iScience, Volume *23*

Supplemental Information

Drosophila PTPMT1 Has a Function in Tracheal Air Filling

Amanda M. Papakyrikos, Min Joo Kim, and Xinnan Wang

Figure S1

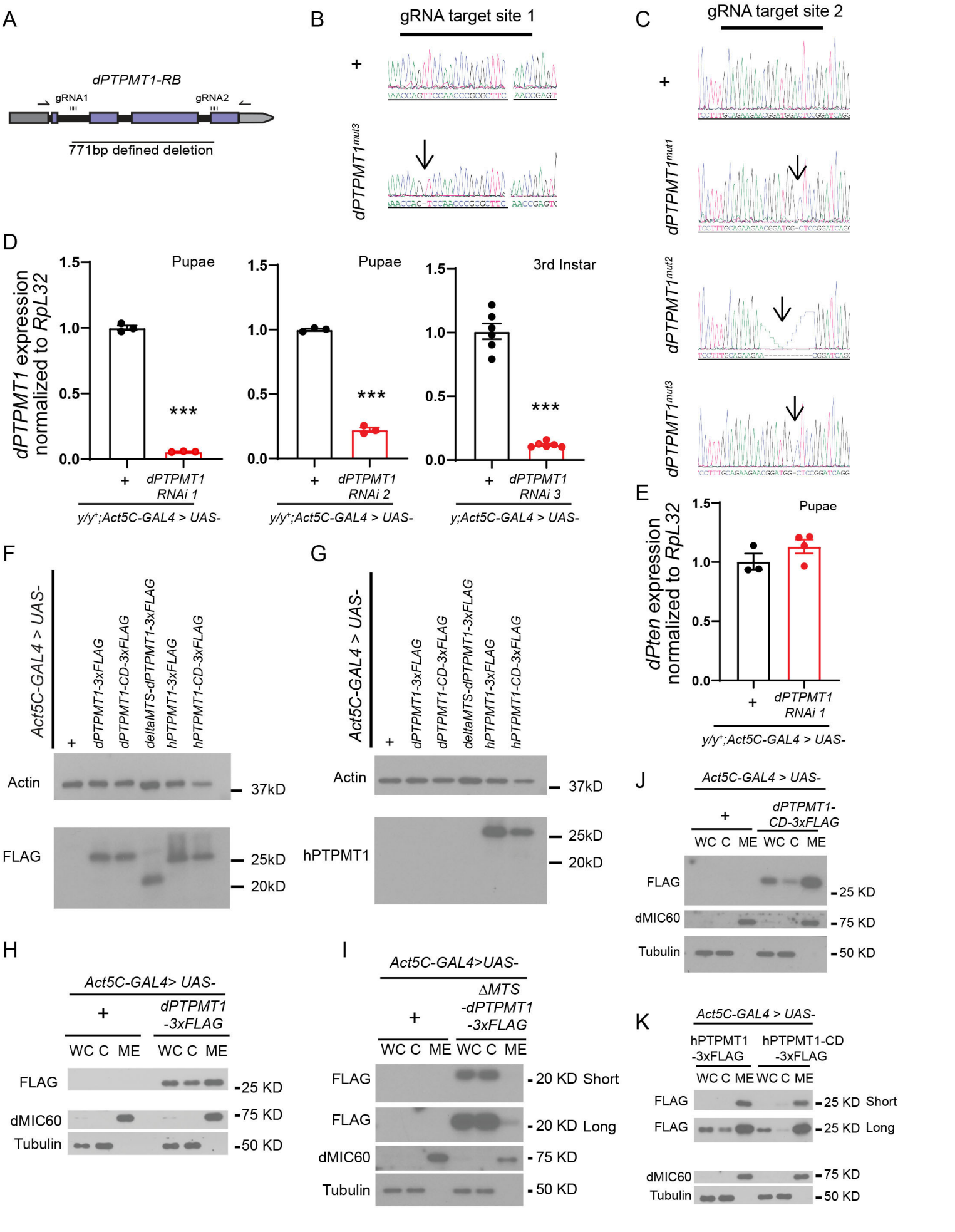

A
dPTPMT1-RB
gRNA1
gRNA2
771bp defined deletion
B
gRNA target site 1
+
dPTPMT1mut3
C
gRNA target site 2
+
dPTPMT1mut1
dPTPMT1mut2
dPTPMT1mut3
D
dPTMT1 expression normalized to RpL32
Pupae
Pupae
3rd Instar
***
+
dPTPMT1 RNAi 1
dPTPMT1 RNAi 2
dPTPMT1 RNAi 3
y/y+;Act5C-GAL4 > UAS-
y/y+;Act5C-GAL4 > UAS-
y;Act5C-GAL4 > UAS-
E
dPten expression normalized to RpL32
Pupae
+
dPTPMT1 RNAi 1
y/y+;Act5C-GAL4 > UAS-
F
Act5C-GAL4 > UAS-
+
dPTPMT1-3xFLAG
dPTPMT1-CD-3xFLAG
deltaMTS-dPTPMT1-3xFLAG
hPTPMT1-3xFLAG
hPTPMT1-CD-3xFLAG
Actin
37kD
FLAG
25kD
20kD
G
Act5C-GAL4 > UAS-
+
dPTPMT1-3xFLAG
dPTPMT1-CD-3xFLAG
deltaMTS-dPTPMT1-3xFLAG
hPTPMT1-3xFLAG
hPTPMT1-CD-3xFLAG
Actin
37kD
hPTPMT1
25kD
20kD
H
Act5C-GAL4> UAS-
+
dPTPMT1 -3xFLAG
WC C ME WC C ME
FLAG
25 KD
dMIC60
75 KD
Tubulin
50 KD
I
Act5C-GAL4>UAS-
+
ΔMTS -dPTPMT1 -3xFLAG
WC C ME WC C ME
FLAG
20 KD Short
FLAG
20 KD Long
dMIC60
75 KD
Tubulin
50 KD
J
Act5C-GAL4 > UAS-
+
dPTPMT1-CD-3xFLAG
WC C ME WC C ME
FLAG
25 KD
dMIC60
75 KD
Tubulin
50 KD
K
Act5C-GAL4 > UAS-
hPTPMT1 -3xFLAG
hPTPMT1-CD -3xFLAG
WC C ME WC C ME
FLAG
25 KD Short
FLAG
25 KD Long
dMIC60
75 KD
Tubulin
50 KD

