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Supplemental Information

Phosphatidylinositol 5 Phosphate 4-Kinase Regulates Plasma-Membrane PIP_3 Turnover and Insulin Signaling

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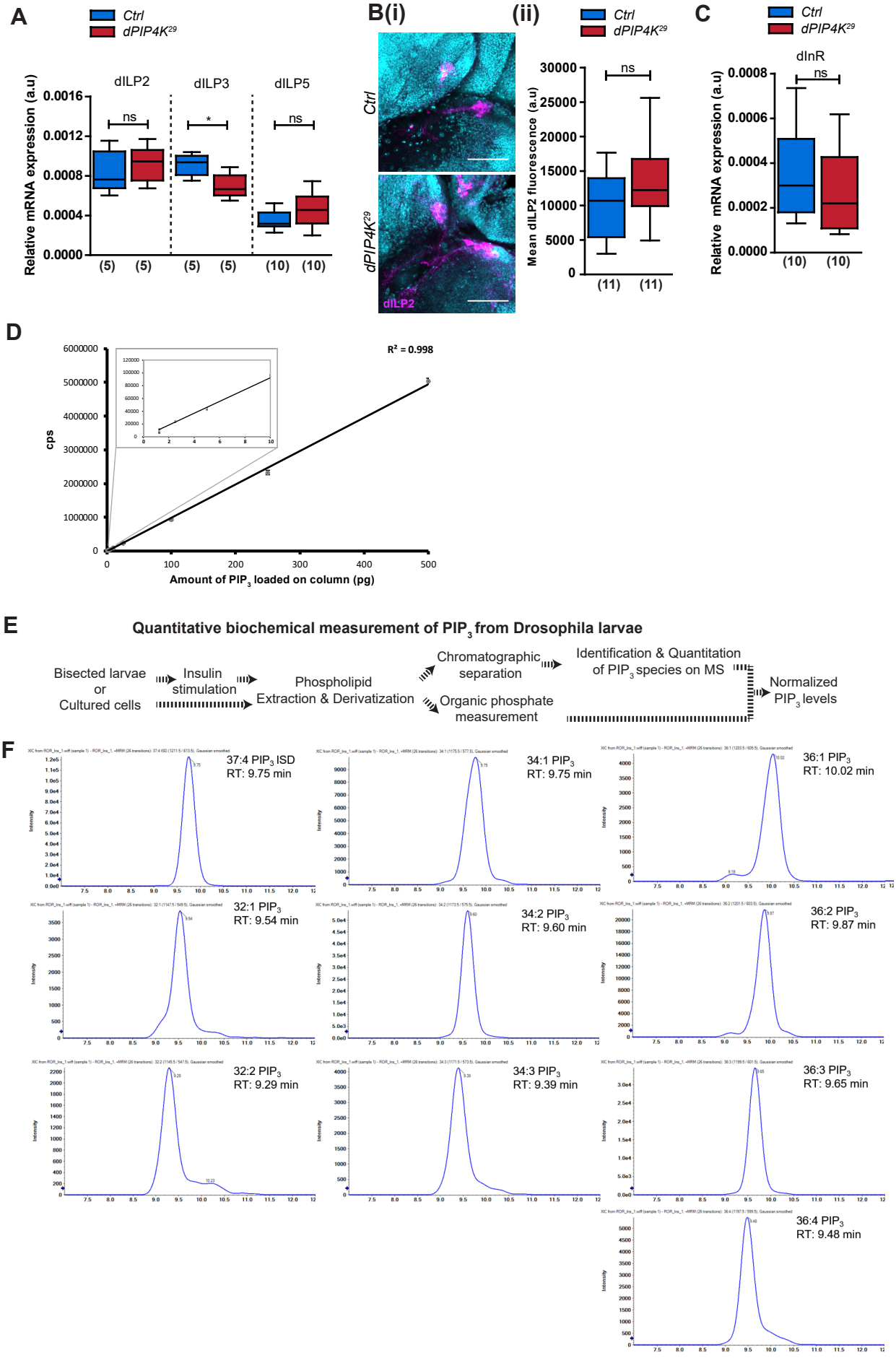
A
Ctrl
dPIP4K29
dILP2
dILP3
dILP5
Relative mRNA expression (a.u)
0.0016
0.0012
0.0008
0.0004
0.0000
ns
*
ns
(5) (5) (5) (5) (10) (10)
B(i)
Ctrl
dPIP4K29
dILP2
(ii)
Ctrl
dPIP4K29
Mean dILP2 fluorescence (a.u)
30000
25000
20000
15000
10000
5000
0
ns
(11) (11)
C
Ctrl
dPIP4K29
dInR
Relative mRNA expression (a.u)
0.0008
0.0006
0.0004
0.0002
0.0000
ns
(10) (10)
D
R² = 0.998
cps
6000000
5000000
4000000
3000000
2000000
1000000
0
0 100 200 300 400 500
Amount of PIP3 loaded on column (pg)
E
Quantitative biochemical measurement of PIP3 from Drosophila larvae
Bisected larvae or Cultured cells
Insulin stimulation
Phospholipid Extraction & Derivatization
Chromatographic separation
Organic phosphate measurement
Identification & Quantitation of PIP3 species on MS
Normalized PIP3 levels
F
37:4 PIP3 ISD
RT: 9.75 min
34:1 PIP3
RT: 9.75 min
36:1 PIP3
RT: 10.02 min
32:1 PIP3
RT: 9.54 min
34:2 PIP3
RT: 9.60 min
36:2 PIP3
RT: 9.87 min
32:2 PIP3
RT: 9.29 min
34:3 PIP3
RT: 9.39 min
36:3 PIP3
RT: 9.65 min
36:4 PIP3
RT: 9.48 min

