

# Supporting Information

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## SI Methods

**Fly Stocks.** All crosses and experiments were kept at 25 °C in light-controlled incubators in 12-h:12-h light-dark cycles. Five- to 10-d-old flies were used for all experiments unless otherwise indicated. Flies were raised using a cornmeal/yeast/agar diet [1.2% (vol/vol) autolyzed yeast, 5.5% (vol/vol) cornmeal, 6% (vol/vol) dextrose, 0.55% agar supplemented with 0.18% Nipagin and 2.9 mL/L Propionic acid]. The transgenic lines used in the study were: *PDF-GAL4* (1), *PdfR*<sup>5304</sup> and *PdfR*<sup>3369</sup> (2), *UAS-Epac1-camps*<sup>50A</sup> (3), *Mef2-GAL4* (4), *24B-GAL4* (5, 6), *UAS-PdfR*<sup>16L</sup> (7), *Myo1a-GAL4* (8), *UAS-eGFP* (9), *UAS-Stinger* (10), *UAS-mCD8-GFP* (11), and *UAS-dTrpA1* (12). All transgenic flies were used in a *yellow*<sup>+</sup> genetic background to prevent physiological phenotypes resulting from absence of *yellow* in intestinal/tubule epithelia. Oregon R (OreR), Canton S or *w*<sup>1118</sup> flies were used as control flies.

**Fixed Tissue Immunohistochemistry.** Adult tissues were dissected, mounted on poly-L-lysine-coated slides and fixed in 4% (wt/vol) paraformaldehyde for 20 min. Subsequent washes and incubations were done in PBS with 0.2% Triton. Tissues were incubated overnight with primary antibody at 4 °C, followed by a 2-h incubation with secondary antibodies at room temperature the next day. Antibodies used were: goat  $\alpha$ -GFP (Abcam; 1:2,000), mouse  $\alpha$ -nc82 (Developmental Studies Hybridoma Bank; 1:50), mouse anti-pigment-dispersing factor (anti-PDF) propeptide (PDF C7, Developmental Studies Hybridoma Bank; 1:50), and rabbit  $\alpha$ -Odd-skipped (13) (1:1,000). Phalloidin-Cy5 or Phalloidin-Alexafluor-633 (Molecular Probes) were used at 1:200. Alexafluor-488-, FITC-, Cy3-, and Cy5-conjugated secondary antibodies were obtained from Jackson Immunolaboratories and used at 1:200 (1:100 for the Cy5-conjugated antibody). All preparations were mounted in Vectashield with or without DAPI (Vectorlabs) and images were acquired using Leica SP5 or Olympus FV1000 confocal microscopes.

**Transmission Electron Microscopy.** Transmission electron microscopy (TEM) was carried out using standard procedures. Briefly, dissected guts and associated tubules were dissected and fixed overnight in 5% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer at 4 °C. The next day, viscera were postfixed in osmium tetroxide, bulk-stained in 2% (wt/vol) uranylacetate, dehydrated, and embedded in araldite. Sections were cut at 50–60 nm with a Leica Ultracut UCT and mounted on 100-mesh copper grids. They were double stained with 2% uranylacetate in 50% (vol/vol) methanol followed by lead citrate, and imaged in an FEI Tecnai G2 TEM operated at 120 kv. Images were captured with an AMT XR60B digital camera running Deben software.

**Visceral Contraction Measurements Using PDF Peptide.** For our initial experiments, flies were anesthetized on ice and adult intestines and their associated Malpighian tubules were dissected into ice-cold Tübingen and Düsseldorf *Drosophila* Ringer's solution containing 46 mM NaCl, 180 mM KCl, 2.2 mM CaCl<sub>2</sub>, and 10 mM Tris (pH 7.2) using fine forceps and microscissors. The dissected viscera were gently unfurled and stuck to the bottom of a 35-mm Grenier culture dish (Grenier Bio One) under 1.8 mL hemolymph-like saline (HL3) (14) containing 70 mM NaCl, 5 mM KCl, 1.5 mM CaCl<sub>2</sub>, 20 mM MgCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub>, 5 mM trehalose, 115 mM sucrose, and 5 mM Hepes (pH 7.1). Viscera were stuck down on the adherent dish with the Malpighian tubules situated at right angles from the gut (Fig. S2). To ensure that the tubule

specificity of PDF's effects was not a result of the use of ice-cold Tübingen and Düsseldorf *Drosophila*, we repeated our bath-applied PDF experiments using CO<sub>2</sub> anesthetization and room temperature HL3 for dissection. These two preparations are referred to as "Cold Ringer's" and "Warm HL3" in the text, figures, and legends.