Figure S2

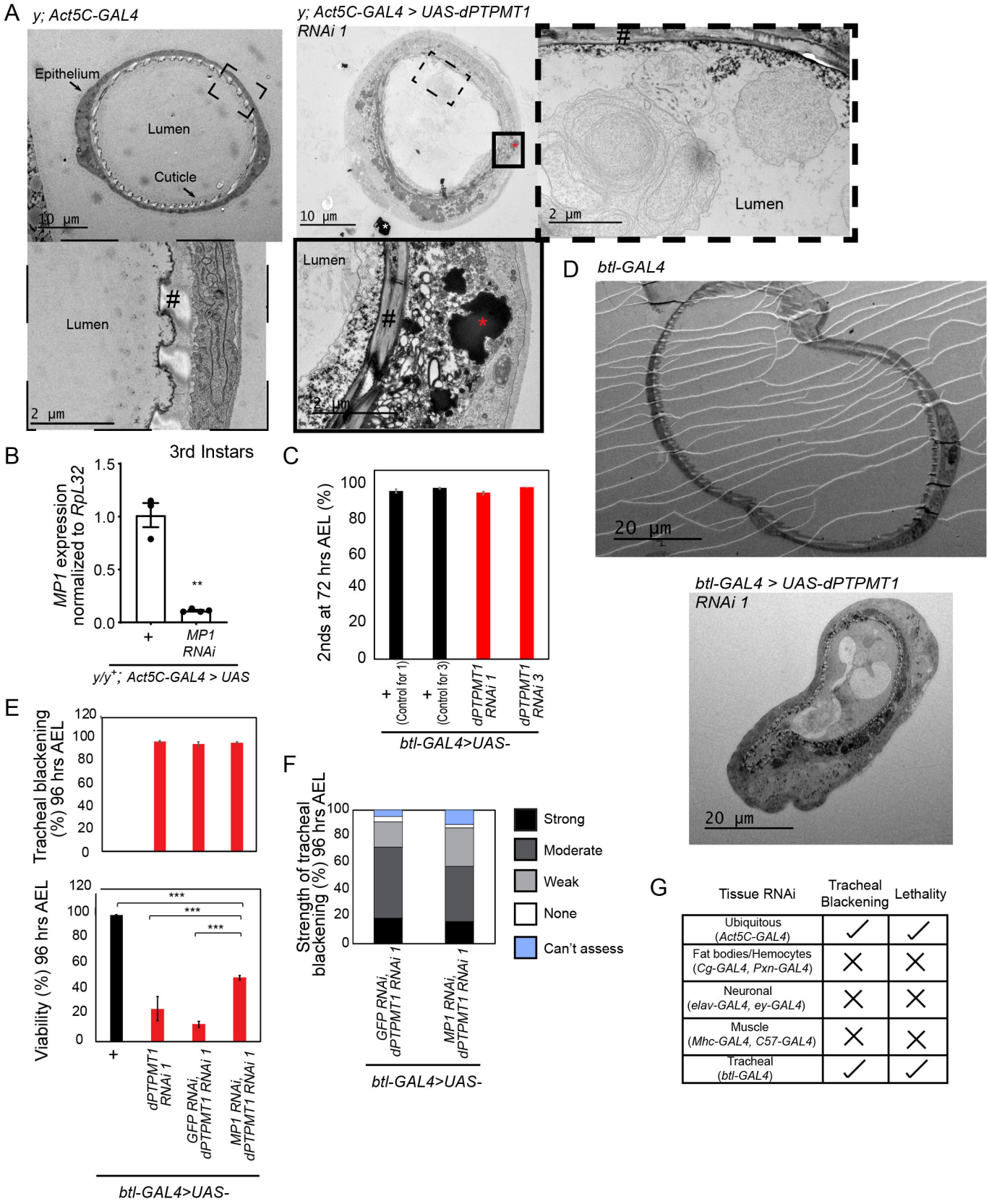

A
y; Act5C-GAL4
Epithelium
Lumen
Cuticle
10 µm
Lumen
#
2 µm
y; Act5C-GAL4 > UAS-dPTPMT1 RNAi 1
10 µm
#
2 µm
Lumen
Lumen
#
2 µm
B
3rd Instars
MP1 expression normalized to RpL32
1.5
1.0
0.5
0.0
**
+
MP1 RNAi
y/y+; Act5C-GAL4 > UAS
C
2nds at 72 hrs AEL (%)
100
80
60
40
20
0
+ (Control for 1)
+ (Control for 3)
dPTPMT1 RNAi 1
dPTPMT1 RNAi 3
btl-GAL4>UAS-
D
btl-GAL4
20 µm
btl-GAL4 > UAS-dPTPMT1 RNAi 1
20 µm
E
Tracheal blackening (%) 96 hrs AEL
120
100
80
60
40
20
0
Viability (%) 96 hrs AEL
120
100
80
60
40
20
0
***
***
***
+
dPTPMT1 RNAi 1
GFP RNAi; dPTPMT1 RNAi 1
MP1 RNAi; dPTPMT1 RNAi 1
btl-GAL4>UAS-
F
Strength of tracheal blackening (%) 96 hrs AEL
100
80
60
40
20
0
Strong
Moderate
Weak
None
Can't assess
GFP RNAi; dPTPMT1 RNAi 1
MP1 RNAi; dPTPMT1 RNAi 1
btl-GAL4>UAS-
G
Tissue RNAi
Tracheal Blackening
Lethality
Ubiquitous (Act5C-GAL4)
Fat bodies/Hemocytes (Cg-GAL4, Pxn-GAL4)
Neuronal (elav-GAL4, ey-GAL4)
Muscle (Mhc-GAL4, C57-GAL4)
Tracheal (btl-GAL4)

