Roles of Major Facilitator Superfamily Transporters in Phosphate Response in *Drosophila*

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Abstract

The major facilitator superfamily (MFS) transporter *Pho84* and the type III transporter *Pho89* are responsible for metabolic effects of inorganic phosphate in yeast. While the *Pho89* ortholog *Pit1* was also shown to be involved in phosphate-activated MAPK in mammalian cells, it is currently unknown, whether orthologs of *Pho84* have a role in phosphate-sensing in metazoan species. We show here that the activation of MAPK by phosphate observed in mammals is conserved in *Drosophila* and, used this assay to characterize the roles of putative phosphate transporters. Surprisingly, while we found that RNAi-mediated knockdown of the fly *Pho89* ortholog *dPit* had little effect on the activation of MAPK in *Drosophila* S2R+ cells by phosphate, two *Pho84/SLC17A1–9* MFS orthologs (*MFS10 and MFS13*) specifically inhibited this response. Further, using a Xenopus oocyte assay, we show that MFS13 mediates uptake of [33P]-orthophosphate in a sodium-dependent fashion. Consistent with a role in phosphate physiology, MFS13 is expressed highest in the *Drosophila* crop, midgut, Malpighian tubule, and hindgut. Altogether, our findings provide the first evidence that *Pho84* orthologs mediate cellular effects of phosphate in metazoan cells. Finally, while phosphate is essential for *Drosophila* larval development, loss of MFS13 activity is compatible with viability indicating redundancy at the levels of the transporters.

Introduction

Inorganic phosphate, the mono- or divalent anion of phosphoric acid [HPO$_4$$^{2-}$, H$_2$PO$_4$$^{-}$], is required for cellular functions such as DNA and membrane lipid synthesis, generation of high-energy phosphate esters, and intracellular signaling [1]. Disturbances of phosphate homeostasis are serious human disorders [2]: the clinical consequences of severe hypophosphatemia, which for example is seen in severe malnutrition or tumor-induced hypophosphatemia [3], include hemolysis, skeletal muscle myopathy, cardiomyopathy, neuropathy, osteomalacia and, in some cases contribute to death. Hyperphosphatemia on the other hand leads to tissue calcifications and metabolic changes, which are to date poorly understood. Hyperphosphatemia is encountered most frequently in patients with chronic kidney disease (CKD), a human disorder that was recently attributed to loss-of-function mutations in the genes encoding fibroblast growth factor 23 (FGF23), UDP-GalNAc transferase 3 (GALNT3), and Klotho (*KL*) [7]. Furthermore, mouse models with hyperphosphatemia due to loss-of-function mutations in Fgf23, *KL* or Galnt3 die prematurely unless they are placed on a phosphate-restricted diet to improve their lifespan [8,9,10] and it is possible that similar mechanisms underlie the known beneficial effects of dietary phosphate-restriction in humans with CKD. An understanding of the molecular basis underlying the metabolic and endocrine phosphate effects is therefore of great significance for human disease.

The intracellular concentration of inorganic phosphate is maintained by membrane transporters which accumulate phosphate against an electrochemical gradient coupled to the plasma membrane H$^+$ [11] or Na$^+$ gradients [12], at concentrations larger than would be predicted if phosphate were distributed passively across the membrane. Much has been learned about phosphate transport in bacteria and in yeast. Bacteria sense phosphate using a four-component Pst-transporter (*PstS, PstA, PstB, PstC*), which is similar to mammalian ABC transporters. Binding of phosphate to *PstABC* represses a two component signaling system composed of the sensory histidine kinase *PhoR* and the winged helix transcription factor *PhoB* [13,14]. Different from bacteria, the main phosphate-sensing transporter *Pho84* in yeast belongs to the major facilitator family (MFS) which regulates the cyclin/cyclin-depen-
dent kinase (CDK) complex Pho89–Pho85 [15]. The activity of Pho80–Pho85 in turn regulates the subcellular localization of the basic helix-loop-helix transcription factor Pho4, which belongs to the myc family. Interestingly, a number of transporters, Pho7, 89, 90 and 91, can compensate for loss of Pho4 under certain conditions in yeast suggesting that signaling is independent of the mode of cellular uptake, and that intracellular phosphate is the signal for gene-regulation [16]. However, the fact that overexpression of a phosphate-transport deficient Pho4 variant can rescue regulation of the extracellular alkaline phosphatease Pho5 by phosphate in Pho84 deficient strains, while overexpression of Pho87, Pho90, Pho91 or Pho4 is ineffective, suggests, that binding of extracellular phosphate alone may be sufficient for some downstream effects of phosphate [16].