The small and fragile nature of the *Drosophila* ureter made mechanical recording of its contractions problematic. We therefore used movie recordings of the midgut/hindgut/ureter junction as a means to quantify such contractions. Movies were captured under an Olympus S2 × 7 stereomicroscope fitted with a DP21 CCD camera and control unit (Olympus). Six-minute, 12-s movies, the maximum movie length for the DP21 control unit, were captured for each experiment. Visceral contractions of the guts and ureters were defined as a perceptible constriction of a segment of gut or ureter and recorded visually during movie playback with the observer blind to treatment and genotype. Motility data were expressed as contractions per minute (cpm) by dividing the total number of contractions observed during each 6-min, 12-s movie by 6.2. Ureter and midgut contractions were recorded in independent viewings.

PDF peptide was added as a 200- $\mu$ L volume of 10 $\times$  peptide in 1% (vol/vol) DMSO in HL3, yielding a final DMSO concentration of 0.1%. Basal contraction rates were recorded for viscera exposed to neither vehicle nor peptide. Vehicle controls were added as 200- $\mu$ L volumes of 1% DMSO. For the PDF dose-response curves wild-type male and female (Canton S) flies were dissected and treated as described above with a series of PDF concentrations or vehicle. Movies of PDF- or vehicle-treated viscera were taken starting 5 min after the addition of peptide/vehicle. Dose-response curves were created by a nonlinear regression analysis using the least squares fitting method in Prism 5 for Macintosh (Graphpad). The resulting curves were used to determine EC<sub>50</sub>s. For the PDF responses of *w*<sup>1118</sup> and *PdfR* mutants to 10<sup>-7</sup> M PDF, 6-min, 12-s baseline movies were recorded before the addition of peptide/vehicle, a second 6-min, 12-s movie was captured 5 min after the addition of peptide/vehicle. We conducted pair-wise comparisons of basal and PDF-treated contraction rates by means of Mann-Whitney *U* tests using Prism 5 (*n* = 7–10 for each condition).

**In Situ Detection of PdfR mRNA.** A digoxigenin-labeled PdfR antisense probe was synthesized using RH51443 cDNA as template, and was purified using mini Quick Spin Columns (Roche). Adult visceral tissues were dissected and fixed on poly-L-lysine-coated slides. In situ hybridization was carried out as previously described (15). Samples were prehybridized for 1 h at 58 °C, and were hybridized overnight at the same temperature. Following incubation with an antidigoxigenin antibody conjugated with alkaline phosphatase, colorimetric detection of the probe was performed with the NBT/BCIP substrates. Wild-type and PdfR mutant digestive tracts were simultaneously dissected and processed on the same slide (*n* = 5 for each genotype).

**Real-Time PCR Analysis of Visceral PdfR Expression.** Primers for PdfR RNA were designed to amplify a 225 base sequence within a PdfR exon that is deleted by the PdfR<sup>5304</sup> mutation. The forward and reverse primers for PdfR were TAATGAAGC-TGCGTCAATCG and CCTCGCCATTTAGAAAGCAG, respectively. PdfR RNA levels were normalized to Rpl32 RNA, which encodes a ribosomal protein that serves as a convenient housekeeping gene (16). The forward and reverse primers for