Figure S3

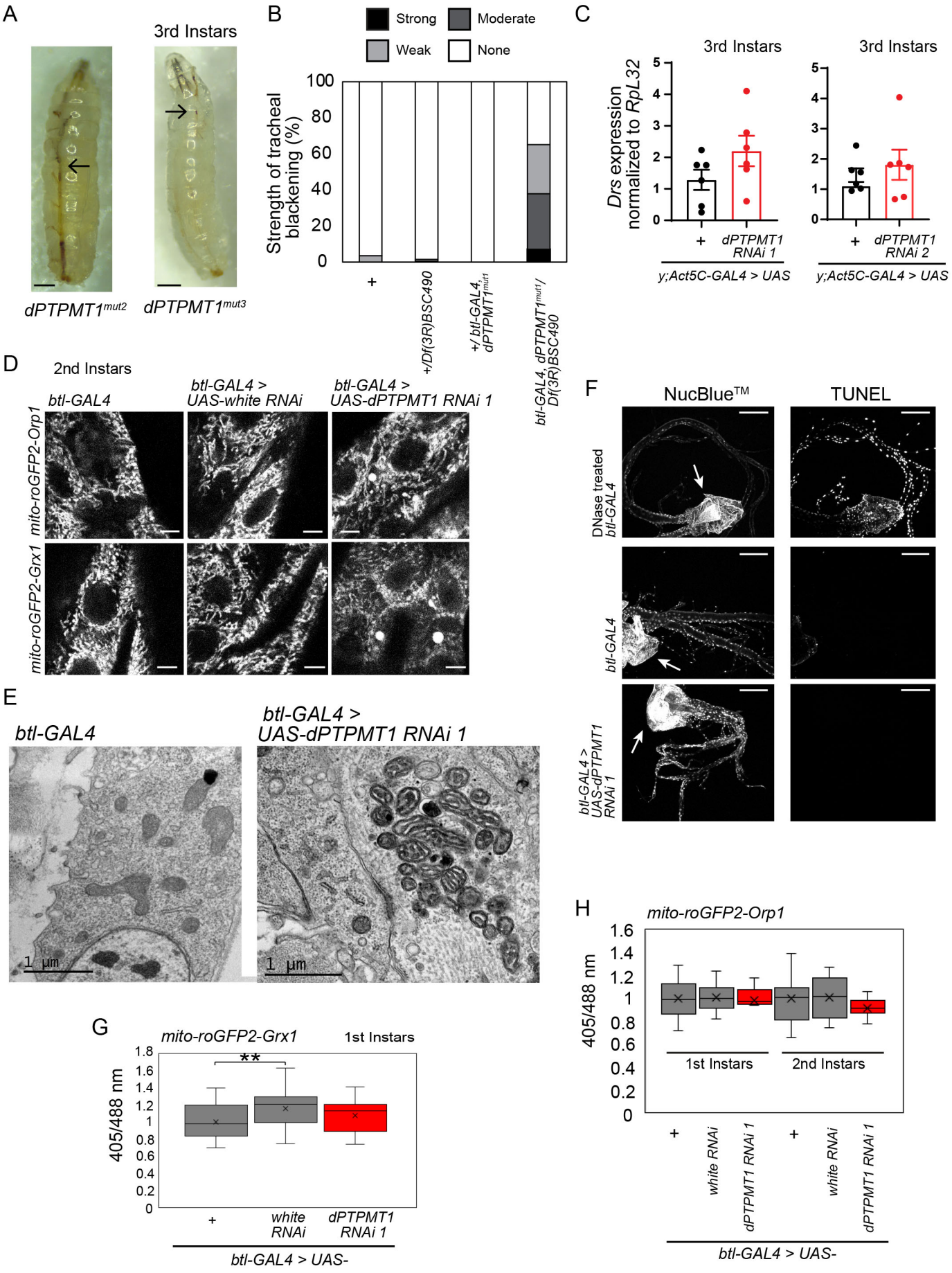
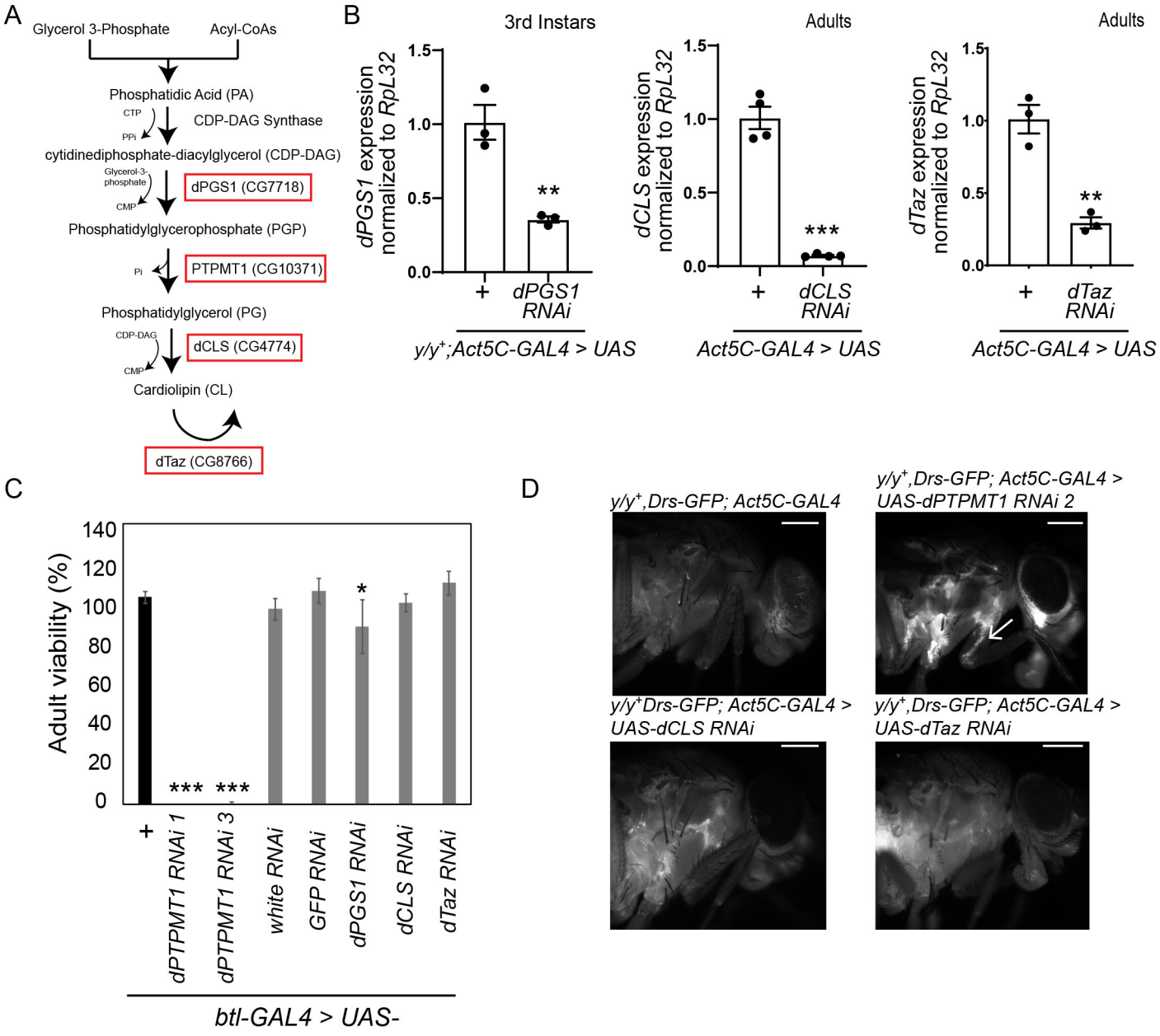

A
3rd Instars
dPTPMT1mut2
dPTPMT1mut3
B
Strong
Moderate
Weak
None
Strength of tracheal blackening (%)
100
80
60
40
20
0
+
+/Df(3R)BSC490
+/ btl-GAL4, dPTPMT1mut1
btl-GAL4, dPTPMT1mut1 / Df(3R)BSC490
C
3rd Instars
3rd Instars
Drs expression normalized to RpL32
5
4
3
2
1
0
+
dPTPMT1 RNAi 1
dPTPMT1 RNAi 2
y;Act5C-GAL4 > UAS
y;Act5C-GAL4 > UAS
D
2nd Instars
btl-GAL4
btl-GAL4 > UAS-white RNAi
btl-GAL4 > UAS-dPTPMT1 RNAi 1
mito-roGFP2-Orp1
mito-roGFP2-Grx1
E
btl-GAL4
btl-GAL4 > UAS-dPTPMT1 RNAi 1
1 µm
1 µm
F
NucBlue™
TUNEL
DNase treated btl-GAL4
btl-GAL4
btl-GAL4 > UAS-dPTPMT1 RNAi 1
G
mito-roGFP2-Grx1
1st Instars
405/488 nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
+
white RNAi
dPTPMT1 RNAi 1
btl-GAL4 > UAS-
H
mito-roGFP2-Orp1
405/488 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1st Instars
2nd Instars
+
white RNAi
dPTPMT1 RNAi 1
+
white RNAi
dPTPMT1 RNAi 1
btl-GAL4 > UAS-