Figure S1. Status of humoral signals in *dPIP4K^{29}* larvae and LCMS method for PIP_3 measurement (Related to Figure 1 and STAR Methods)

A. qPCR measurements for mRNA levels of *dILP 2, 3* and *5* from whole larvae. **B(i, ii).** Confocal z-projections for dILP2 immunostaining in larval IPCs and quantification of dILP2 staining intensity in the third instar wandering larval brains; Scale: 50 µm. **C.** qPCR measurements for *dInR*. Transcript levels for each gene were normalized to the mRNA levels of *rp49* in the same sample. For A-C, Mann-Whitney test used for statistical analysis; *p - *value* <0.05. **D.** Linearity of mass spectrometer response for increasing amounts of PIP_3 standard (17:0, 20:4) injected. Each point on the curve indicates the mean ± SD of three replicate injections. **E(i).** Steps involved in extraction, separation and detection of PIP_3 from larvae. **E(ii).** Chromatograms showing the elution profiles and retention times for various PIP_3 species detected from whole larval lipid extracts of wildtype *(ROR)* larvae stimulated *ex vivo* with 100 µM insulin for 10 min. Note the changing retention times with increase in no. of double bonds and increase in length of acyl chains. Increase in double bonds for a fixed acyl chain length results in earlier elution. Increase in length of acyl chain delays elution. Genotypes: A, C. *tGPH/tGPH* and *tGPH/tGPH ; dPIP4K^{29}*. B. *ROR* and *dPIP4K^{29}*.