*Rpl32* were CGGATCGATATGCTAAGCTGT and GCGC-TTGTTTCGATCCGTA, respectively. The RNA extraction protocol used was previously described (17). Briefly, individual flies were dissected within 2-min windows and tissues of interest were placed into ice cold TRIzol (Invitrogen). All tissues were homogenized within two hours of dissection. RNA was extracted with chloroform (Acros Organics) and precipitated with isopropanol (Sigma-Aldrich). RNase-free conditions were maintained by cleaning all tools and surfaces with Ambion RNaseZap (Invitrogen) and using RNase free solutions. Following extraction, RNA was stored at  $-20^{\circ}\text{C}$  for 3 d or less before it was used for cDNA synthesis, which was performed using the SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen). Real-time PCR was performed using Absolute Blue qPCR SYBR Green Low ROX Mix (Thermo Fisher Scientific) in a 7500 Fast Real-Time PCR System (Applied Biosystems).

**cAMP Live-Imaging.** Epac1-camps FRET imaging was performed on the cold Ringer's visceral preparation described above using male *w<sup>1118</sup>;Mef2-GAL4/UAS-Epac1camps<sup>50</sup>*; and *PdfR<sup>5304</sup>;Mef2-GAL4/UAS-Epac1camps<sup>50A</sup>* flies. Ureters were imaged with an Olympus FV 1000 scanning laser confocal microscope, through a LUMPL 20 $\times$ /0.50 water objective with immersion cone and correction collar (Olympus). Frames were scanned once every 5 s with a 440-nm laser using a DM405-440/515 dichroic mirror and CFP/YFP emission was separated by means of a SDM510 dichroic. For each region of interest (ROI), an average spillover-corrected FRET ratio was determined for each time-point by the following equation:

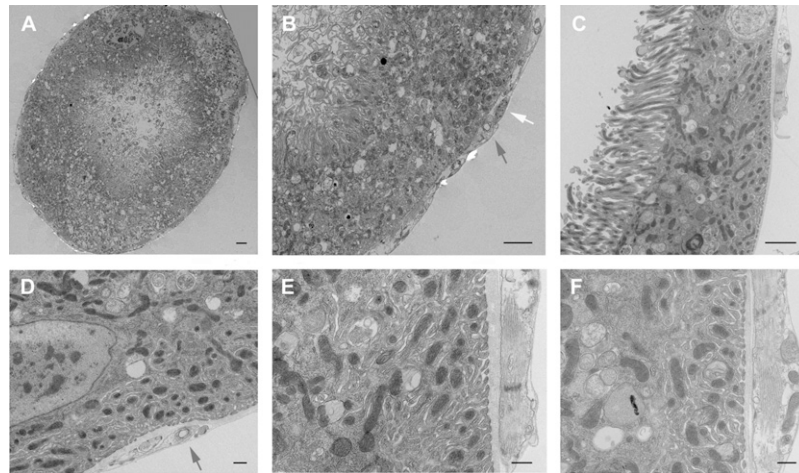
$$\text{Spillover Corrected FRET} = (\text{YFP} - (\text{CFP} * 0.444)) / (\text{CFP}), \quad [\text{S1}]$$

where CFP and YFP are the CFP and YFP emission intensities and 0.444 is the proportion of CFP emission spillover into the YFP channel on our imaging system. Using Olympus's Fluoview software, ROIs were selected over individual muscles of a single ureter, making sure to make each ROI large enough to ac-

