8.1 ± 1.0, 1.4 ± 0.4 and 9.7 ± 1.6 ng/fly/60 min, respectively. However, the

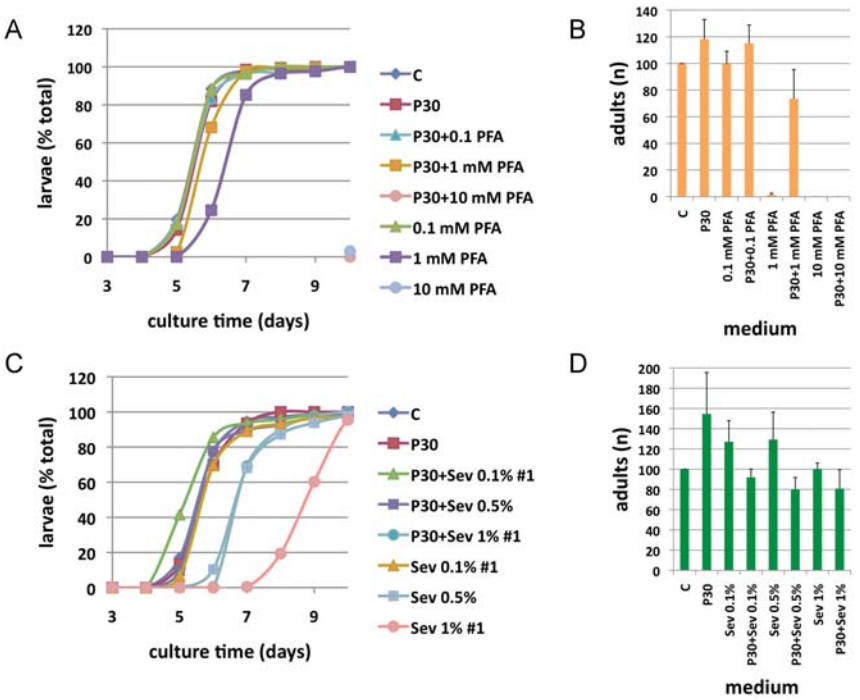

Fig. 1. The effect of sevelamer and PFA on larval development is reversible by addition of 30 mM phosphate to the medium. To quantify larval development on different media, we prepared culture vials with SM containing vehicle or supplements as described in the text. Care was taken to avoid variation in the fluid content between media. Twenty mated virgin females were cultured at 25°C for 48 h to permit laying of 150–200 eggs per vial. Subsequently, adults were removed and vials inspected daily to determine the number of animals that had emerged from the medium. (**A**, **C)** The number of larvae which emerged from the medium (% of total). P30 = SM supplemented with 30 mM sodium phosphate (pH 6.0), PFA = phosphonoformic acid 0.1–10 mM, Sev = sevelamer 0.1–1%; Means, CV<10%, (**B**, **D):** Number of adults eclosed are shown. Means ± SEM of one representative experiment using *yw* control flies are shown, performed in triplicate (modified after Supplementary Fig. S4 in [19]).