Figure S4


A
Glycerol 3-Phosphate
Acyl-CoAs
Phosphatidic Acid (PA)
CTP
PPi
CDP-DAG Synthase
cytidinediphosphate-diacylglycerol (CDP-DAG)
Glycerol-3-phosphate
CMP
dPGS1 (CG7718)
Phosphatidylglycerophosphate (PGP)
Pi
PTPMT1 (CG10371)
Phosphatidylglycerol (PG)
CDP-DAG
CMP
dCLS (CG4774)
Cardiolipin (CL)
dTaz (CG8766)
B
3rd Instars
dPGS1 expression normalized to RpL32
1.5
1.0
0.5
0.0
**
+
dPGS1 RNAi
y/y+;Act5C-GAL4 > UAS
Adults
dCLS expression normalized to RpL32
***
dCLS RNAi
Act5C-GAL4 > UAS
Adults
dTaz expression normalized to RpL32
dTaz RNAi
Act5C-GAL4 > UAS
C
Adult viability (%)
140
120
100
80
60
40
20
0
***
***
*
+
dPTPMT1 RNAi 1
dPTPMT1 RNAi 3
white RNAi
GFP RNAi
dPGS1 RNAi
dCLS RNAi
dTaz RNAi
btl-GAL4 > UAS-
D
y/y+,Drs-GFP; Act5C-GAL4
y/y+,Drs-GFP; Act5C-GAL4 > UAS-dPTPMT1 RNAi 2
y/y+Drs-GFP; Act5C-GAL4 > UAS-dCLS RNAi
y/y+,Drs-GFP; Act5C-GAL4 > UAS-dTaz RNAi

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Generation of *dPTPMT1* Mutants and Transgenes, Related to Figure 1. (A) Design for CRISPR-mutagenesis. Exons of *dPTPMT1* transcript are indicated with wide purple bars and introns with black lines. *vas-Cas9*-expressing embryos were injected with two guide RNAs (dashed lines) in order to create a defined deletion. Locations of primers for screening are indicated with half arrows. (B-C) The direct sequencing chromatograms of $dPTPMT1^{mut}$ lines are shown. Arrows point to deletions. $dPTPMT1^{mut3}$ has a 1 bp deletion within the first gRNA site (B). $dPTPMT1^{mut1-3}$ have deletions in the second gRNA site (C). (D-E) qPCR evaluation of *dPTPMT1* (D) or *dPten* (E) transcript levels. Pupae were mixed-sex, females are heterozygous for *y* and males are hemizygous for *y*. n=3-6 replicates, 15-20 pupae for each repeat (RNAi 1&2) or 3rd instar males (RNAi 3). Data are represented as mean ± SEM and *** $p<0.001$, by one-tailed Student's t-test. (F-G) Validation of *PTPMT1* transgene expression. Whole fly lysates of 0- to 2-day-old adults were immunoblotted with the antibodies indicated. (H-K) Cellular localization of ubiquitously expressed *dPTPMT1-3×FLAG* (H), Δ*MTS-dPTPMT1-3×FLAG* (I), *dPTPMT1-CD-3×FLAG* (J), *hPTPMT1-3×FLAG* and *hPTPMT1-CD-3×FLAG* (K). Whole cell fraction (WC), cytosolic fraction (C), mitochondrial-enriched fraction (ME) were prepared and immunoblotted with the antibodies indicated. Results were observed 3 times and representative blots are shown. Equal total protein from each fraction was loaded. "Short" = short exposure, "Long" = long exposure.

Figure S2. **dPTPMT1 Depletion Results in Tracheal Blackening and Lethality, Related to Figure 2.** (A) TEM images of transverse sections of the dorsal trunk of the trachea in a 3rd instar male with ubiquitous *dPTPMT1* RNAi. Cuticle indicated by the # symbol. Red asterisk indicates electron dense regions. White asterisk indicates dust on the thin section. Dashed boxes show higher

magnifications of key areas. Scale bars (low magnification), 10 μm, scale bars (high magnification), 2 μm. (B) qPCR validation of *MP1* RNAi. Pupae were mixed-sex, females are heterozygous for *y* and males are hemizygous for *y*. n= 3-4 replicates, 20 for each repeat. Data are represented as mean ± SEM and ** $p < 0.01$, by one-tailed Student's t-test. (C) Percentage of larvae at 72 hours AEL which are 2nd instars, in tracheal-specific *dPTPMT1* RNAi. n=2-3 replicates, 40-94 for each repeat. Data are represented as mean ± SEM and $p > 0.05$, by Chi-square test. (D) TEM images of transverse sections of the dorsal trunk of the trachea in a 3rd instar male with tracheal-specific *dPTPMT1* RNAi and a control larva. Note the collapsed trachea and electron dense lumen in the mutant. Crosses were grown at 25ºC for three days and then shifted to 30ºC to drive a stronger knockdown. Scale bars, 20 μm. (E) Percentage of larvae with tracheal blackening and viability in tracheal-specific RNAi of *MP1* and *dPTPMT1*. n=2-3 replicates, 54-100 for each repeat. Control data (left two bars) is the same as in Figure 2F. Data are represented as mean ± SEM and *** $p < 0.001$, by Chi-square test. (F) Strength of tracheal blackening in tracheal-specific RNAi of *MP1* and *dPTPMT1*. n=179-199 larvae, 2 independent crosses. (G) Screen for phenotypes following tissue-specific *dPTPMT1* RNAi. Crosses were grown at 25ºC for three days and then shifted to 30ºC to drive a stronger knockdown.

Figure S3. dPTPMT1 Depletion Results in Tracheal Blackening, *Drs* Upregulation, and Mitochondrial Defects, Related to Figure 2, Figure 3, and Figure 4. (A) $dPTPMT1^{mut2}$ and $dPTPMT1^{mut3}$ mutants show tracheal blackening. Scale, 250 μm. (B) Strength of tracheal blackening in $dPTPMT1^{mut1}$ 3rd instar larvae. n=81-226, 3-4 independent crosses. (C) qPCR evaluation of *Drs* expression in 3rd instar male larvae with ubiquitous *dPTPMT1* RNAi. n=6 replicates, 20 for each repeat. Data are represented as mean ± SEM and $p > 0.05$, by one-tailed

Student's t-test. (D) Enlarged mito-roGFP puncta visualized by live imaging mitochondrially localized ROS reporters in tracheal epithelial cells of 2nd instars with tracheal-specific *dPTPMT1* RNAi. Scale, 5 μm. (E) TEM images show a cluster of mitochondria in a 3rd instar male larva with tracheal-specific *dPTPMT1* RNAi. Crosses were grown at 25ºC for three days and then shifted to 30ºC to drive a stronger knockdown. Scale, 1 μm. (F) TUNEL staining of the tracheae of 2nd instar larvae. NucBlue™ stains nuclei. Arrows point to the larval cuticle. DNase treatment causes double strand breaks (top row), serving as a positive control. Percentage of TUNEL positive nuclei: *btl-GAL4* (Control for RNAi 1): 1% ±1%, *btl-GAL4>UAS-dPTPMT1* RNAi 1: 2% ±1%, *btl-GAL4>UAS-GFP* RNAi: 3% ±1%, *btl-GAL4* (Control for RNAi 3): 1% ±1%, *btl-GAL4>UAS-dPTPMT1* RNAi 3: 1% ±1%. n=8-18 larvae, 2 independent crosses. Scale, 100 μm. Data are represented as mean ± SEM and $p > 0.05$, by one-tailed Student's t-test. (G-H) Quantification of the fluorescence ratios of mitochondrially localized roGFP-Grx1 (G) and roGFP2-Orp1 (H) expressed in the trachea of 2nd instars. n=8-30. Boxes show 25th/75th percentiles, whiskers are the minimum and maximum values, and x is the median marker and ** $p < 0.01$, by one-tailed Student's t-test.