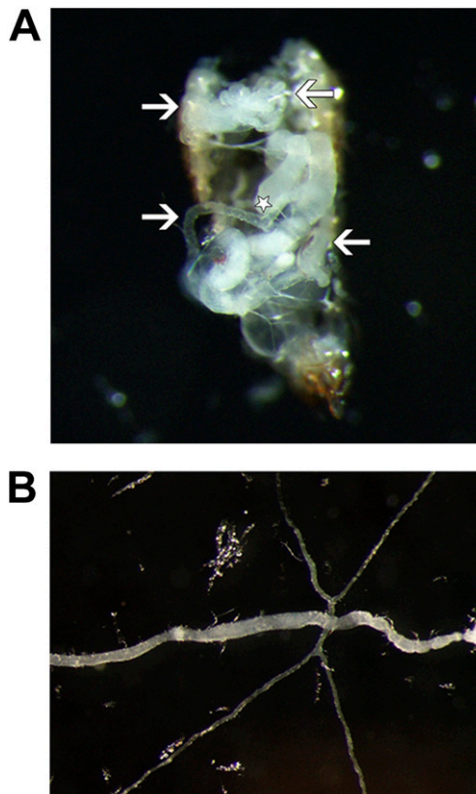
commodate the movement of the muscle during PDF-induced contractions (Fig. S5). Thus, each ROI contained pixels corresponding to both background Epac1-camps expressing ureter muscle. Confocal apertures for CFP and YFP emission were increased relative to the optimal confocal diameters to increase the thickness of the optical section as an additional means to accommodate the moving ureters. FRET traces were filtered with a six-timepoint moving average, and normalized to the value of the first timepoint (= 1.0). We created average traces in Excel (Microsoft) by calculating the average ratio and SE at each timepoint for each treatment and genotype. Pooled time-courses were compared by means of repeated measures ANOVAs with Bonferroni posttests using Prism 5 for Macintosh (Graphpad). Peptide or vehicle was added as described for the contraction assays above.

***Drosophila* TrpA1 Activation of Visceral PDF Axons.** Flies for this experiment were grown at  $18^{\circ}\text{C}$ . After eclosion, flies were maintained at  $22^{\circ}\text{C}$  for 5–8 d and were then transferred (without using  $\text{CO}_2$ ) to room-temperature HL3 buffer, in which their digestive tracts and Malpighian tubules were dissected. Explanted viscera were stuck to the bottom of a 35-mm Grenier culture dish (or Falcon Easy Grip) as described above under visceral contraction measurements. The dish was kept in the dark at room temperature for 10 min, and was then placed onto a  $33^{\circ}\text{C}$  water-heated Plexiglass chamber for 3 min, until the temperature in the Petri dish had reached  $31^{\circ}\text{C}$ . Explanted viscera were allowed to stand for another 5 min at  $31^{\circ}\text{C}$ . Five-minute movies were then captured at  $31^{\circ}\text{C}$  under a Leica MZ16F stereomicroscope fitted with a Leica DFC420C camera. Visceral contractions and motility data were recorded and annotated, respectively, as described above for visceral contraction measurements. Pooled experiments were tested for normality using the Shapiro–Wilk test. This revealed a requirement for nonparametric analyses and the Friedman rank-sum ANOVA with an asymptotic general independence test and paired-comparisons was using the R statistical environment.