Humans have three types of membrane-bound phosphate transporters: The type I transporters SLC17A1–9 that belong to the MFS group. MFS are widely expressed and some also mediate transport of organic anions, such as uric or sialic acid, or certain antibiotics [17,18]. Conversely, the human type II phosphate transporters NPT2a, NPT2b, and NPT2c, and type III phosphate transporters Pit1 and Pit2 are thought to be exclusively transporting phosphate [12,19]. NPT2a, NPT2c, and Pit2 are expressed in the renal proximal tubule and mediate re-absorption of phosphate from the urine, NPT2b and Pit2 mediate absorption of phosphate from the diet in the gut, and Pit1 is ubiquitously expressed and facilitates uptake of phosphate from the circulation to supply cellular functions [20,21]. Pho4 belongs to the MFS group, Pho87, 90 and 91 are related to metazoan sodium-sulfate transporters (SLC13A1–4), and Pho89 is related to the type III sodium-phosphate transporters SLC20A1 (Pit-1) and SLC20A2 (Pit-2) [22,23].

Compared to bacteria and yeast, little is known about the metabolic effects of phosphate in metazoan species [20,21]. Over the past decade, activation of MAPK by inorganic phosphate at concentrations between 5–10 mM alone was demonstrated in multiple cell lines including MC3T3 mouse fibroblast cells [24,25], chondrogenic ATDC5 cells, MC3T3-E1 osteoblasts and ST2 murine bone marrow stromal cells [26], HEK293 human proximal tubular cells [27], and lung alveolar cells [28]. Although some cell lines, for example C2C12 or L929 cells, are less responsive than others [26], activation of MAPK by phosphate appears to be quite universal. Addition of phosphonoformic acid (PFA), a competitive antagonist of phosphate transporters and antibiotics [17,18]. Conversely, the human type II phosphate transporters NPT2b, NPT2c, and NPT2a mediate absorption of phosphate from the diet in the gut, and Pit1 is ubiquitously expressed and facilitates uptake of phosphate from the circulation to supply cellular functions [20,21]. Pho4 belongs to the MFS group, Pho87, 90 and 91 are related to metazoan sodium-sulfate transporters (SLC13A1–4), and Pho89 is related to the type III sodium-phosphate transporters SLC20A1 (Pit-1) and SLC20A2 (Pit-2) [22,23].

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Phosphate-uptake studies

We downloaded protein sequences from Ensembl version 56 [37] and used BLAST to cluster all known yeast, *Drosophila* and human proteins containing the MFS protein domain PF07690 [38] into five main families. Next using Bayesian phylogenetic reconstruction [MrBayes v3.1.2 [39]] we identified 29 fly orthologs that are most closely related to yeast Pho84 and human SLC17A1–9, an anion transporter subfamily with members known to mediate phosphate transport. Refer to Table S2 for all blast hits *S. cerevisiae* vs. *D. melanogaster* (Table S2.1), *D. melanogaster* vs. *D. melanogaster* (Table S2.2), *D. melanogaster* vs. human (Table S2.3).