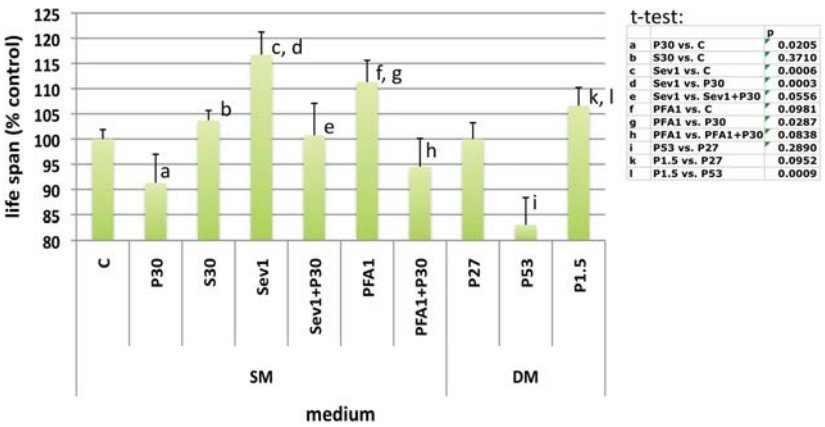

Fig. 2. Effect of phosphate supplementation or inhibitors of phosphate uptake on adult lifespan in flies with normal renal function. Lifespan was evaluated by culturing 30–40 young adult *yw* males on the SM or DM with different supplements. Vials were changed twice weekly and dead flies counted every other day. The median lifespan was calculated for each vial using Prism 5.0d (GraphPad Software, CA), averaged between multiple vials: Control (**C**, $n = 550$), 30 mM sodium phosphate (P30, $n = 465$), 30 mM sodium sulfate (S30, $n = 245$), 1% sevelamer (Sev1, $n = 207$), 1 mM PFA1 ($n = 130$) and the combinations: Sev1+P30 ($n = 115$), PFA1+P30 ($n = 137$), or Roberts' defined medium (DM) [66] supplemented with 1.5, 27 or 53 mM sodium phosphate (P1.5, $n = 202$; P27, $n = 200$; P53, $n = 194$). Gender-specific effects of dietary phosphate supplementation were ruled out in initial tests [67].

hemolymph phosphate concentration (19.6 ± 2.8, 21.3 ± 5.1 and 21.8 ± 1.0 mg/dL in SM, Sev1% and Pi30) and whole fly phosphate content (295.3 ± 3.0, 288.2 ± 20 and 287.9 ± 5.7 ng/fly in SM, Sev1% and Pi30) of these flies were indistinguishable across all culture conditions (Figure 3). Similar results were obtained for w^{1118}, CS and OR control strains.

These data suggest that homeostatic mechanisms exist in flies just as in higher species to keep circulating phosphate levels and body phosphate within the normal range.

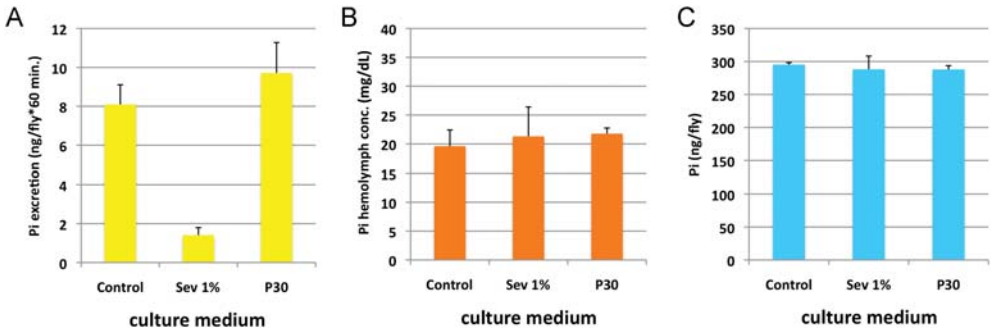

Fig. 3. Adult hemolymph Pi, Pi excretion and whole fly Pi. Thirty *yw* female flies per treatment were used within 2–3 days of eclosing and incubated at 29°C for 5 days to induce RNAi-mediated knockdown. On day 5, flies were transferred to a 1.5 mL Eppendorf vial, and excretions deposited on the vial wall were collected for exactly 60 min. Flies were removed from this vial and anesthetized with CO_2, heads were removed and fly bodies were centrifugated at 5000 rpm for 3 min. at 4°C in a 200 µL Eppendorf vial with a punctured bottom, allowing for the collection of the clear, cell-free hemolymph. Whole fly aqueous extracts were prepared directly in 100 µL ammonium molybdate phosphate assay reagent (Phospho Liqui-UV, Stanbio 0851-250), cleared and along with excretion and hemolymph samples assayed for phosphate concentration using a spectrophotometer at 340 nm. **(A)** excretion of phosphate after culture for 5 days on the SM (**C**) alone or supplemented with 1% sevelamer (Sev1%) and 30 mM sodium phosphate (P30) ($n = 3$ pooled collections of 15–20 flies ea.). (**B**) Hemolymph phosphate concentration ($n = 3$ pooled collections of 15 flies ea.) and (C) whole fly phosphate ($n = 10$) of flies cultured as described for A [67].