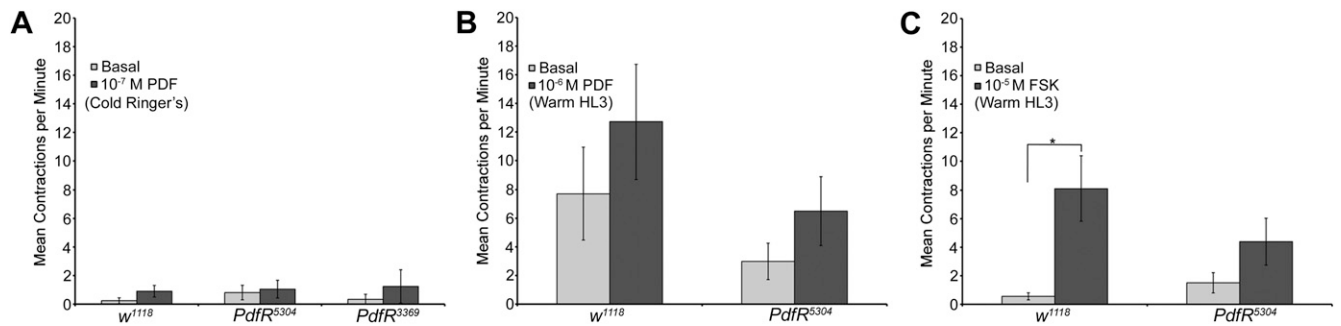
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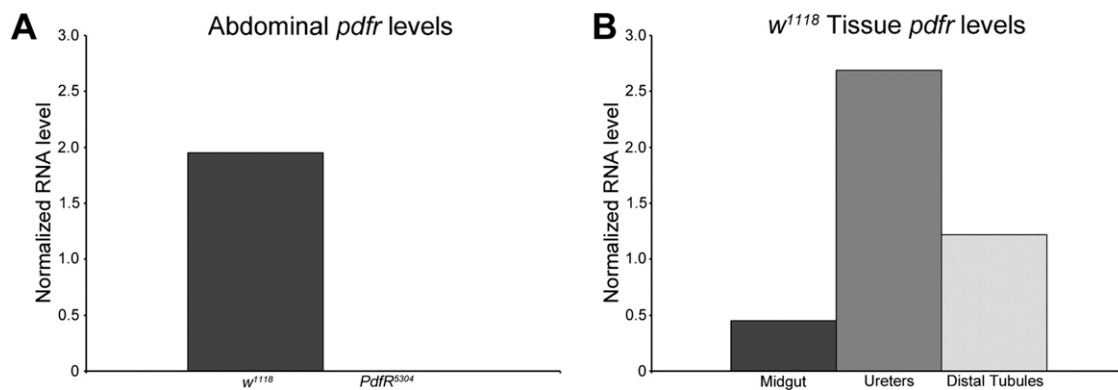
**Fig. S1.** Transmission electron micrographs of ureter cross-sections reveal its lack of innervation. (A) Representative whole ureter cross-sections. (B) Two types of cells surround the renal epithelium: visceral muscle cells (white arrow) and trachea (gray arrow). (C) Renal epithelial cell with an apical brush border (Left). A visceral muscle cell abuts the extracellular matrix on its basal side. (D) A tracheal branch (gray arrow) is surrounded by extracellular matrix on the basal side of an epithelial cell, and it is flanked on both sides by visceral muscles. (E and F) Detail of the basal side of an epithelial cell (Left), its basal lamina and a visceral muscle section (Right; note the myofilaments in its cytoplasm) in two different ureters. A lack of innervation is indicated by the absence of cytoplasm with the characteristic microtubular ultrastructure of neurons, dense core vesicles, synapses or glial wrappings in all these micrographs. (Scale bars: 2  $\mu$ m in A–C; 500 nm in D–F.)



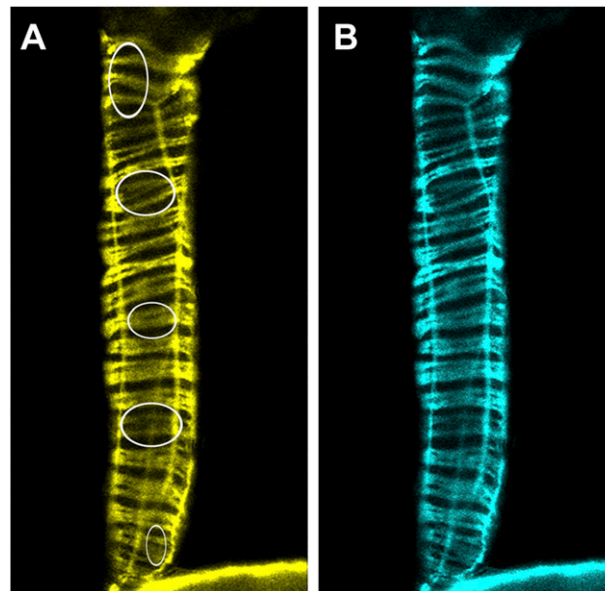
**Fig. S2.** Preparation of viscera for movie recording. (A) Abdomen of a  $w^{1118}$  male fly with the ventral cuticle removed before it has been fully dissected. Arrows indicate branches of the Malpighian tubules and the star indicates one of the ureters. Note that one ureter/tubule runs to the anterior portion of the intestine (Upper two arrows) and the other is associated with the posterior portion of the intestine and the reproductive organs (Lower two arrows). (B) An image of  $w^{1118}$  male viscera arranged for movie recording. The Malpighian tubules have been dissociated from anterior and posterior viscera and the reproductive organs have been removed. (Magnification: A, 5.6 $\times$ ; B, 4.5 $\times$ .)