RNAi knockdown experiments

RNAi knockdown experiments were performed in S2R+ cells as described previously [40]. We used the SnapDragon tool from the Flybase (http://flybase.org/) notes only one transcript and Genome Resource Center (DGRC, https://dgrc.cgb.indiana.edu/). FBgn0030452 of the eight MFS type I transporters expressed in S2R+ cells/well in 48-multiwell plates were incubated with 3 μg dsRNA per well in 250 ul serum-, phosphate- and antibiotic-free medium supplemented with 10 mM HEPES (pH 7.4) for 45 min. At 25 C. Transfection was stopped by the addition of 250 μl phosphate-free medium containing 20% heat-inactivated FBS, 2 μg penicillin/streptomycin and 10 mM HEPES (pH 7.4). After culture for three days cells were challenged with 10 nM sodium-phosphate (pH 7.4) or 25 μg/ml Insulin for 3 min. Lysates were analyzed by Western analysis as described above. For quantitative RT-PCR analysis, lysates were prepared using 300 ul RLT-PLUS per well according to the manufacturer’s instructions for RNeasy-micro PLUS kit (Qiagen). Following reverse transcription using the Omni-script kit (Qiagen), quantitative PCR using the Cybr Green kit (Qiagen) was performed using intron-overlapping primers, which had been chosen so to not overlap the dsRNA target sequences (see Table S1 for primer sequences). To calculate efficiency of knockdown, target mRNA expression corrected for the actin mRNA expression of cells treated with target RNAi was compared to target mRNA expression corrected for actin mRNA expression of cells treated with RNAi targeting luciferase, a gene that is not expressed in fly cells, and thus serves to control for non-specific RNAi effects. qRT-PCR of dPit mRNA was unaffected by RNA-mediated knockdown of MFS10 and MFS13 and vice versa. To exclude off-target effects of the dsRNA affecting phosphate-induced MAPK independent of the transporters under investigation we furthermore showed that several independent dsRNAs targeting MFS10, 13 and Pit1 have similar effects.

Phosphate-uptake studies

Plasmids encoding full-length cDNAs of FBgn0010497, FBgn0030452, and FBgn0260793 were obtained from the *Drosophila* Genome Resource Center (DGRC, https://dgrc.cgb.indiana.edu/). Flybase (http://flybase.org/) notes only one transcript and protein for each transporter genes and nucleotide sequence analysis was used to independently confirm presence of initiation codon and poly-adenylation signal in the cDNAs obtained from the DGRC prior to preparation of cRNA for expression in *Xenopus* oocytes using the mMessage Machine T7 and polyA-tailing kits (Ambion), and PCR-based templates generated using a sense primer containing the T7 promoter sequence (for primer sequences see Table S1). Full length of cRNA transcripts was confirmed using denaturing gel electrophoresis, and thus it is unlikely that non-functional splice variants or incomplete proteins were expressed.

For phosphate-uptake experiments, *Xenopus* oocytes were harvested from female frogs by C-section, de-folliculated and injected with 50 ng capped and poly-adenylated RNA in 100 nl per oocyte, prepared with the mMessage Machine kit (Ambion) as previously described [43]. Following incubation in ND96++ buffer (NaCl 0.192 M; KCl 4 mM; HEPES pH 7.4, 20 mM; CaCl2 1.8 mM; MgCl2 1 mM; 1× penicillin/streptomycin) for three days at 18 C to permit protein expression, phosphate uptake was measured in ND100 (100 mM NaCl, 2 mM KCl, 1.3 mM CaCl2, 1 mM MgCl2, 1 mM Pi, 10 mM Hepes-Tris (pH 7.4), supplemented with [33P]-orthophosphoric acid (Perkin Elmer) (final specific activity, 5–50 μCi/mmol) at room temperature for 60 min., followed by four washes in ND+2 mM Pi, lysis and detection of single-oocyte uptake using a scintillation counter. For sodium-free conditions 100 mM choline-chloride was substituted for 100 mM sodium-chloride to obtain ND0.

Fly culture and crosses

Standard fly culture was performed at 25°C on 17 g/l yeast, 9.8 g soy flour, 71 g/l corn meal, 5.6 g/l agar, 5.6 g/l malt, 75 ml/l corn syrup, 4 ml/l propionic acid and 250 mg/l tegosept ( Spectrum M1187). This medium was supplemented with 30 mM sodium-phosphate (pH 6.0), sodium-sulfate (pH 6.0), 10 mM phosphonoformic acid (Sigma P8610), or 1% sevelamer (gift from Dr. Yves Sabbagh, Genzyme, Inc.).