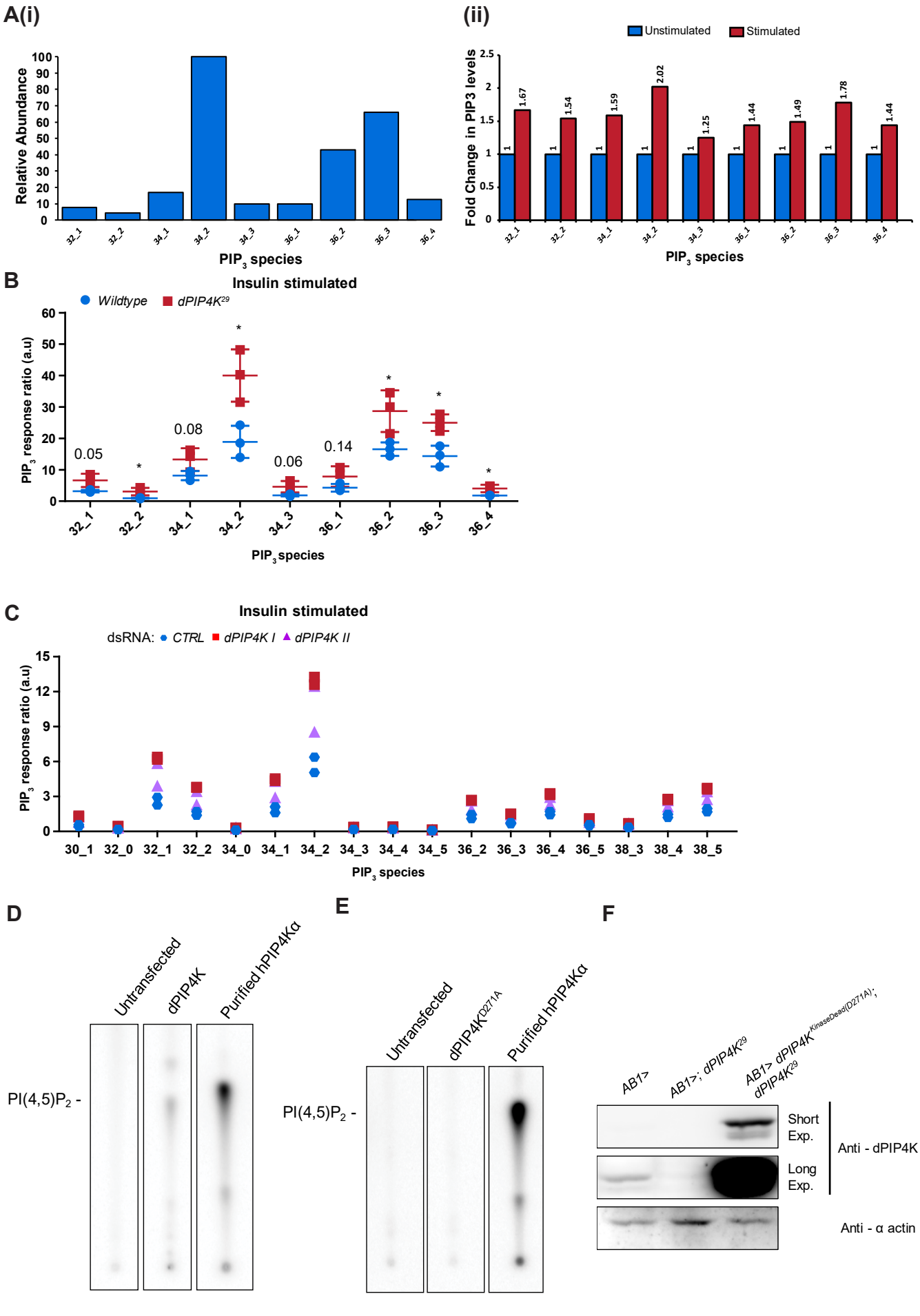
A(i)
Relative Abundance
PIP3 species
(ii)
Unstimulated
Stimulated
Fold Change in PIP3 levels
PIP3 species
B
Insulin stimulated
Wildtype
dPIP4K29
PIP3 response ratio (a.u)
0.05
0.08
0.06
0.14
PIP3 species
C
Insulin stimulated
dsRNA: CTRL dPIP4K I dPIP4K II
PIP3 response ratio (a.u)
PIP3 species
D
Untransfected
dPIP4K
Purified hPIP4Kα
PI(4,5)P2 -
E
Untransfected
dPIP4KD271A
Purified hPIP4Kα
PI(4,5)P2 -
F
AB1>
AB1>; dPIP4K29
AB1> dPIP4KKinaseDead(D271A); dPIP4K29
Short Exp.
Long Exp.
Anti - dPIP4K
Anti - α actin

Figure S2. Mass spectrometry to measure PIP_3 levels and generation of kinase-dead dPIP4K (Related to Figure 1, 2 and STAR Methods)

A(i). Relative abundance of various PIP_3 species in whole larval lipid extracts of wildtype larvae stimulated *ex vivo* with 100 μM insulin for 10 min. **A(ii).** An experiment showing changes in the levels of various larval PIP_3 species upon insulin stimulation (100 μM, 10 min). B**.** Levels of various PIP_3 species in insulin-stimulated *wildtype* (*ROR*) and *dPIP4K*^{29} whole larval lipid extracts determined using LCMS. Student's t-test used for statistical comparison between same species across genotypes; * *p - value* <0.05. C**.** Levels of various PIP_3 species in lipid extracts from insulin-stimulated S2R+ cells with or without knockdown of dPIP4K determined using LCMS. The graphs show mean PIP_3 levels (normalized to spiked internal standards and total lipid phosphates recovered). Error bars depict SD. Representative thin layer chromatographs from PI5P-4-kinase activity assays showing - D. presence of activity in wildtype dPIP4K expressing lysates and E. no activity in dPIP4K^{D271A} expressing lysates. Bacterially expressed and purified PIP4Kα was used as a positive control. F. Immunoblots from 3rd instar larval salivary glands showing levels of dPIP4K^{KinaseDead} upon reconstitution. On each panel, numbers inside the parentheses indicate the no. of biological replicates used for the measurement.