**Fig. S3.** The effects of PDF and forskolin (FSK) on the rates of midgut contraction in wild-type and *PdfR* mutant viscera. (A) Mean basal and PDF-treated (10<sup>-7</sup> M) midgut contraction rates from viscera dissected from wild-type (*w<sup>1118</sup>*) and *PdfR* mutants (*PdfR<sup>5304</sup>* and *PdfR<sup>3369</sup>*) using cold Ringer's (see *SI Methods*). There were no statistically significant differences in basal and PDF-treated midgut contraction rates for any genotype ( $P = 0.2500$  for *w<sup>1118</sup>*,  $P = 0.7500$  for *PdfR<sup>5304</sup>*, and  $P = 0.5000$  for *PdfR<sup>3369</sup>*). (B) Mean basal and PDF-treated (10<sup>-7</sup> M) midgut contraction rates from viscera dissected from wild-type (*w<sup>1118</sup>*) and *PdfR<sup>5304</sup>* mutants using warm HL3 (see *SI Methods*). Neither genotype displayed significant differences between basal and PDF-treated contraction rates ( $P = 0.2188$  for *w<sup>1118</sup>* and  $P = 0.1994$  for *PdfR<sup>5304</sup>*). (C) The response of wild-type and *PdfR<sup>5304</sup>* mutant midguts to bath-applied FSK from viscera dissected using warm HL3. The midguts of wild-type (*w<sup>1118</sup>*) viscera responded to 10  $\mu$ M FSK with significant increases in contraction rates compared with the basal contraction rates ( $P = 0.0151$ ). Although the mean contraction rate of *PdfR<sup>5304</sup>* mutant midguts was slightly higher in the presence of 10  $\mu$ M FSK, the difference was not statistically significant ( $P = 0.1138$ ). \* $P < 0.05$ .



**Fig. S4.** Real-time PCR analysis of *PdfR* RNA levels in visceral tissues of wild-type flies. (A) Comparison of *PdfR* levels from whole abdomen extracts of *w<sup>1118</sup>* and *PdfR<sup>5304</sup>* flies ( $n = 12$  for both). The amount of RNA, normalized to the housekeeping gene *Rpl32*, was negligible in the mutant (0.0029), indicating that the primers designed to amplify a C-terminal region of *PdfR* specifically amplify *PdfR* RNA. (B) A comparison of normalized *PdfR* RNA levels in the midguts ( $n \sim 70$ ), ureters ( $n \sim 120$ ), and distal renal tubules ( $n \sim 100$ ) of *w<sup>1118</sup>* flies.

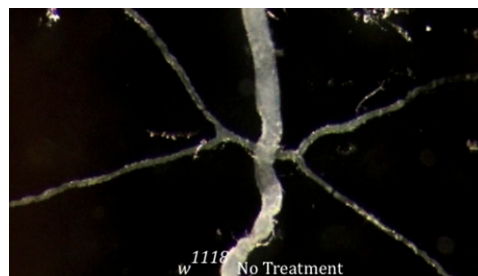


**Fig. S5.** Epac1-camps expression in the ureter muscles. Images showing YFP (A) and CFP (B) expression in the ureter of a *w;UAS-Epac1-camps/+;Mef2-GAL4/+* fly. The ovals on the YFP image represent the typical size and distribution of ROIs from which we calculated the change in FRET. The ureter was imaged using a 20 $\times$  dipping cone objective with a 2 $\times$  digital zoom.



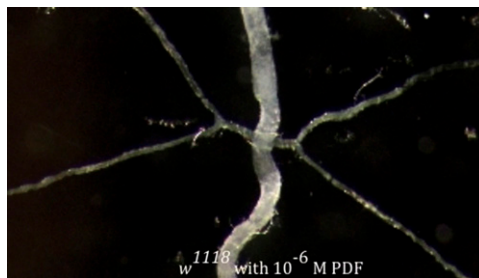
**Movie S1.** A representative movie of a Canton S intestine that has had no treatment played at 2 $\times$  speed. (Magnification: 4.5 $\times$ .)

