

Supplemental Methods

Fly stocks and culture

Additional stocks used in the study include *yellow white* (*yw*), Canton S (BSCD stock ID #1), *w*¹¹¹⁸, and Oregon R, and *w*⁻; *Uro-Gal4/flyCyO*; *tub-Gal80*^{ts7} (*Uro-Gal4*^{ts}, *w*⁻; *Uro-Gal4/CyO* to generate this stock was kindly provided by Julian Dow, University of Glasgow, UK). Transgenic flies carrying RNAi constructs were obtained from the Transgenic RNAi Project (TRiP) [74] and Vienna Drosophila RNAi Center (VDRC) [75]. Additional stocks used: *w*⁻, *UAS-rpr(l)*(Bloomington),

Standard fly culture was performed at 25°C on 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, 75 ml/l corn syrup, 4 ml/l propionic acid and 250 mg/l Tegosept (Spectrum M1187). This standard medium (SM) was supplemented with 30 mM sodium phosphate (pH 6.0)(P30), 30 mM sodium sulfate (pH 6.0)(S30), 0.1-10 mM phosphonoformic acid (Sigma P6801)(PFA0.1-10), or 0.1-1% sevelamer (gift from Dr. Yves Sabbagh, Genzyme, Inc.)(Sev0.1-1%).

Supplementation of SM with 30 mM sodium sulfate was tolerated, while 30 mM sodium phosphate reduced life span. However, 60 mM sodium sulfate did reduce life span (Supplemental Fig. S3B), which is why 30 mM sodium phosphate was used for all subsequent experiments.

Defined medium (DM) was prepared according to Roberts [22] in which casein as a protein source is replaced with egg whites (30 g/l agar, 55 g/l egg white (MP Biomedicals 901633), 7.5 g/l sucrose (Sigma S0389), 0.3 g/l cholesterol (Sigma C3045), 0.24 g/l choline chloride (Acros 110290500), 0.64 g/l inosine (MP 102049), 0.57 g/l uridine (Alfa Aesar A15227), 1 g/l NaHCO₂ (Sigma S5761), 0.71 g/l KH₂PO₄ (Fisher BP362-500), 3.73 g/l K₂HPO₄ (Fisher BP363-500), 0.62 g/l MgSO₄·7H₂O (Sigma M9397), 0.1218 g/l K₂SO₄ (Sigma P0772), and a multivitamin mixture containing 0.002 g/l thiamine (MP Biomedicals 194749), 0.01 g/l riboflavine (Sigma R9504), 0.012 g/l nicotinic acid (Acros 128290050), 0.016 g/l Ca-pantothenate (Acros 243300050), 0.0025 g/l pyridoxine (MP Biomedicals 194733), 0.0002 g/l biotin (Acros 230095000), 0.003 g/l folic acid (MP Biomedicals 101725)). We added 0.003% propionic acid (Malincrodt 7179-04) and 1 mg/ml Tegosept (Spectrum M1187) to inhibit fungal growth.

To quantify larval development on different media, we prepared culture vials with SM and vehicle or supplements as described above. Care was taken to avoid variation in fluid content. Specifically, all supplements were premixed as 10X aqueous solutions and added to SM to a final concentration of 1X in 5 ml SM/culture vial).

Dye-feeding assay

The dye-feeding assay was modified from Edgecomb et al. (1994). Briefly, male

flies aged 3–10 days were switched from SM to SM supplemented with 1% FD&C Blue No. 1 (Sigma) and 1% Phenol Red (Sigma) for 1 hr or 18 h at 25°C. As described by Edgecomb et al. [24], FD&C Blue No. 1 remains in the digestive tract and is not affected by gut pH and enzymes, whereas Phenol Red is

absorbed and excreted with the urine. To determine the dye consumption rate after feeding for 60 min., flies were transferred to SM w/o dyes for 60 min. Fly heads were removed to prevent eye pigment from interfering with measurement of the dye. Fly bodies were individually homogenized using a Kontes pestle homogenizer in 100 μ l water. To evaluate dye excretion rate, flies fed on SM with dye overnight were transferred to SM for 60 min., followed by 60 min. collection of blue/red excretions in a 1.5 ml vial. Flies were discarded and excretions dissolved in 100 μ l water prior to measurement. Cleared supernatants were measured at 430 nm (phenol red) and 630 nm (FD&C blue-1) using a Nano-drop 6000 (Fisher). A theoretical ratio of 5.2 (OD₄₃₀/630 nm) is expected, if no additional Phenol Red was excreted with urine, whereas ratios of more than 5.2 may indicate excretion of Phenol Red in excess of the amount present in the feces.