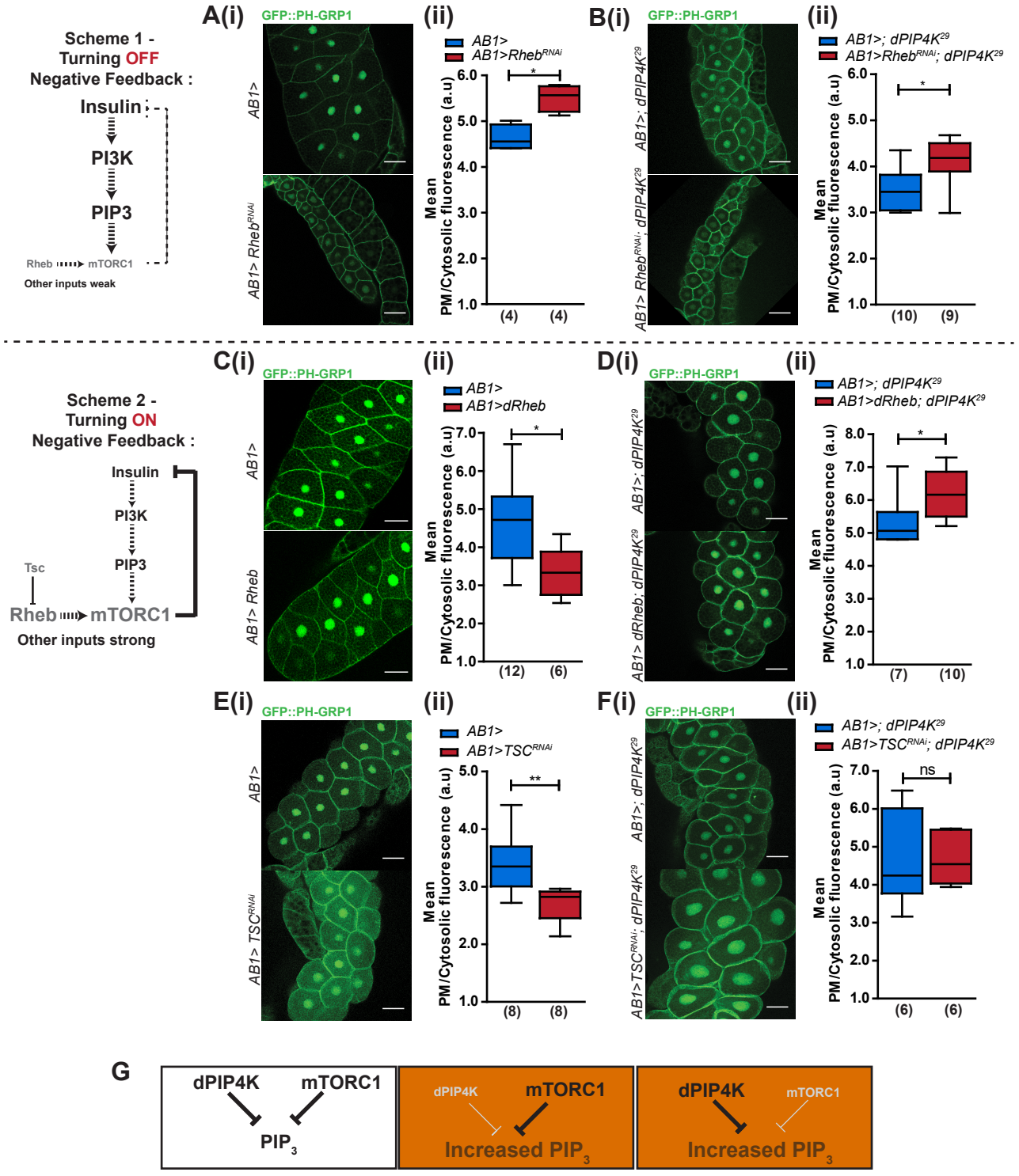

Figure S3. dPIP4K and TORC1-mediated negative feedback independently regulate plasma-membrane PIP_3 (Related to Figure 3)