[Movie S1](#)



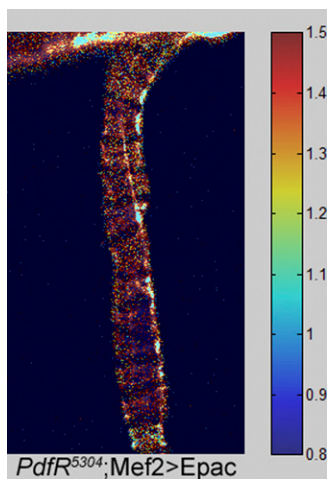
**Movie S2.** A representative movie of a *w<sup>1118</sup>* intestine with no treatment played at 2 $\times$  speed. (Magnification: 4.5 $\times$ .)

[Movie S2](#)



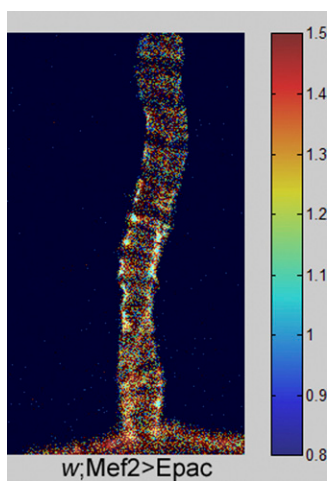
**Movie S3.** A movie of the intestine from [Movie S2](#), treated with bath applied  $10^{-6}$  M PDF. The movie is also at 2 $\times$  speed. (Magnification: 4.5 $\times$ .)

[Movie S3](#)



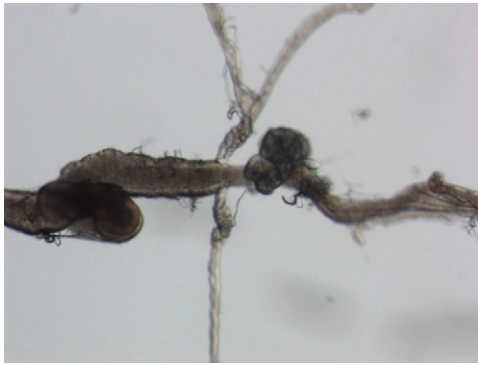
**Movie S4.** A movie showing the FRET response of *PdfR<sup>5304</sup>;UAS-Epac1-camps/+;Mef2-GAL4/+* to bath-applied  $10^{-6}$  M PDF. The movie was captured using a 20 $\times$  dipping cone objective and a 2 $\times$  digital zoom.

[Movie S4](#)



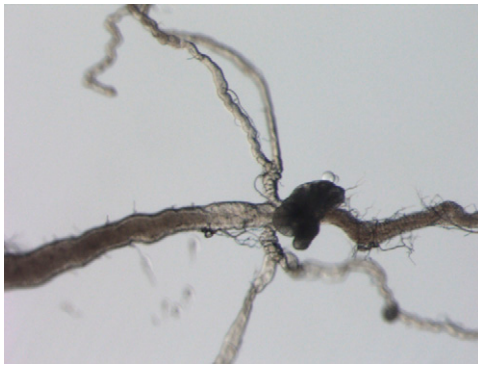
**Movie S5.** A movie showing the FRET response of *w;UAS-Epac1-camps/+;Mef2-GAL4/+* to bath-applied  $10^{-6}$  M PDF. A decrease in FRET indicates an increase in cAMP. PDF was added in the second second of this 40-s movie (representing about 40 s of actual time). The movie was captured using a 20 $\times$  dipping cone objective and a 2 $\times$  digital zoom.

[Movie S5](#)



**Movie S6.** A representative movie of a *UAS-dTrpA1/+* control intestine 5 min after the shift to 31 °C, played at 2× speed. (Magnification: 8×.)

[Movie S6](#)



**Movie S7.** A representative movie of a *PDF > dTrpA1* intestine 5 min after the shift to 31 °C, played at 2× speed. (Magnification: 8×.)

[Movie S7](#)