Genome-wide *Drosophila* cell-based RNAi screen

The genome-wide dsRNA screening collection of the *Drosophila* RNAi Screening Center (DRSC) covering >95% of the *Drosophila* genome has been described previously [25]. This library contains one or two unique dsRNAs per gene for approximately 14,000 genes. The library was designed based on current rules for minimizing off-target effects and is annotated based on a recent FlyBase release (see also www.flyrnai.org). Our assay was based on the assay used in a previous screen using insulin, EGF and PVF as stimulus [29]. Briefly, two copies each of sixty-two unique 384-well plates pre-aliquoted with the DRSC library were seeded with S2R+ cells, a variant adherent S2 line [26], at 20,000 cells/well and incubated in serum-free medium for 45 minutes. Next, serum-containing medium was added and the cells were incubated for four days at 25°C. On day four, 60 mM Na-phosphate buffer (pH 7.4) was added in 10 μ l/well for a final concentration of 10 mM. Following stimulation at 25°C for 10 min., cells were fixed by the addition of 10 μ l/well of 28% formaldehyde for a final concentration of 4% for 10 minutes and washed twice with PBS+0.1%Triton (PBST). A monoclonal antibody directed against dual-phosphorylated mammalian ERK1/2 was obtained from Cell Signaling Technologies, Inc (#CS4370; see also [29]). The antibody was added in PBST+3% bovine serum albumin (BSA) at a final concentration of 1:400 in 10 μ l/well. After incubation for 18-24 hrs at 4°C, the wells were rinsed three times with 50 μ l PBST, followed by incubation with PBST+3% BSA containing an anti-rabbit secondary antibody directly conjugated to Alexa 647 (Invitrogen #A21246, working concentration 1:2000) and 1 ng/ml DAPI (Roche #10 236 276 001) at room temperature for 2 hrs. In initial experiments, Dead Red (Molecular Probes Kit# L7013, Invitrogen) was used as a protein stain according to the manufacturer's instructions to validate cell counts obtained with DAPI. Following three additional rinses with PBST, the plates were read in 30 μ l PBST/well using a Molecular Devices Analyst GT micro-well plate fluorometer and Typhoon 9410 Imager (Amersham/ GE Healthcare) to detect phospho-ERK, protein and DNA content. Knockdown of *Ras85D*, and *Dsor1/MEK* upstream of ERK reduced fluorescence, whereas knockdown of *Rho* increased fluorescence in control wells of each assay plate, supporting the idea that the screen assay was working as expected. The chemical MEK inhibitor UO126 (final concentration 30 μ M), and *thread* RNAi which causes cell death were used as an additional controls on each assay plate (Fig. S-6A-C).

Primary screen data analysis

Our data analysis pipeline was directed towards removing as many sources of noise as possible. Many of the issues we faced have been recently reviewed [76] and variations of the approaches described in the review were applied to this work. Raw dpERK, DAPI and Dead Red intensities were first background subtracted to account for autofluorescence of the plastic screening plates. To correct for spatial irregularities in the fluorescence measurements across individual screening plates (*e.g.* “edge-effects”), dpERK values were corrected by dividing by the moving median of each well’s column and row. Corrected dpERK values were then divided by corrected DAPI or Dead Red values for each channel to yield a normalized dpERK value. Median and IQR of each 384-well plate were used for Z-score transformation of normalized corrected dpERK values, an approach that is less sensitive to outliers than use of standard deviation and means. Z-scores for replicate wells for each unique dsRNA were then averaged. Genes whose average Z-scores met a cutoff of ± 1.0 with at least one of the targeting RNAi-amplicons were subjected to further evaluation using option #1 of the screen analysis tool RNAi-Cut (<http://groups.csail.mit.edu/cb/RNAiCut/>) [77], which automatically defines a cut-off depending on hit-connectivity using protein-protein interaction (PPI) networks and lowered the final z-score cut-off to approximately ± 1.3 . By comparison, control dsRNAs targeting *drk/GRB2*, and *Tao1*, as well as the chemical MEK inhibitor UO126 (30 μ M) achieved larger z-scores (Supplemental Fig. S6B-C). We next annotated our hits list for expression in the screening cell line, molecular function, conservation, and other features by cross-referencing with publicly-available databases *i.e.* DAVID [27], fly-MINE [28] and manual curation of the list as described in Methods. Care was taken to use follow-up dsRNAs without significant predicted off-target effects (OTEs) [78,79]. To identify targets that might preferentially regulate phosphate-induced MAPK, in the validation round, we assayed 12 replicate plates each with cells treated with 10 mM Na-phosphate buffer or with 25 μ g/ml human insulin as stimulus. After wandering median correction, corrected normalized dpERK values were again transformed into z-scores as above and subjected to a two-sided t-test. Of a total of 555 genes 146 could be verified at $p < 0.05$. Of these, 84 preferentially interfered with phosphate-induced MAPK at the concentrations used. From the remaining 62 non-selective genes we eliminated 43 “frequent hitters” in RNAi screens because they were identified in more than three of 44 prior DRSC screens and further evaluated 103 genes.