Scheme 1 and 2 depict the feedback regulation of insulin signalling by TORC1 activity in **OFF** and **ON** state respectively. The font and arrow sizes are indicative of the extent of molecular activity. **A-F.** PIP_3 quantification – Salivary gland images showing the distribution of the PIP_3 binding probe GFP-PH-GRP1 in cells. The distribution was quantified as the ratio of probe fluorescence on the plasma membrane to that in the cytosol. In all these experiments, the genetic manipulation was restricted to the salivary glands using *AB1*Gal4. **A, C, E.** In a wildtype background, **A(i, ii).** downregulation of TOR signalling by RNAi for *Rheb*; **C(i, ii).** upregulation of TOR signalling through overexpression of Rheb and **E(i, ii).** downregulation of TSC. **B, D, F.** In *dPIP4K*^{29} background, **B(i, ii).** downregulation of TOR signalling by RNAi for *Rheb*; **D(i, ii).** upregulation of TOR signalling through overexpression of Rheb and **F(i, ii)**. downregulation of TSC. Scale: 50 µm. **G.** Schematic

showing basal PIP_3 levels is subject to dual negative regulation by dPIP4K and TORC1 output. Decreasing TOR activity weakens negative feedback thereby increasing plasma membrane PIP_3 levels in both *wildtype* and *dPIP4K*^{29}. Increasing TOR activity engages the negative feedback loop and brings down PIP_3 levels in wildtype cells but is ineffective and cannot do so in the absence of dPIP4K. Thus, conditions that either reduce dPIP4K levels or TORC1 activity both lead to a dysregulated increase in PIP_3 levels. Whiskers in the box plots represent minimum and maximum values, with a line at the median. Numbers inside the parentheses below the plots indicate the no. of biological replicates used for the measurement. Mann Whitney test used for statistical analysis of the distributions. **p-value* < 0.05. ***p-value* <0.01. Genotypes: A. *AB1Gal4, tGPH/+* and *AB1Gal4, tGPH/ UAS-Rheb*^{RNAi}. B. *AB1Gal4, tGPH /+; dPIP4K*^{29} and *AB1Gal4, tGPH/ UAS-Rheb*^{RNAi}*; dPIP4K*^{29}. C. *AB1Gal4, tGPH /+* and *UAS-dRheb/+; AB1Gal4, tGPH/+*. D. *AB1Gal4, tGPH /+; dPIP4K*^{29} and *UAS-dRheb/+; AB1Gal4, tGPH/+; dPIP4K*^{29}. E. *AB1Gal4, tGPH/ UAS-TSC*^{RNAi}. F. *AB1Gal4, tGPH/ UAS-TSC*^{RNAi}*; dPIP4K*^{29}.