*P*-element insertions and deficiency mutants targeting the genomic locus of MFS13 (FBgn0010497) were obtained from the Bloomington *Drosophila* Stock Center (BDBC, http://bystocks.bio.indiana.edu/), and the Exelixis collection (http://drosophila.med.harvard.edu/). P*{PZ}l(2)0181001810*/CyO; y*596 (Bloomington stock 11076), y1 w*; *Mi{MIC}l(2)01810 MI00602, P*{XP}l(2)007000 (Bloomington stock 20492), y1 w*; M{MIC}l(2)01810/+, P{XP}.l1(2)01810/+, PBac{RB}l(2)01810/+, w*1111; Df{2L}BSC326/SM6a (Bloomington stock 27900), w*1118; Df{2L}BSC327/CyO (Bloomington stock 24348) (for genomic location of these insertions see Figure S1A and B).

While four *P*-element insertions were homozygous viable, P*{XP}l(2)01810/+, all the deficiencies were lethal. To examine, whether lack of viability of these stocks is related to homozygous loss of MFS13, we generated flies of the genotypes P*{XP}l(2)01810/+ /Df{2L}BSC326 and P*{XP}l(2)01810/+ /Df{2L}BSC325, which were viable. Loss of MFS13 in these heterozygous flies was confirmed by quantitative RT-PCR. Briefly, 5 flies were collected for total RNA preparation using Trizol reagent (Invitrogen). cDNA was synthesized using the Omniscript cDNA reverse transcription kit (Qiagen). The levels of mRNA for different genes were measured by using SYBR-GREEN QuantiTect (Qiagen) on a StepOnePlus real time PCR system (Applied Biosystems). For these experiments RpL32 (FBgn0002626) was used for normalization, which, unlike actin5C, is not influenced by culture temperature of the flies [44]. The primers used are listed in Table S1.

Statistical analysis

Assay variability was generally less than 10% for Westernblot, qPCR and phosphate uptake experiments. Means±SEM of at least three independent experiments performed in duplicate are shown.
### Table 1. *Drosophila* orthologs of yeast Pho84 and human SLC17A1–9.

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Transporter protein IDs used for BLAST and Bayes phylogenetic analysis.
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Results

Phosphate activates MAPK in S2R+ cells in a time and dose-dependent fashion

To establish an assay for phosphate sensing in Drosophila, we investigated whether, as observed in mammalian cells, phosphate can activate MAPK in Drosophila cells. Drosophila S2R+ cells were exposed to 10 mM sodium-phosphate buffer (pH 7.4) and a phosphate-specific ERK antibody was used to detect MAPK activation (Fig. 1A). Phosphate activates MAPK rapidly within 3 min, and desensitizes over the course of 15 min (Fig. 1B). Activation is dose-dependent, and reaches a maximum at 10 mM (Fig. 1C). Activation of MAPK is not seen with an iso-osmolar stimulus of 10 mM sodium-sulfate (Fig. 1B). The time course of activation by phosphate is similar to activation of MAPK by insulin, with a peak at 3 min and desensitization over the course of 15 min (data not shown). Long-term exposure to 10 mM phosphate or insulin over 24 hrs does not lead to significant activation of MAPK above baseline (data not shown).

Similar time-dependent activation of MAPK is seen in a second Drosophila hemocyte-like cell line, Kc167 (Fig. 1D). Different, however, from S2R+ cells, activation of MAPK is followed by suppression below baseline after 10 and 15 min, with return to baseline after 30 min.

Addition of phosphonoformic acid (PFA) blocks activation of MAPK by phosphate in mammalian cell lines [29,30], indicating that binding or cellular uptake of phosphate is required for the activation of MAPK. Similarly, exposure to PFA for 60 min prior to exposure to phosphate blocks activation of MAPK by phosphate in mammalian cell lines [5–10 mM, [29,30]], indicating that binding or cellular uptake of phosphate is required for the activation of MAPK by phosphate in mammalian cell lines [29,30]. Importantly, PFA blocks MAPK by phosphate in mammalian cell lines [29,30], indicating that binding or cellular uptake of phosphate is required for the activation of MAPK by phosphate in mammalian cell lines [29,30].