Figure S4. **Mutants of the CL Pathway Do not Mimic Phenotypes of *dPTPMT1* Deficiency, Related to Figure 4.** (A) Biosynthesis of cardiolipin, inspired by Zhang et al. 2011. (B) qPCR data for RNAi of enzymes in CL biosynthesis. n=3-4 replicates, 20 for each repeat. *dCLS* = adults females, 0-2 days old. *dTaz* = half male and half female adults, 0-2 days old. *dPGS1* = 3rd instar larvae, mixed sex, females are heterozygous for *y* and males are hemizygous for *y*. Data are represented as mean ± SEM and ** $p < 0.01$, *** $p < .001$ by one-tailed Student's t-test. (C) Viability of tracheal RNAi of CL pathway enzymes. n=3 replicates. 0- to 2-day-old adults. Crosses

were grown at 25ºC for three days and then shifted to 30ºC to drive a stronger knockdown. For simplicity only one background control is shown. Genotypic frequency is calculated for the genotype of interest and then normalized to its expected genotypic frequency. Statistical significance is given relative to respective controls. *dPGS1* RNAi viability was significantly decreased relative to its control. Data are represented as mean ± SEM and * $p<0.05$, *** $p<0.001$ by Chi-square test. (D) *Drs-GFP* expression in females with ubiquitous RNAi of *dTaz* and *dCLS*. Control images and ubiquitous *dPTPMT1* RNAi are the same as in Figure 3C. Scale, 250 μm.

TRANSPARENT METHODS

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly Stocks

dPTPMT1 (CG10371) RNAi stocks included *UAS-dPTPMT1* RNAi 1(VDRC 47624GD), *UAS-dPTPMT1* RNAi 2 (VDRC 47623GD), *UAS-dPTPMT1* RNAi 3 (BDSC 40913). For *dPTPMT1* RNAi controls, drivers were crossed to background controls specific to the RNAi line used. For *dPTPMT1* RNAi 1 and 2 controls ("Control for 1&2"), drivers were crossed to VDRC 60000GD. For *dPTPMT1* RNAi 3 controls ("Control for 3"), drivers were crossed to BDSC 36303. Additional RNAi stocks included *UAS-dPGS1* RNAi (VDRC 109405KK), *UAS-dCLS* RNAi (BDSC 77150), *UAS-dTaz* RNAi (BDSC 31099), *UAS-GFP* RNAi (BDSC 41557), *UAS-white* RNAi (a gift from Dr. Bingwei Lu's Lab), and *UAS-MP1* RNAi (VDRC 18970GD). BDSC 36303 was used as a background control for *dCLS*, *dTaz*, and *MP1* RNAi experiments. VDRC 60100KK was used as a background control for *dPGS1* RNAi. Additional stocks included *y, Drs-GFP* (BDSC 55707), *UAS-mito-HA-GFP* (BDSC 8442), *UAS-mito-roGFP2-Orp1* (BDSC 67667), *UAS-mito-roGFP2-*

Grx1 (BDSC 67664), *btl-GAL4* (BDSC 78328, the *y* background mutation was removed by outcrossing), *y,w*;*Act5C-GAL4* (Wang lab stock), *C57-GAL4* (Wang lab stock), *Mhc-GAL4* (Wang lab stock), *ey-GAL4* (a gift from Dr. Bingwei Lu's Lab), *elav-GAL4* (BDSC 458, a gift from Liqun Luo Lab), *Cg-GAL4* (BDSC 7011), *Pxn-GAL4* (a gift from Dr. Clarissa Cheney Lab), *Df(3R)BSC490* (BDSC 24994), *Df(3R)Exel9013* (BDSC 7991), *vas-Cas9* (BDSC 51323), *dCLS* mutants (BDSC 17917, BDSC 10741) (Thibault et al., 2004), $Pink1^{rv}$ (Park et al., 2006), and $Pink1^{B9}$ (Park et al., 2006), $dMIC60^{mut}$ (Tsai et al., 2017), *e/TM3* (*ActGFP, ser*) (BDSC 7408), and *Dr/TM6B* (Wang Lab stock).

Flies were grown on a standard molasses media except where otherwise stated in the methods. For *Act5C-GAL4*-driven crosses, flies were grown at 25ºC for 3 days and then switched to 30ºC to drive a stronger knockdown. In all other experiments, flies were maintained at 25ºC, except where otherwise stated in the figure legends. Because there is evidence that the presence of yellow mutations (*y*) impact immune phenotypes (Tang et al., 2008), we indicated the presence of this mutation in all experiments. Developmental stage, age, and sex of samples are given in the figure legends. All adults were between 0-2 days old, except for the *Pink11* mutants which were aged to 5 days and the adult flies used for mitochondrial enrichments, which were 1-3 days old. Where possible, we used males in our assessments of adult phenotypes, except in the case of adults on a *Drs-GFP* background, as males in this background showed reduced viability. For qPCR on adults, we used equal ratios of males and females, except for *dCLS* RNAi where we used females only because males had reduced viability. Additionally, for the assessment of *Drs* levels in adults with ubiquitous *dPTPMT1* RNAi, we used males only in order to control for the presence of yellow mutations in this background. Between 1-376 adults were used for each genotype in each experiment. Between 1-699 larvae were used for each genotype in each experiment.

METHOD DETAILS

CRISPR/Cas9: *dPTPMT1* mutants were generated using the methods of the O'Connor-Giles, Wildonger, and Harrison labs (Gratz et al., 2013, Gratz et al., 2015) for creating defined deletions. Transgenic flies expressing Cas9 protein in their ovaries under the control of *vas* regulatory sequences (BDSC 51323) were sequenced for SNPs. Two gRNAs (gRNA1 5'-CTTCGTTGAAGCGCGGGTTGGAAC-3', gRNA2 5'-CTTCGCAGAAGAACGGATGGACTC-3') were identified using the flyCRISPR Optimal Target Finder (http://tools.flycrispr.molbio.wisc.edu/targetFinder/). These gRNAs would be expected to generate a defined deletion of ~770bp. gRNAs were selected in regions that did not contain SNPs and did not overlap with neighboring genes. gRNAs were cloned into pU6-BbsI-chiRNA using restriction-enzyme cloning (Gratz et al., 2013). pU6-BbsI-chiRNA was a gift from Melissa Harrison, Kate O'Connor-Giles, and Jill Wildonger (Addgene plasmid # 45946; http://n2t.net/addgene:45946; RRID:Addgene_45946). gRNA oligonucleotides were designed with a 4bp overhang complementary to those generated by BbsI (see above). Oligonucleotides were purchased pre-phosphorylated from Integrated DNA Technologies (Redwood City, CA) and were subsequently annealed at 95 ^{o}C for 5 minutes in T4 ligation buffer (NEB, B0202S). pU6-BbsI-chiRNA was digested with BbsI (NEB, R0539S), dephosphorylated with Calf Intestinal Alkaline Phosphatase (NEB, M0290), and then ligated with oligonucleotides using T4 DNA ligase (NEB, M0202S). The resulting constructs were midi-prepped and sequenced by Genewiz (South San Francisco, CA) using T3 and T7 primers (5'-GCAATTAACCCTCACTAAAGG-3', 5'-TAATACGACTCACTATAGGG-3'). gRNAs were injected into Cas9 transgenic flies by the BestGene Inc. (Chino Hills, CA) at 100 ng/µl and 250 ng/µl per plasmid. Progeny from injections