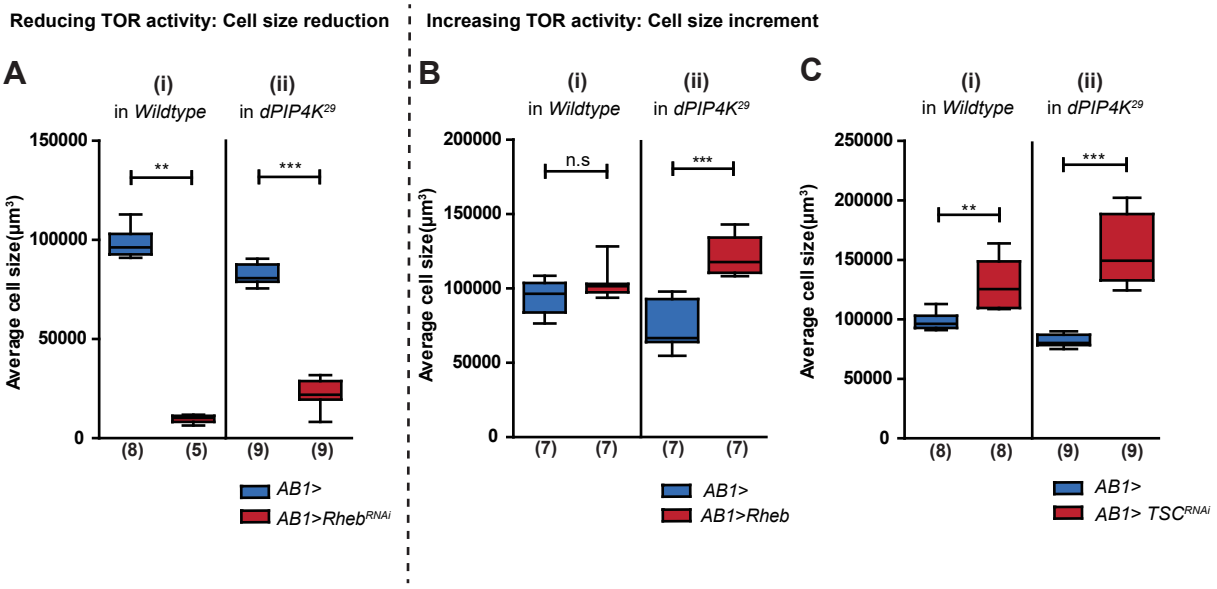

Figure S4. Changes in TORC1 activity mediate changes in the size of larval salivary gland cells (Related to Figure 3)

Cell size measurements in salivary glands upon **A.** knockdown of Rheb, **B.** overexpression of dRheb, **C.** knockdown of TSC, in *wildtype* and *dPIP4K*^{29} backgrounds. Whiskers in the box plots represent minimum and maximum values, with a line at the median. Numbers inside the parentheses below the plots indicate the no. of biological replicates used for the measurement. Mann Whitney test used for statistical analysis of the distributions. ***p-value* <0.01, ****p-value* <0.001. Genotypes: A(i). *AB1Gal4/+ and AB1Gal4/UAS-Rheb*^{RNAi} and (ii). *AB1Gal4/+; dPIP4K*^{29} *and AB1Gal4/UAS-Rheb*^{RNAi}*; dPIP4K*^{29} B(i). *AB1Gal4/+ and UAS-dRheb/+; AB1Gal4/+* and (ii). *AB1Gal4/+; dPIP4K*^{29} *and UAS-dRheb/+; AB1Gal4/+; dPIP4K*^{29}. C(i). *AB1Gal4/+ and UAS-Tsc1*^{RNAi}*/+; AB1Gal4/+*. C(ii). *AB1Gal4/+; dPIP4K*^{29} *and UAS-Tsc1*^{RNAi}*/+; AB1Gal4/+; dPIP4K*^{29}. Controls for A(ii) and C(ii) represent values from the same dataset.