Furthermore, the data suggest that the toxic effects of dietary phosphate may be the result of this homeostatic response, rather than being mediated by elevated hemolymph phosphate. Interestingly, the fly phosphate hemolymph concentrations we observe are about 3-fold higher than serum phosphate concentrations in mice and about 5-fold higher than serum phosphate concentrations in humans. Thus, in *Drosophila*, phosphate in the hemolymph may serve as a readily accessible extracellular store, consistent with the fact that flies lack a skeleton, which in higher species is thought to be a source for rapid release of phosphate in times of starvation. It is also possible that mammals have more efficient mechanisms of phosphate uptake which are utilized to supply cellular processes, thus requiring a lower circulating phosphate concentration, or that higher hemolymph levels reflect higher intracellular phosphate stores in this species.

Conclusions

Evolutionarily well-conserved nutrient-sensing pathways are important for the lifespan extension seen during DR, and modulation of these nutrient-sensing pathways was recently shown to regulate adult physiology and lifespan independent of DR. Gaining an understanding of the specific pathways and mediators that regulate cellular and organismic phosphate levels and how they interact with known nutrient-sensing pathways using *Drosophila* as a model organism might ultimately lead to the development of improved dietary and therapeutic approaches to the treatment of human disorders of hypo- and hyperphosphatemia.

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References

1. Bringhurst FR, Leder BZ. Regulation of calcium and phosphate homeostasis. In: DeGroot LJ, Jameson JL (eds). *Endocrinology.* Philadelphia: W.B. Saunders Co., 2006, pp. 805–843.
2. Bergwitz C, Juppner H. Disorders of posphate homeostasis and tissue mineralisation. *Endocr Dev* 2009; 16: 133–156.
3. Razzaque MS. Does *FGF23* toxicity influence the outcome of chronic kidney disease? *Nephrol Dial Transplant* 2009; 24: 4–7.
4. Gutierrez OM, Mannstadt M, Isakova T *et al*. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. *N Engl J Med* 2008; 359: 584–592.
5. Mizobuchi M, Towler D, Slatopolsky E. Vascular calcification: the killer of patients with chronic kidney disease. *J Am Soc Nephrol* 2009; 20: 1453–1464.
6. Wahl P, Xie H, Scialla J *et al*. Earlier onset and greater severity of disordered mineral metabolism in diabetic patients With chronic kidney disease. *Diabetes Care* 2012; 35: 994–1001.
7. Sprecher E. Familial tumoral calcinosis: from characterization of a rare phenotype to the pathogenesis of ectopic calcification. *J Invest Dermatol* 2010; 130: 652–660.
8. Stubbs JR, Liu S, Tang W *et al*. Role of hyperphosphatemia and 1,25-dihydroxyvitamin D in vascular calcification and mortality in fibroblastic growth factor 23 null mice. *J Am Soc Nephrol* 2007; 18: 2116–2124.
9. Morishita K, Shirai A, Kubota M *et al*. The progression of aging in klotho mutant mice can be modified by dietary phosphorus and zinc. *J Nutr* 2001; 131: 3182–3188.
10. Ohnishi M, Razzaque MS. Dietary and genetic evidence for phosphate toxicity accelerating mammalian aging. *FASEB J* 2010; 24: 3562–3571.
11. Bevington A, Kemp GJ, Graham R *et al*. Phosphate-sensitive enzymes: possible molecular basis for cellular disorders of phosphate metabolism. *Clin Chem Enzym Comms* 1992; 4: 235–257.
12. Oshima Y. The phosphatase system in Saccharomyces cerevisiae. *Genes Genet Syst* 1997; 72: 323–334.
13. Kaffman A, Herskowitz I, Tjian R *et al*. Phosphorylation of the transcription factor PHO4 by a cyclin-CDK complex, PHO80-PHO85. *Science* 1994; 263: 1153–1156.