were kept at 18ºC for the duration of the screen, in order to promote survival of weak *dPTPMT1* mutants. F0s were crossed to *Dr/TM6B* balancer flies (Wang Lab Stock) in order to create stable F2 stocks. Homozygous lethal F2 lines were screened over deficiency lines covering PTPMT1 (BDSC 24994, BDSC 7991). Homozygous larvae, from stocks which were lethal over deficiency lines, were sequenced for mutations in *dPTPMT1*. The *dPTPMT1* gene region was amplified by PCR (5'-GTTATGCAAAGGAACGCGCA-'3, 5'-CGTAACAAAGGTGGTGCCTG-'3). PCR products were gel-extracted using the QIAquick Gel Extraction Kit (Qiagen, 28706) and sent for sequencing.

Fly DNA preparations and polymerase chain reaction (PCR): A single larva was manually crushed in 50-100 µl of fly squishing buffer (FSB: 10mM Tris-HCl pH8, 1mM EDTA, 25mM NaCl) and 0.2 mg/ml of Proteinase K (NEB, P8107S). Lysates were incubated at 37ºC for 30 minutes and then boiled for 10 minutes at 95ºC to inactivate the Proteinase K. PCR reactions were performed using 1-4 µl of crude DNA. Platinum™ Green Hot Start PCR Master Mix (2X) (Thermo Fischer, 13001012) or Q5® High-Fidelity 2X Master Mix (NEB, M0492S) were used for PCR reactions, following manufacturer's instructions.

Quantitative real-time PCR (qPCR): Total RNA was extracted by homogenization in TRIzol (Thermo Fisher, 15596026) and then mixed with chloroform vigorously and centrifuged at 12,000g at 4°C for 15 minutes. To precipitate the RNA, the aqueous phase was mixed with 100% isopropanol at 1:1 ratio. RNA pellets were washed with 70% ethanol and then resuspended in nuclease-free water. RNA was cleaned with chloroform (1:1), vortexed, and then centrifuged at 10,000g at 4°C. The aqueous phase was removed and mixed with ice-cold ethanol (1:3) and sodium

acetate (pH 4.8, 10:1) and precipitated overnight at -20°C. The RNA pellet was washed in 70% ethanol, dried, and then resuspended in DNase, RNase free water. To remove DNA contamination, RNA was treated with DNase (NEB, M0303S), following manufacturer's instructions. cDNA synthesis was performed with iScript Reverse Transcription Supermix (Bio-RAD, 1708841) using 500 ng of DNase-treated RNA. cDNA was diluted by 4.5-fold with DNase/RNase free water for qPCR. qPCR was performed using Taqman Fast Advanced Master Mix (Applied Biosystems, 4444557) and commercially available TaqMan Gene Expression Assay probes (Applied Biosystems: *Drs*: Dm01822006_s1, *MP1*:Dm02136644_g1, *dPTPMT1*:Dm02143827_g1, *RpL32RpL32*:Dm02151827_g1, *dPten*:Dm01844965_g1, *dCLS*:Dm02144883_g1, *dTaz*:Dm01793307_g1, *dPGS*1: Dm02142030_g1) and analyzed by a Step One Plus Real-Time PCR System (Applied Biosystems) following manufacturer's instructions. Analysis was performed using the ΔΔCT method (Livak and Schmittgen, 2001). Expression was normalized to the house-keeping gene *RpL32*. A minimum of 3 replicates were performed for each experiment. For qPCR evaluation of *Drs* expression, individuals were collected at the same time of day, to control for possible circadian variation in expression of immune genes, as recommended by others (Neyen et al., 2014).

Generation of *dPTPMT1* transgenic flies: pUASTattB-dPTPMT1-3×FLAG, pUASTattB-ΔMTS-dPTPMT1-3×FLAG and pUASTattB-hPTPMT1-3×FLAG were generated by GenScript (Piscataway, NJ). WT *dPTPMT1* (isoform RB), Δ*1-31-dPTPMT1*, and codon optimized *hPTPMT1* (isoform 1) were synthesized by GenScript (Piscataway, NJ) and cloned into pUASTattB (Groth et al., 2004) using NotI/XbaI. A modified kozak sequence was added to the 5' end (Pfeiffer et al., 2012) to enhance gene expression and a codon-optimized 3×FLAG tag was added to the 3' end to

facilitate localization studies. Site-directed mutagenesis, performed by GenScript, was used to generate pUASTattB-dPTPMT1-CD-3×FLAG and pUASTattB-hPTPMT1-CD-3×FLAG. We used PhiC31 integrase-mediated transgenesis to generate transgenic fly lines (Markstein et al., 2008). All constructs were injected by the Bestgene Inc (Chino Hills, CA) into y^{1}, w^{67c23}; P{CaryP}attP40 flies (the Bestgene Inc), with an estimated insertion at 25C6 (Markstein et al., 2008).

Inserted sequences for *dPTPMT1* constructs: NotI sequence is in red, XbaI sequence is in blue, modified Kozak (Pfeiffer et al., 2012) is underlined, and 3×FLAG sequence is in bold.

dPTPMT1-3×FLAG **(Isoform RB):**

GCGGCCGCAACTTAAAAAAAAAAATCAAAATGGAGATGAGTGCTGCCATGTTCGCA
CGCGTTTCCTTCTACCCCACCCTGCTGTACAATGTCCTGATGGAAAAGGCATCGGCC
AGGAATTGGTACGATCGCATCGATGAGCATGTGATACTGGGAGCACTGCCCTTTCGC
AGCCAGGCCAATGACCTCATTGAAAAGGAGAACATGAAGGCGGTGGTGTCGATGAA
CGAGGACTATGAGCTGACCGCCTTCTCCAACAACACGGAGAAGTGGCGAAAGCTTG
GCATTGAGTTCCTGCAGCTGGCCACCACCGACATCTTTGAGTCGCCCAATCAAGAAA
AGCTCTTCCGCGGCGTGGAATTCATAAACAAGTTCCTGCCTCTAAAGCAAAGAATTG
GTGGCCTAAGTTCCTCCTACCAGCCGGAGAACGTGGGTTCTGTCTATGTGCACTGCA
AGGCTGGTAGGACGCGAAGTGCCACTTTGGTGGGATGCTACCTCATGATGAAGAAC
GGATGGACTCCGGATCAGGCGGTTGACCACATGCGTAAGTGCCGACCGCACATTCT
GCTGCACACCAAACAATGGGATGCCCTCCGGTTATTCTACACAAACAATGTGGAGA