Different, Drosophila hemocyte-like cell line, Kc167 (Fig. 1D). MAPK activation by phosphate is similar to activation of MAPK by insulin appears to be more sustained and returns back to baseline after 30–60 min (data not shown). Long-term exposure to 10 mM sodium-phosphate buffer (pH 7.4) can activate MAPK in mammalian cells, although higher doses were required (Fig. 1B). The time course of activation by phosphate is similar to activation of MAPK by sodium-phosphate co-transporter [19], indicating that this type III sodium-phosphate co-transporter [19] is required for the activation of MAPK in this cell line. However, RNAi knockdown of the dPit mRNA level was reduced 100-fold when compared to baseline, i.e., cells transfected with dsRNA targeting luciferase, a gene not expressed in S2R+ cells and thus serving as a control for non-specific RNAi effects (Fig. 3C).

RNAi knockdown of MSF transporters blocks phosphate- but not insulin-induced MAPK in S2R+ cells

siRNA-mediated knockdown of the Psl sodium-phosphate co-transporter blocks activation of MAPK by phosphate in human embryonic kidney (HEK295) cells [27] indicating that this type III sodium-phosphate co-transporter [19] is required for the activation of MAPK in this cell line. However, RNAi knockdown of the dPit only reduced phosphate-induced MAPK by 20% in S2R+ cells (Fig. 3B). Findings were similar with three independent dsRNAs and quantitative RT-PCR confirmed that the Psl mRNA level was reduced 100-fold when compared to baseline, i.e., cells transfected with dsRNA targeting luciferase, a gene not expressed in S2R+ cells and thus serving as a control for non-specific RNAi effects (Fig. 3C).

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Individual knockdown of two of the eight expressed MFS transporters, MFS10 and MFS13 (encoded by FBgn003452 and FBgn0010497, respectively) resulted in 40% reduction of phosphate-induced MAPK, which exceeds the effect seen by knockdown of dPit (Fig. 3B). Knockdown of these MFS transporters was specific for phosphate, since insulin continued to be able to stimulate MAPK. These results were reproducible by two independent sets of dsRNAs and quantitative RT-PCR confirmed that the Psl mRNA level was reduced 100-fold when compared to baseline, i.e., cells transfected with dsRNA targeting luciferase, a gene not expressed in S2R+ cells and thus serving as a control for non-specific RNAi effects (Fig. 3C).

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MFS13 (FBgn0010497) mediates Na-dependent phosphate-uptake when expressed in Xenopus oocytes

To test whether MFS10 and MFS13 facilitate cellular phosphate uptake, we injected capped and polyadenylated sense RNA encoding these transporters into Xenopus oocytes. Following injection of 50 ng/oocyte and culture at 18 C for three days to allow for expression of the transporter protein in the oocyte plasma membranes, we performed a radioactive-phosphate uptake experiment in the absence or presence of sodium and PFA at pH 5.5, 7.4, and 8.5.

MFS13 showed significant uptake of phosphate, while no significant uptake was seen when expressing MFS10. Uptake mediated by MFS13 was similar in magnitude to that seen with
dPit but 10% when compared to that seen with oocytes expressing human SLC34A3 (NaPi-IIc; data not shown). Radioactive phosphate uptake was dependent on sodium and blocked by PFA or low pH, while transport was maximal at physiological pH 7.4 and at pH 8.5 (Fig. 4). Altogether, these results indicate that MSF13, but not MSF10, mediates uptake of [33P]-orthophosphate in a sodium-dependent fashion.

Phosphonoformic acid and sevelamer impair larval development in fly

A search in FlyAtlas [47] reveals that MFS10 (FBgn0030452) mRNA is expressed highest in the male accessory gland, two-fold enriched in brain and four-fold enriched in the Malpighian tubule, the renal tubule equivalent in fly, when compared to whole fly expression. MFS13 (FBgn0010497) mRNA is expressed highest in the crop, midgut, Malpighian tubule, and hindgut, where it is three-fold enriched when compared to whole fly (Fig. S3). No entry is found for dPit.

To explore the role of phosphate during larval development of Drosophila we cultured wild-type flies in 0.5% sevelamer to inhibit absorption of dietary phosphate [48] and 1 mM PFA to block phosphate transport into cells [49]. This treatment delayed embryonic and larval development (Fig. S4A and B). The effect of sevelamer and PFA was reversed by addition of 30 mM sodium phosphate.