14. Lenburg ME, O'Shea EK. Signaling phosphate starvation. *Trends Biochem Sci* 1996; 21: 383–387.
15. Nair D, Misra RP, Sallis JD *et al*. Phosphocitrate inhibits a basic calcium phosphate and calcium pyrophosphate dihydrate crystal-induced mitogen-activated protein kinase cascade signal transduction pathway. *J Biol Chem* 1997; 272: 18920–18925.
16. Julien M, Magne D, Masson M *et al*. Phosphate stimulates matrix Gla protein expression in chondrocytes through the extracellular signal regulated kinase signaling pathway. *Endocrinology* 2007; 148: 530–537.
17. Yamazaki M, Ozono K, Okada T *et al*. Both *FGF23* and extracellular phosphate activate Raf/MEK/ERK pathway via FGF receptors in HEK293 cells. *J Cell Biochem* 2010; 111: 1210–1221.
18. Chang SH, Yu KN, Lee YS *et al*. Elevated inorganic phosphate stimulates Akt-ERK1/2-Mnk1 signaling in human lung cells. *Am J Respir Cell Mol Biol* 2006; 35: 528–539.
19. Bergwitz C, Rasmussen MD, DeRobertis C *et al*. Roles of major facilitator superfamily transporters in phosphate response in Drosophila. *PLoS One* 2012; 7: e31730.
20. Mansfield K, Teixeira CC, Adams CS *et al*. Phosphate ions mediate chondrocyte apoptosis through a plasma membrane transporter mechanism. *Bone* 2001; 28: 1–8.
21. Suzuki A, Ammann P, Nishiwaki-Yasuda K *et al*. Effects of transgenic Pit-1 overexpression on calcium phosphate and bone metabolism. *J Bone Miner Metab* 2010; 28: 139–148.
22. Beck L, Leroy C, Beck-Cormier S *et al*. The phosphate transporter PiT1 (Slc20a1) revealed as a new essential gene for mouse liver development. *PLoS One* 2010; 5: e9148.
23. Festing MH, Speer MY, Yang HY *et al*. Generation of mouse conditional and null alleles of the type III sodium-dependent phosphate cotransporter PiT-1. *Genesis* 2009; 47: 858–863.
24. Wang C, Li Y, Shi L *et al*. Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. *Nat Genet* 2012; 44: 254–256.
25. Salaun C, Leroy C, Rousseau A *et al*. Identification of a novel transport-independent function of PiT1/SLC20A1 in the regulation of TNF-induced apoptosis. *J Biol Chem* 2010; 285: 34408–34418.
26. Aoki Y, Niihori T, Narumi Y *et al*. The RAS/MAPK syndromes: novel roles of the RAS pathway in human genetic disorders. *Hum Mutat* 2008; 29: 992–1006.
27. Liu S, Tang W, Fang J *et al*. Novel regulators of Fgf23 expression and mineralization in Hyp bone. *Mol Endocrinol* 2009; 23: 1505–1518.
28. Zhang MY, Ranch D, Pereira RC *et al*. Chronic inhibition of ERK1/2 signaling improves disordered bone and mineral metabolism in hypophosphatemic (Hyp) Mice. *Endocrinology* 2012; 153: 1806–1816.
29. Nechama M, Ben-Dov IZ, Briata P *et al*. The mRNA decay promoting factor K-homology splicing regulator protein post-transcriptionally determines parathyroid hormone mRNA levels. *FASEB J* 2008; 22: 3458–3468.
30. Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V *et al*. The parathyroid is a target organ for *FGF23* in rats. *J Clin Invest* 2007; 117: 4003–4008.
31. Krajisnik T, Bjorklund P, Marsell R *et al*. Fibroblast growth factor-23 regulates parathyroid hormone and 1alpha-hydroxylase expression in cultured bovine parathyroid cells. *J Endocrinol* 2007; 195: 125–131.
32. Slatopolsky E, Finch J, Denda M *et al*. Phosphorus restriction prevents parathyroid gland growth. High phosphorus directly stimulates *PTH* secretion in vitro. *J Clin Invest* 1996; 97: 2534–2540.
33. Almaden Y, Canalejo A, Hernandez A *et al*. Direct effect of phosphorus on *PTH* secretion from whole rat parathyroid glands *in vitro*. *J Bone Miner Res* 1996; 11: 970–976.
34. Bikle D, Adams J, Christakos C. Vitamin D: production, metabolism, mechanism of action, and clinical requirements. In: Rosen C (ed). *Primer on Metabolic Bone Disease and Disorders of Mineral Metabolism*. Philadelphia, PA: Lippincott-Raven, 2008, pp. 141–149.
35. Brown MR, Kornberg A. Inorganic polyphosphate in the origin and survival of species. *Proc Natl Acad Sci USA* 2004; 101: 16085–16087.
36. Ghorbel S, Kormanec J, Artus A *et al*. Transcriptional studies and regulatory interactions between the phoR-phoP operon and the phoU, mtpA, and ppk genes of Streptomyces lividans TK24. *J Bacteriol* 2006; 188: 677–686.
37. Rao NN, Kornberg A. Inorganic polyphosphate regulates responses of Escherichia coli to nutritional stringencies, environmental stresses and survival in the stationary phase. *Prog Mol Subcell Biol* 1999; 23: 183–195.
38. Kulaev I, Kulakovskaya T. Polyphosphate and phosphate pump. *Annu Rev Microbiol* 2000; 54: 709–734.
39. Auesukaree C, Homma T, Tochio H *et al*. Intracellular phosphate serves as a signal for the regulation of the PHO pathway in Saccharomyces cerevisiae. *J Biol Chem* 2004; 279: 17289–17294.
40. Docampo R, de Souza W, Miranda K *et al*. Acidocalcisomes - conserved from bacteria to man. *Nat Rev Microbiol* 2005; 3: 251–261.
41. Kumble KD, Kornberg A. Inorganic polyphosphate in mammalian cells and tissues. *J Biol Chem* 1995; 270: 5818–5822.
42. Schmelzle T, Hall MN. TOR, a central controller of cell growth. *Cell* 2000; 103: 253–262.
43. Tang H, Hornstein E, Stolovich M *et al*. Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol-3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. *Mol Cell Biol* 2001; 21: 8671–8683.
44. Jacinto E, Hall MN. Tor signalling in bugs, brain and brawn. *Nat Rev Mol Cell Biol* 2003; 4: 117–126.
45. Shiba T, Nishimura D, Kawazoe Y *et al*. Modulation of mitogenic activity of fibroblast growth factors by inorganic polyphosphate. *J Biol Chem* 2003; 278: 26788–26792.
46. Murshed M, Harmey D, Millan JL *et al*. Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. *Genes Dev* 2005; 19: 1093–1104.
47. Rutsch F, Nitschke Y, Terkeltaub R. Genetics in arterial calcification: pieces of a puzzle and cogs in a wheel. *Circ Res* 2011; 109: 578–592.
48. Gonlusen G, Akgun H, Ertan A *et al*. Renal failure and nephrocalcinosis associated with oral sodium phosphate bowel cleansing: clinical patterns and renal biopsy findings. *Arch Pathol Lab Med* 2006; 130: 101–106.
49. Moe SM, Drueke T, Lameire N *et al*. Chronic kidney disease-mineral-bone disorder: a new paradigm. *Adv Chronic Kidney Dis* 2007; 14: 3–12.
50. Faul C, Amaral AP, Oskouei B *et al*. *FGF23* induces left ventricular hypertrophy. *J Clin Invest* 2011; 121: 4393–4408.
51. Lim K, Lu TS, Molostvov G *et al*. Vascular klotho deficiency potentiates the development of human artery calcification and mediates resistance to fibroblast growth factor 23. *Circulation* 2012; 125: 2243–2255.
52. Price PA, Roublick AM, Williamson MK. Artery calcification in uremic rats is increased by a low protein diet and prevented by treatment with ibandronate. *Kidney Int* 2006; 70: 1577–1583.
53. Ishimura E, Okuno S, Taniwaki H *et al*. Different risk factors for vascular calcification in end-stage renal disease between diabetics and nondiabetics: the respective importance of glycemic and phosphate control. *Kidney Blood Press Res* 2008; 31: 10–15.
54. Kurosu H, Yamamoto M, Clark JD *et al*. Suppression of aging in mice by the hormone Klotho. *Science* 2005; 309: 1829–1833.
55. Swinnen E, Wanke V, Roosen J *et al*. Rim15 and the crossroads of nutrient signalling pathways in Saccharomyces cerevisiae. *Cell Div* 2006; 1: 3.
56. Sabbagh Y, Carpenter TO, Demay MB. Hypophosphatemia leads to rickets by impairing caspase-mediated apoptosis of hypertrophic chondrocytes. *Proc Natl Acad Sci USA* 2005; 102: 9637–9642.
57. Miedlich SU, Zalutskaya A, Zhu ED *et al*. Phosphate-induced apoptosis of hypertrophic chondrocytes is associated with a decrease in mitochondrial membrane potential and is dependent upon Erk1/2 phosphorylation. *J Biol Chem* 2010; 285: 18270–18275.
58. Magne D, Bluteau G, Faucheux C *et al*. Phosphate is a specific signal for ATDC5 chondrocyte maturation and apoptosis-associated mineralization: possible implication of apoptosis in the regulation of endochondral ossification. *J Bone Miner Res* 2003; 18: 1430–1442.