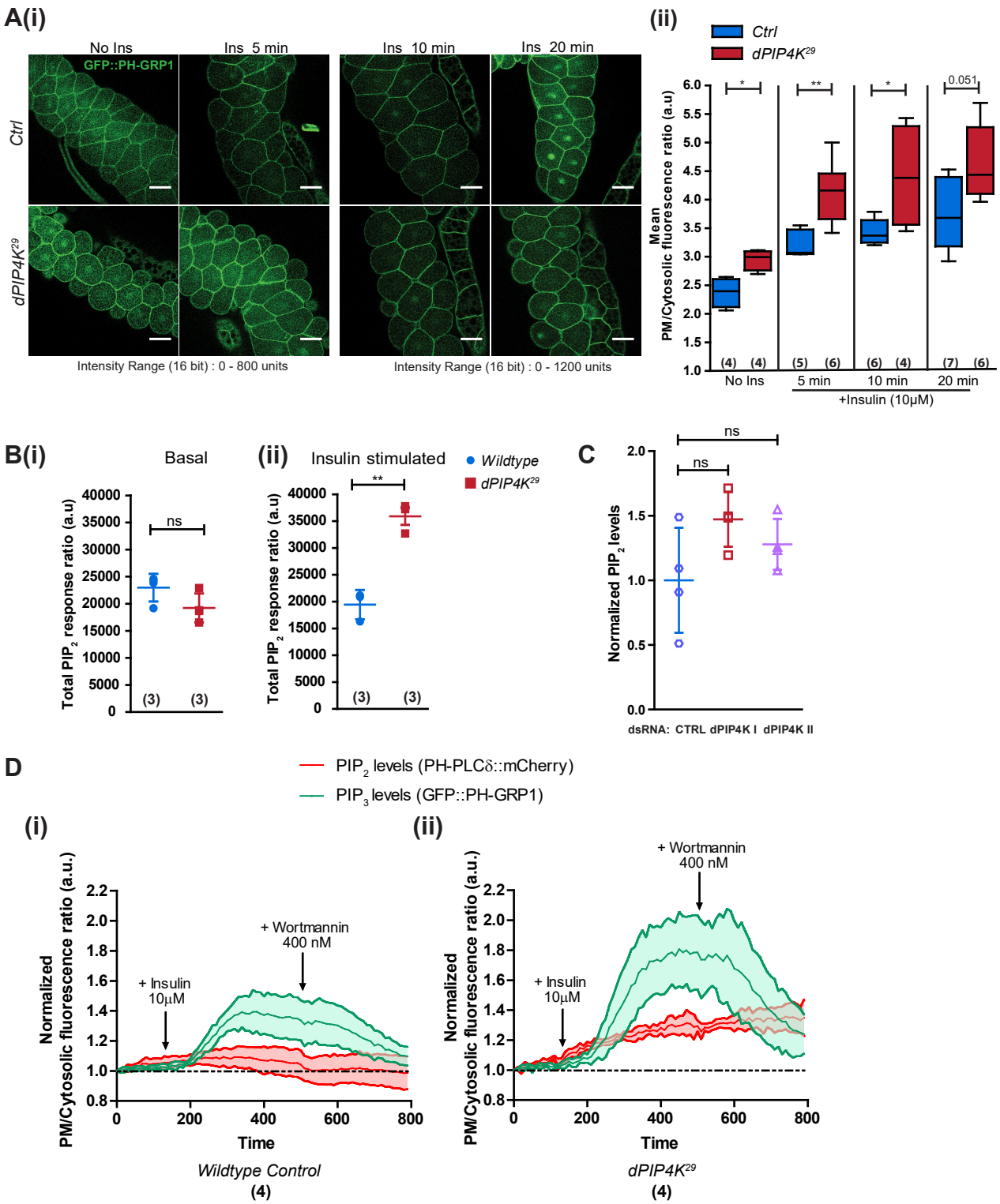

Figure S5. PIP_2 and PIP_3 measurements in *Drosophila* cells (Related to Figure 4)
A(i). Confocal z-projections of salivary glands expressing GFP-PH-GRP1 in wildtype and *dPIP4K*^{29} backgrounds. Salivary glands dissected from wandering 3 rd instar larvae were stimulated or not with 10 µM bovine insulin for indicated times, fixed and imaged. **(ii)**. Relative PIP_3 levels were measured as a ratio of mean fluorescence intensity at the plasma membrane to that in the cytosol. **B.** Measurement of total PIP_2 levels in lipid extracts using LCMS. **(i).** basal PIP_2 levels **(ii).** insulin-stimulated PIP_2 levels from wildtype (*ROR*) and *dPIP4K*^{29}. **C.** insulin-stimulated PIP_2 levels from control and PIP4K knockdown cells. **D.** Live imaging traces from salivary glands expressing both PIP_2 and PIP_3 binding probes **(i).** in wildtype controls **(ii).** in *dPIP4K*^{29} larvae. On each panel, numbers inside the parentheses indicate the no. of biological replicates used for the

measurement. For A, Mann Whitney test used for statistical analysis of the distributions. *p-value <0.05, **p-value <0.01. Student's unpaired t-test used for statistical analysis of the distributions in B. **p-value <0.01. Error bars depict SD. One-way ANOVA with posthoc Tukey's pairwise comparison used for statistical analysis in C. Genotypes: A. *AB1Gal4, tGPH/+* and *AB1Gal4, tGPH /+; dPIP4K^{29}*. B. *wildtype (ROR)* and *dPIP4K^{29}*. D. *AB1Gal4, tGPH/UAS-PH-PLC::mCherry* and *AB1Gal4, tGPH/UAS-PH-PLC::mCherry; dPIP4K^{29}*.