To further evaluate the role of MFS13 in vivo, we obtained a P-element insertion in MFS13 (P{PZ}l(2)0181001810) that was viable over two deficiencies of the region (Df(2L)BSC826, Df(2L)BSC323). qPCR analysis of adult flies of the genotype P{PZ}l(2)0181001810/Df(2L)BSC826 or P{PZ}l(2)0181001810/Df(2L)BSC323 revealed that MFS3 expression is most likely completely absent, suggesting that MSF13 mediates phosphate transport together with other transporter(s) (Fig. S1C).

Discussion

In this study, we show that activation of MAPK is part of the down-stream events stimulated when two Drosophila hemocyte-like cell lines, S2R+ and Kc167, are exposed to phosphate. Just like in mammalian cell lines, we furthermore found that PFA blocks phosphate induced MAPK in S2R+ Drosophila cells. Activation of MAPK by phosphate, which thus far has only been shown in

**Figure 1. Western blot analysis of phosphate-induced MAPK in S2R+ and Kc167 cells.** A: Dose response and time course of phosphate-activated MAPK in S2R+ cells. B: Dose response of phosphate-activated MAPK in S2R+ cells. C: Time course of phosphate-activated MAPK in S2R+ cells. D: Time course of phosphate-activated MAPK in Kc167 cells. E: Effect of PFA on activation of MAPK in S2R+ cells. Shown are one representative Western blot autoradiogram (A), or pooled densitometric data of at least three independent Western blot experiments (B–E). Abbreviations: Pi = inorganic phosphate, Ins = human insulin, Ly = Ly294002 (PI3K-inhibitor, 50 uM), PFA = phosphonoformic acid (30 mM, unless otherwise noted), S10 = sodium sulfate (10 mM), P10 = sodium phosphate (10 mM). doi:10.1371/journal.pone.0031730.g001
Figure 2. Blast and Bayes analysis of MFS transporters. Heatmap of pairwise BLAST bit scores for all known yeast, Drosophila and human proteins containing the MFS protein domain PF07690 [38] (left panel) sorted by a hierarchical clustering (middle panel). Bayesian phylogenetic reconstruction (dendogram) was used to identify 29 fly orthologs that are most closely related to yeast Pho84 (YML123C) and human SLC17A1–9. Posterior probabilities are indicated above each branch. Fly transporters found to be expressed in S2R+ cells are shown in bold/italic script.
doi:10.1371/journal.pone.0031730.g002

Figure 3. Effect of RNAi knockdown of MFS transporters and dPit on MAPK. A: mRNA expression of MFS and Pit transporters in S2R+ cells. Data of three replicate experiments are shown as mean ± SEM expression relative to actin 5 C. B: Effect of RNAi knockdown of MFS transporters and dPit on MAPK. Data of three replicate experiments are shown as mean ± SEM relative to cells transfected with dsRNA targeting luciferase (luc). C: RNAi knockdown efficiency. To calculate efficiency of knockdown, parallel wells prepared for pERK1/2 Western analysis above (Fig. 2B) were used for total RNA extraction and quantitative RT-PCR. Shown are mean ± SEM of three replicate experiments after expression was corrected for actin 5 C mRNA. Cells treated with dsRNA targeting luciferase are set 100% for each specific primer pair.
doi:10.1371/journal.pone.0031730.g003
mammalian cell lines (reviewed in: [20,21]), consequently appears to be evolutionarily conserved.

Activation of the MAPK pathway by phosphate in metazoan species is likely relevant for cellular functions as has been shown for the regulation of RANK/RANK-L signaling [50], mRNA expression of bone matrix proteins osteopontin [24], and matrix gla protein [51] or down-regulation of type III transporters Pit1 and Pit2 [52], all of which are blocked by UO126, an inhibitor of the upstream MAPK-kinase MEK. Yet, it is poorly understood, whether phosphate needs to enter metazoan cells to stimulate intracellular signaling events as suggested by the inhibitory action of PFA, or whether it binds and activates a cell surface receptor.