CGAAATCA**GACTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGAT**

AAGGACTACAAGGATGACGATGATAAGTGATCTAGA

***dPTPMT1-C141S-3×FLAG* (Isoform RB)**:

GCGGCCGCAACTTAAAAAAAAAAATCAAAATGGAGATGAGTGCTGCCATGTTCGCA

CGCGTTTCCTTCTACCCCACCCTGCTGTACAATGTCCTGATGGAAAAGGCATCGGCC

AGGAATTGGTACGATCGCATCGATGAGCATGTGATACTGGGAGCACTGCCCTTTCGC

AGCCAGGCCAATGACCTCATTGAAAAGGAGAACATGAAGGCGGTGGTGTCGATGAA

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GCATTGAGTTCCTGCAGCTGGCCACCACCGACATCTTTGAGTCGCCCAATCAAGAAA

AGCTCTTCCGCGGCGTGGAATTCATAAACAAGTTCCTGCCTCTAAAGCAAAGAATTG

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AGGCTGGTAGGACGCGAAGTGCCACTTTGGTGGGATGCTACCTCATGATGAAGAAC

GGATGGACTCCGGATCAGGCGGTTGACCACATGCGTAAGTGCCGACCGCACATTCT

GCTGCACACCAAACAATGGGATGCCCTCCGGTTATTCTACACAAACAATGTGGAGA

CGAAATCA**GACTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGAT**

AAGGACTACAAGGATGACGATGATAAGTAATCTAGA

Δ*MTS-dPTPMT1-3×FLAG* (Isoform RB):

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CATGAAGGCGGTGGTGTCGATGAACGAGGACTATGAGCTGACCGCCTTCTCCAACA

ACACGGAGAAGTGGCGAAAGCTTGGCATTGAGTTCCTGCAGCTGGCCACCACCGAC

ATCTTTGAGTCGCCCAATCAAGAAAAGCTCTTCCGCGGCGTGGAATTCATAAACAAG

TTCCTGCCTCTAAAGCAAAGAATTGGTGGCCTAAGTTCCTCCTACCAGCCGGAGAAC

GTGGGTTCTGTCTATGTGCACTGCAAGGCTGGTAGGACGCGAAGTGCCACTTTGGTG

GGATGCTACCTCATGATGAAGAACGGATGGACTCCGGATCAGGCGGTTGACCACAT

GCGTAAGTGCCGACCGCACATTCTGCTGCACACCAAACAATGGGATGCCCTCCGGTT

ATTCTACACAAACAATGTGGAGACGAAATCA**GACTACAAGGATGACGACGATAAG**

GATTACAAGGATGACGACGATAAGGACTACAAGGATGACGATGATAAGTAATC

TAGA

***hPTPMT1-3×FLAG* (Isoform 1):**

GCGGCCGCAACTTAAAAAAAAAAATCAAAATGGCCGCCACCGCCCTGCTGGAGGCC

GGACTGGCCCGCGTGCTGTTTTACCCAACCCTGCTGTATACCCTGTTTCGCGGAAAG

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GGAGCCCTGCCCCTGCGCTCGCTGACCCGCCAGCTGGTGCAGGATGAGAACGTGCG

CGGCGTGATCACCATGAATGAGGAGTACGAGACGCGCTTCCTGTGCAACTCGTCGC

AGGAGTGGAAGCGCCTGGGAGTGGAGCAGCTGCGCCTGAGCACCGTGGATATGACC

GGCATCCCAACCCTGGACAATCTGCAGAAGGGCGTGCAGTTCGCCCTGAAGTACCA

GTCCCTGGGCCAGTGCGTGTACGTGCACTGCAAGGCCGGACGCTCGCGCAGCGCCA

CGATGGTGGCCGCCTACCTGATCCAGGTGCACAAGTGGTCCCCAGAGGAGGCCGTG

CGCGCCATTGCCAAGATCCGCTCGTACATTCACATTCGCCCGGGACAGCTGGATGTG

CTGAAGGAGTTCCACAAGCAGATCACCGCCCGCGCCACCAAGGATGGAACCTTCGT

GATCTCCAAGACC**GACTACAAGGATGACGACGATAAGGATTACAAGGATGACGA**

CGATAAGGACTACAAGGATGACGATGATAAGTGATCTAGA

***hPTPMT1-C132S-3×FLAG* (Isoform 1):**

GCGGCCGCAACTTAAAAAAAAAAATCAAAATGGCCGCCACCGCCCTGCTGGAGGCC

GGACTGGCCCGCGTGCTGTTTTACCCAACCCTGCTGTATACCCTGTTTCGCGGAAAG

GTGCCCGGACGCGCCCACCGCGATTGGTACCACCGCATCGACCCCACCGTGCTGCTG

GGAGCCCTGCCCCTGCGCTCGCTGACCCGCCAGCTGGTGCAGGATGAGAACGTGCG

CGGCGTGATCACCATGAATGAGGAGTACGAGACGCGCTTCCTGTGCAACTCGTCGC

AGGAGTGGAAGCGCCTGGGAGTGGAGCAGCTGCGCCTGAGCACCGTGGATATGACC

GGCATCCCAACCCTGGACAATCTGCAGAAGGGCGTGCAGTTCGCCCTGAAGTACCA

GTCCCTGGGCCAGTGCGTGTACGTGCACAGCAAGGCCGGACGCTCGCGCAGCGCCA

CGATGGTGGCCGCCTACCTGATCCAGGTGCACAAGTGGTCCCCAGAGGAGGCCGTG

CGCGCCATTGCCAAGATCCGCTCGTACATTCACATTCGCCCGGGACAGCTGGATGTG

CTGAAGGAGTTCCACAAGCAGATCACCGCCCGCGCCACCAAGGATGGAACCTTCGT

GATCTCCAAGACC**GACTACAAGGATGACGACGATAAGGATTACAAGGATGACGA**

CGATAAGGACTACAAGGATGACGATGATAAGTGATCTAGA

Mitochondrial enrichment: To generate subcellular fractions enriched for mitochondria, forty 1-3 day-old adult flies (20 male and 20 female or 40 larvae for *Act5C-GAL4 > UAS-ΔMTS-dPTPMT1* flies) were homogenized in 1 ml mitochondrial isolation buffer (MIB: 70 mM sucrose, 210 mM Mannitol, 50 mM Tris/HCl pH 7.5, 10 mM EDTA/Tris pH 7.5) with protease inhibitors (Roche, 04693116001) at 4°C. Lysate was centrifuged twice at 600g for 10 minutes at 4°C to remove cell debris and nuclei. A portion of this lysate was removed for the "whole cell fraction." Samples were subsequently centrifuged at 7,000g for 10 minutes at 4°C to pellet mitochondria.

The mitochondrial pellet was resuspended three times in MIB to wash the pellet and then finally re-suspended in MIB. The supernatant was saved as the "cytosolic fraction". Protein concentration was measured using Pierce^{TM} BCA Protein Assay kit (Thermo Scientific, 23225), according to manufacturer's instructions. Equal total protein from each fraction was (1 μg) was loaded. Mitochondrial enrichment experiments were repeated three times using adults/larvae from independent crosses.