59. Fontana L, Partridge L, Longo VD. Extending healthy life span—from yeast to humans. *Science* 2010; 328: 321–326.
60. Linford NJ, Kuo TH, Chan TP *et al*. Sensory perception and aging in model systems: from the outside in. *Annu Rev Cell Dev Biol* 2011; 27: 759–785.
61. Vellai T, Takacs-Vellai K, Sass M *et al*. The regulation of aging: does autophagy underlie longevity? *Trends Cell Biol* 2009; 19: 487–494.
62. Kabil H, Kabil O, Banerjee R *et al*. Increased transsulfuration mediates longevity and dietary restriction in Drosophila. *Proc Natl Acad Sci USA* 2011; 108: 16831–16836.
63. Tenenhouse HS, Klugerman AH, Neal JL. Effect of phosphonoformic acid, dietary phosphate and the Hyp mutation on kinetically distinct phosphate transport processes in mouse kidney. *Biochim Biophys Acta* 1989; 984: 207–213.
64. Chertow GM, Dillon M, Burke SK *et al*. A randomized trial of sevelamer hydrochloride (RenaGel) with and without supplemental calcium. Strategies for the control of hyperphosphatemia and hyperparathyroidism in hemodialysis patients. *Clin Nephrol* 1999; 51: 18–26.
65. Edgecomb RS, Harth CE, Schneiderman AM. Regulation of feeding behavior in adult Drosophila melanogaster varies with feeding regime and nutritional state. *J Exp Biol* 1994; 197: 215–235.
66. Roberts DM. *Drosophila: A Practical Approach (The Practical Approach Series)*. New York: Oxford University Press, 1998.
67. Bergwitz C, Huang J, Wee MJ *et al*. SA-PO2712 dietary phosphate modifies life span in Drosophila. *JASN* 2011; 22: 744A.