In yeast the major facilitator superfamily transporters Pho84 and the type III transporter Pho89 have been implicated in phosphate-sensing in yeast [16]. Recent evidence suggests that the mammalian ortholog of Pho89, Pit1, mediates cellular effects of phosphate, however, we found in S2R+ cells that knockdown of the fly ortholog dPit only reduced activation of MAPK by phosphate by 20% when compared to control, while it reduced dPit mRNA by more than 90%. Since orthologs of the type II cotransporters are absent from the Drosophila genome, we therefore postulated that a type I co-transporter ortholog related to Pho84 may be involved in phosphate sensing in Drosophila S2R+ cells. Despite sequence divergence and size of this transporter family we were able to identify eight fly Pho84 candidates based on sequence homology to the human MFS transporters SLC17A1–9, and expression in our cell line. These eight transporters are highly supportive of their universal role for phosphate-sensing. Evaluation of these eight MFS members using phosphate-induced MAPK as readout provides evidence that four Drosophila type I (MFS) transporters are positive regulators, while one transporter is a negative regulator of phosphate-induced MAPK. Three of these five transporters specifically affect phosphate, while insulin-induced MAPK was unaffected. We decided to further investigate the two positive and specific regulators MFS10 and MFS13 (encoded by FBgn0030452 and FBgn0010497), which are required for the activation of MAPK by phosphate in S2R+ cells. Further evaluation after expression in X. oocytes indicates that one of these two transporters (MFS13, encoded by FBgn0010497) shows significant phosphate conductance, which is comparable in magnitude to that seen with dPit. Consistent with the mechanism of transport known for human SLC17A1–9, this phosphate conductance is sodium-dependent and inhibited by PFA or low pH. Our findings therefore provide first evidence for the presence of multiple Pho84 orthologs in a multicellular organism, which along with the Pho89 ortholog dPit are involved in phosphate-sensing. The sequence alignment highlights conserved domains and residues which may be involved in these functions (Fig. S2C).

Since 5 mM PFA is sufficient to inhibit the MFS13 transporter after expression in X. oocytes, lower potency of PFA on MFS10, dPit or possibly other transporters may explain the high concentration of 30 mM PFA is needed to block phosphate-induced MAPK in S2R+ cells.

Loss of Pho84 reduces proliferation and survival in yeast, which can be rescued by over-expressing the related phosphate transporter Pho89 [16], suggesting that members of different superfamilies permit cellular uptake of phosphate in yeast that then is sensed intracellularly. However, the fact that overexpression of a phosphate-transport deficient Pho84 variant can rescue regulation of the extracellular alkaline phosphatase Pho5 by phosphate in Pho84 deficient strains, while overexpression of Pho89 is ineffective, suggests, that binding of extracellular phosphate alone may be sufficient, at least for some down-stream effects of phosphate in yeast [16]. Since multiple transporter are involved in S2R+, our findings support the possibility that cellular uptake of phosphate is required, and that also in metazoan cells intracellular phosphate is what is sensed and what leads to activation of MAPK.

This study has several limitations that require future investigation: only 29 out of 219 known Drosophila pho84 orthologs were examined and it is possible that other orthologs are expressed and involved in phosphate-induced MAPK in S2R+ cells. Phosphate transport data shown here are qualitative in nature and future experiments have to include quantification of surface expression of the fly transporters. Since transport for phosphate by MFS13 and dPit was in our hands less efficient when compared to the human type II sodium-phosphate co-transporter NaPi-IIc (data not shown), and we were unable to show phosphate-conductance for the second type I transporter MFS10 (encoded by FBgn0030452) it is possible that extracellular binding of phosphate to these transporters leads to activation of intracellular events independent of phosphate-uptake. Based on studies in mammalian cells, it is possible, that Pit1 is the sole functional paralog of yeast Pho84 and Pho89 in higher species [17,19]. Indeed, targeted deletion, hypomorphic and overexpression mutants of Pit1 support a fundamental role of this transporter in liver growth and phosphate homeostasis of mice [32,33,34]. However, additional mechanisms for phosphate-sensing possibly involving Pho84 orthologs may exist since Pit1 null mice exhibit normal embryonic development and morphogenesis. Consistent with an important role for phosphate in metabolism and endocrine regulation we found that PFA and sevelamer impair larval development of Drosophila. However, just like in mice we also found that deletion of MFS13 is compatible with larval development and metamorphosis of flies indicating that loss of a single transporter can be compensated by others in vivo.