in vivo Drosophila RNAi screen

Transgenic RNAi lines targeting 103 genes were obtained from the Transgenic RNAi Project (TRiP, <http://www.flyrnai.org/TRiP-HOME.html>) and the Vienna Drosophila RNAi Center (VDRC, <http://stockcenter.vdrc.at/control/main>). The “virginator” stock *w⁻; hs-hid(y)/w⁻; tub-Gal80^{ts20}; da-Gal4* was used to obtain large numbers of virgin females for the initial screen that were crossed with transgenic males from the TRiP or VDRC collections. F1 offspring of the genotypes: *UAS-RNAi/w⁻; tub-Gal80^{ts20}/+; da-Gal4/+*, *w⁻; UAS-RNAi/tub-Gal80^{ts20}; da-Gal4/+*, or *w⁻; tub-Gal80^{ts20}/+; UAS-RNAi/da-Gal4* were then used for subsequent experiments and hereafter, will be referred to using simply the “driver>RNAi line ID or target gene name” convention. The TRiP collection used three different RNAi vectors for hairpin expression in the soma: valium 10 contains long dsRNAs with homology to the

targeted gene over several hundred base pairs, while valium 20 and 22 contain short hairpins containing a 21 bp homology domain for the target gene. All vectors were inserted in a single AttP2 site on chromosome 3. The VDRC library uses long dsRNAs in a modified pUAST vector, pMF3 (“GD” library; randomly integrated), or pKC26 and 43 (“KK” library; inserted in a single AttP VIE-260B landing site on chromosome 2). Hairpin effects were comparable between males and females, and all experiments were repeated at 18°C to show that effects observed at the inducing temperature were specific to the RNAi (Fig. S1A, Fig. S9).

Results with additional control lines were: Life span of F1 males generated with the attP2 landing site strain *y sc v* (TRiP #TB00018) was 38+/-1.4 days. Life span of F1 males generated with *w¹¹¹⁸* isogenic flies (VDRC #60000) was 42+/-0.9 days, and of *y w[1118];P{attP,y[+],w[3]}* (VDRC #60100) was 43+/-1.4 days. Median±IQR of median life spans across the entire screen for F1 males generated with TRiP lines was 34+/-8 days (n=165, 15794 flies), for VDRC GD lines was 38+/-5 days (n=34, 2450 flies), and for VDRC KK lines was 40+/-3 days (n=53, 3554 flies).

Hemolymph phosphate of F1 generation females for the control strain *y sc v* (TRiP TB00018) was 23.5+/-1.5 mg/dl. Hemolymph phosphate of F1 females of *w¹¹¹⁸* isogenic females (VDRC #60000) was 32+/-1.2 mg/dl, and of *y w[1118];P{attP,y[+],w[3]}* (VDRC #60100) was 30+/-1.3 mg/dl. Median±IQR of hemolymph concentrations across the entire screen for F1 females generated with TRiP lines was 29+/-6 mg/dl (n=146), for VDRC-GD lines was 32+/-5 mg/dl (n=32), and for VDRC-KK lines was 32+/-5 mg/dl (n=53).

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