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New players in the pathogenesis of focal segmental glomerulosclerosis

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Abstract
Focal segmental glomerulosclerosis (FSGS) is the most common primary glomerular disorder causing end-stage renal disease. Since the first description of this clinicopathological entity in the early 1930s, various studies have identified numerous underlying pathogenetic mechanisms. Nevertheless, FSGS is still a complex, only partially understood and in its classification sometimes confusing disease. A unifying pathophysiological concept has not been identified and might not even exist. However, research efforts of past decades identified FSGS as a podocytopathy with several podocyte molecules being key players in the development and the course of FSGS. Podocytes are crucially involved in the formation of the glomerular barrier and any assault on their delicate physiological balance and architecture can result in the development of proteinuria. The following review article will introduce most recent examples identifying novel players in the complex pathogenesis of FSGS.

Keywords: Arhgap24; CD2AP; FSGS; podocyte; suPAR

Introduction

The term focal segmental glomerulosclerosis (FSGS) stands for a complex clinicopathological entity, which integrates various clinical presentations as well as different underlying pathophysiological aetiologies [1–5]. However, FSGS is uniformly characterized by the occurrence of proteinuria and the involvement of the podocyte, a specialized epithelial cell, which is essential for the formation and the integrity of the glomerular barrier. For decades, FSGS was mainly investigated in terms of histological description and these efforts culminated in the classification in five morphological subtypes [6]. From an aetiological and functional standpoint, FSGS can be divided into primary and secondary FSGS. The difference between these two groups has major therapeutic implications, as primary FSGS is usually treated empirically with immunomodulatory agents. This treatment concept is mainly based on the theory that a dysregulated immune system contributes to the pathogenesis of FSGS. However, recent studies also identified off-target effects of established immunomodulatory drugs, such as rituximab and cyclosporine A, that can directly affect and modulate podocyte function [7–9].

While primary FSGS subsumes all idiopathic cases of FSGS, the group of secondary FSGS presents a kaleidoscopic array of different diseases, all resulting in different degrees of nephron loss and podocyte damage. The greatest contribution in understanding how the podocyte is particularly affected was done by the identification of causative genes in hereditary forms of nephrotic syndrome. Here we will only briefly review some of the most important genes