In conclusion, our findings suggest that activation of MAPK by phosphate is evolutionarily conserved from fly to man. MFS
transporters mediate cellular effects of phosphate in fly S2R+ cells along with dPit, which may be relevant for higher species and humans. Further studies are required to better understand the role of these transporters in Drosophila phosphate-homeostasis.

Supporting Information

Figure S1 P-element and deficiency stocks for MFS13 (l(2)10180, FBgn0010497). The insertion sites of the P-elements obtained from flybase (www.flybase.org) is shown in A, the location of available chromosome 2 deficiency mutants surrounding the genetic locus and including FBgn0010497 is shown in B. qRT-PCR to confirm complete loss of MFS13 transcripts in P[P^H2]l(2)10180/0, l(2)BSC326 (11076/27900) or P[P^H2]l(2)10180/^l(2)BSC323 (11076/24348) adult flies when compared to heterozygous stocks and wild-type flies (CTRL) (C). (PDF)

Figure S2 Sequence comparison between ph84 and MFS transporters expressed in S2R+ cells. A: Global alignment. Amino acid sequence identity in % between the sequence shown in column and row (alignment length in brackets). B: Local alignment. Amino acid sequence identity in % between sequence shown in column and row (alignment length in brackets). C: Clustal W alignment of fly transporters expressed in S2R+ cells along with Pho84 using Jalview (http://www.jalview.org/download.html). (PDF)

Figure S3 FlyAtlas tissue distribution of MFS10 (FBgn0030452) and MFS13 (FBgn0010497). Using FlyAtlas [46], mRNA expression of MFS10, and MFS13 (encoded by FBgn0030452, FBgn0010497, respectively) is shown for various larval and adult fly tissues. (TIF)

Figure S4 Phosphonoformic acid and sevelamer impair larval development. Yellow white flies were cultured on standard medium at 25°C. This medium was supplemented with 30 mM sodium-phosphate (pH6.0/030), 1 mM phosphonoformic acid (PFA), or 0.5% sevelamer (Sev) or in combinations thereof. Number of larvae emerged from the medium over time are shown. (TIF)

Table S1 Primer sequences used for dsRNA synthesis, cRNA synthesis and qRT-PCR. Primer sequences are displayed 5¢ to 3¢ and include the T7-RNA-polymerase promoter when used to generate PCR templates for dsRNA or cRNA synthesis. (XLS)

Table S2 Tables of all blast hits S. cerevisiae vs. D. melanogaster (S2.1), D. melanogaster vs. D. melanogaster (S2.2), D. melanogaster vs. human (S2.3) with the following format. 1. Query ID (Ensembl), 2. Subject ID (Ensembl), 3. % identity between query and subject, 4. alignment length between query and subject, 5. mismatches between query and subject, 6. gap openings between query and subject, 7. query start, 8. query end, 9. subject start, 10. subject end, 11. e-value, 12. bit score, 13. query family ID, 14. subject family ID. (XLS)

Table S3 MFS expression in S2R+ cells. Cell-specific RNA expression profiling for S2R+ cells using high-density genome tiling microarrays (next generation RNAseq technology) was obtained from ModENCODE [45], and using Affymetrix Flychip Drosophila expression array technology was obtained from FLIGHT [46]. Expression cut-offs are given in brackets and expressed genes are high-lighted in green. (XLS)

Table S4 Expression data for Drosophila MFS transporters in S2R+ cells. Cell-specific RNA expression profiling of 17 Drosophila cell lines using high-density genome tiling microarrays (next generation RNAseq technology) was obtained from ModENCODE [45], and using Affymetrix Flychip Drosophila expression array technology was obtained from FLIGHT [46]. Expressed genes are highlighted in green (or blue for modest expression). (XLS)

Author Contributions

Conceived and designed the experiments: CB MDR NP. Performed the experiments: CB CDJ HHC SSJH. Analyzed the data: CB MDR NP. Contributed reagents/materials/analysis tools: CB MDR NP. Wrote the paper: CB.

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