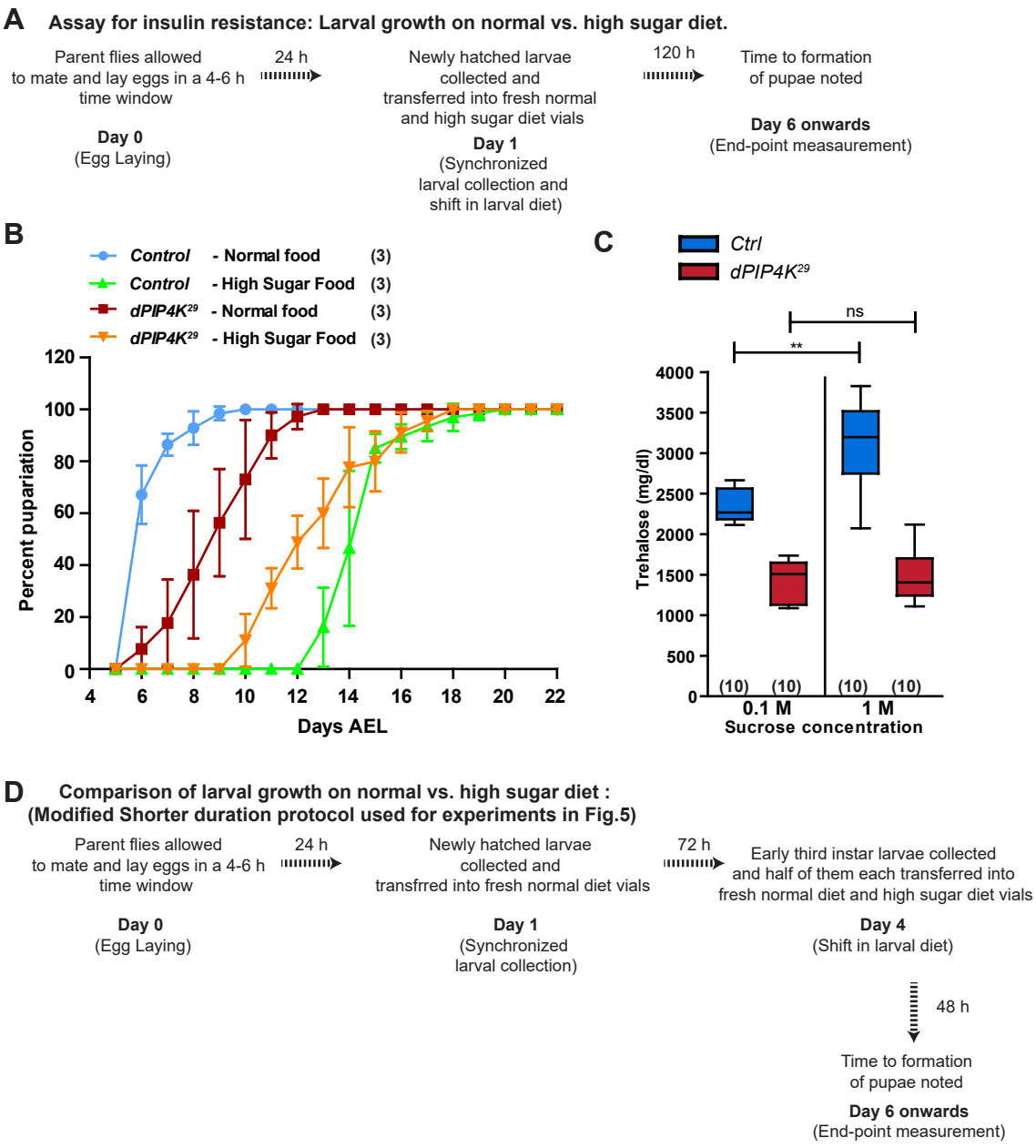

Figure S6. Insulin resistance model in *Drosophila* larvae (Relate to Figure 5)

A. Schematic depicting a longer protocol for induction of insulin resistance-like phenotype in *Drosophila* larvae using a high sugar diet. **B.** The graph represents the mean percentage of pupariation (After egg laying, AEL) observed over time on indicated diets. Data collected from 3 independent batches of about 12-25 larvae per batch (for *ROR and dPIP4K*^{29}). Error bars indicate SD. **C.** Mean hemolymph trehalose levels measured of hemolymph pooled from 5-8 larvae each per genotype (*ROR and dPIP4K*^{29}). Mann Whitney test used for statistical analysis of the distributions. ***p-value* <0.01. **D.** A schematic depicting a short protocol for induction of insulin resistance-like phenotype in *Drosophila* larvae on a high sugar diet. Whiskers in the box plots represent minimum and maximum values, with a line at the median. Numbers inside the parentheses below the plots indicate the no. of biological replicates used for the measurement.