Western blotting: To verify expression of *dPTPMT1* transgenes, *Act5C-GAL4* virgins were crossed to males carrying each transgene. Ten 3rd instar larvae were collected for each genotype and homogenized in 100 μl of RIPA buffer (Thermo Scientific, 89900) with protease inhibitors (1:1000, Millipore Sigma, 539134). Lysates were centrifuged at 17,000g for 20 minutes at 4°C and then diluted 1:8 in Laemmli buffer (BioRad, 1610737) and RIPA. Primary antibodies used include anti-FLAG (Sigma Aldrich, F7425) at 1:1000, anti-Actin (Abcam, ab8224) at 1:3000, anti-Tubulin (Invitrogen, 62204) at 1:3000**,** anti-hPTPMT1 (Thermo Scientific, PA5-60557) at 1:1000, and anti-dMIC60 (Tsai et al., 2018) at 1:6000. Secondary antibodies used include HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 115035166) and HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 111035144) at 1:5000 for chemiluminescent imaging. For representative chemiluminescent images, immunoblots were imaged on an SRX-101A film processor (Konica Minolta).

Quantification of tracheal air filling: To assess tracheal air filling during the 1st to 2nd instar molt, 50-100 virgin females were crossed to 10-30 males in egg laying chambers on plates containing standard sugar-yeast media (SY: 15 g/L agar, 50 g/L sugar, 100 g/L autolysed yeast, 6 g/L nipagin

and 3 mL/L propionic acid) (Kinghorn et al., 2015). Flies were fattened with yeast for 3 days. On the day of collection, females laid for 2 hours on a fresh plate. These embryos were discarded, and a new plate was placed in the chamber. Eggs were collected for 3 hours and then the plates were removed from the chamber and placed back in the incubator at 25ºC. Forty-eight hours AEL, large 1st instar larvae were selected and transferred to individual wells in a 24-well plate filled with SY media at room temperature (22 ºC). Larvae were observed every 30 minutes. The onset of molting was marked by the presence of a double vertical plate (dVP) and was considered complete once the outer cuticle and 1st instar mouthparts were shed (Park et al., 2002). At this timepoint, if the trachea was not filled with air it was counted as having incomplete air filling. Air filling was assessed in the dorsal trunk of the trachea using a transmitted light microscope. Trachea that are filled with air have a silvery appearance that makes the trachea readily visible. For *dPTPMT1*^{mut1} mutants, air filling was assessed in 3rd instar escapers grown in standard molasses media vials. Vials were flipped every 3-4 days. 3rd instar escapers were resuspended in 20% sucrose, washed in distilled water and their trachea were examined under transmitted light for air-filling defects in the dorsal trunk of the trachea. Each experiment was repeated a minimum of 2 times using independent crosses.

Quantification of tracheal blackening: For quantification of tracheal blackening in adults with ubiquitous *dPTPMT1* RNAi, parents laid eggs for 3 days at 25ºC after which their progeny were transferred to 30ºC for the remainder of development. Adult males were assessed for tracheal blackening 0-2 days post-eclosion. For assessments of the strength of tracheal blackening in adults, we used the following scale. Adults were scored as “none” if they had no visibly blackened trachea, “weak” if they had light blackening in one tracheal region, “moderate” if they had one heavily

blackened tracheal region or multiple light to moderately blackened regions, and "strong" if they had multiple heavily blackened tracheal regions. For assessment of tracheal blackening in adults with ubiquitous expression of *hPTPMT1* in a wild-type background, equal number of males and females were assessed 0-2 days after eclosion. For assessments of tracheal blackening in 3rd instars with ubiquitous *dPTPMT1* RNAi, parents were allowed to lay for 24 hours and then removed from the vial. Vials were transferred to 30°C. Once 3rd instar larvae were visible on the side of the vial, larvae were re-suspended in 20% sucrose and washed briefly in phosphate buffered saline (PBS) in preparation for assessment. For assessment of tracheal blackening in 2nd instars with *btl-GAL4*-driven RNAi of *dPTPMT1* see "Viability Assays" below. For assessment of tracheal blackening in $dPTPMT1^{mut1}$ 3rd instar escapers, crosses were grown at 25°C and flipped once every three days. 3rd instar escapers were resuspended following the criteria for larvae outlined above. For assessment of the strength of tracheal blackening in larvae, we used the following scale. Larvae were scored as "strong" if more than 50% of the dorsal trunk of the trachea was blackened, "moderate" if less than 50% but greater than 10% of the dorsal trunk of the trachea was blackened, "weak" if less than 10% of the dorsal trunk of the trachea was blackened, and "none" if they had no visible tracheal blackening. For tracheal-specific RNAi experiments, decomposed larvae were omitted from the assessment and categorized as "can't tell". Each experiment was repeated a minimum of 2 times using independent crosses.

Survival assays: For survival assays with *Act5C-GAL4* RNAi (Figure 1D), 3 independent crosses were set up at 25°C. After 3 days, parents were removed from the vial and the remaining progeny were transferred to 30°C. 3rd instar larvae were floated out of the media with 20% sucrose, washed in PBS, and then transferred to fresh vials. Three replicates (1 vial per independent cross) of 25-

26 3rd instar male larvae per vial were performed for RNAi 1& 2 and its background control. Six replicates (2 per independent cross) with 4-25 3rd instar male larvae per vial were performed for RNAi 3 and its control. The percentage of 3rd instars surviving to pupal stage and adult stage was determined.

Viability assays: For early larval viability assays with CRISPR mutants (Figure 1B), eggs were collected for 3 hours following the methods described above, see "Quantification of tracheal air filling". Twenty-four hours AEL, the number of viable and non-viable 1st instar larvae were counted to assess early survival in *dPTPMT1*^{mut1} mutants. If a larva did not respond to mechanical stimulus it was counted as non-viable. This assay was repeated three times using independent crosses. For viability experiments with *btl-GAL4* RNAi (Figure 2E), eggs were collected for 3 hours following the methods described above. 1st instar larvae were collected 24 hours AEL and placed in vials containing SY media for the continuation of development. Larvae were floated out of the media with 20% sucrose and then poured out onto filter paper for assessment. Larvae were assayed cross-sectionally for viability at 48 hours, 72 hours, 96 hours, and 120 hours post-egg collection. If a larva did not respond to mechanical stimulus it was counted as non-viable. The percentage of viable larvae in each vial was determined at each time point. Larvae were also assessed for tracheal blackening and developmental stage at 48 hours, 72 hours, and 96 hours. Assays were repeated 2-3 times using independent crosses with 2-4 vials per cross, with 13-30 larvae per vial. To quantify adult viability (Figure 1C, 4I, and S4C), the number of adults of each genotype was counted within 0-2 days of eclosion. Genotypic frequency was calculated and then normalized to the expected genotypic frequency for that genotype. In cases where the genotype of interest had a viability of above 100%, the siblings of this genotype (for example, *UAS-*

RNAi/*TM3*(*ActGFP*, *ser*)) showed slight reductions in viability, which is to be expected given the multiple inversions present within balancer chromosomes. For these assays, tracheal blackening was also assessed in all adults, 0-2 days post-eclosion. A minimum of three independent crosses were quantified for each genotype.

ATP assay: ATP levels were measured using a luciferase-based bioluminescence assay (ATP Bioluminescence Assay Kit HS II, 11699709001, Roche Applied Science) following manufacturer's instructions. For each measurement, a single fly was homogenized in 100 µl of lysis buffer (provided by the kit). The lysate was boiled for 5 minutes, briefly placed on ice, and then cleared by centrifugation at 17,000g for 1 minute. Lysates were diluted 1:50 with dilution buffer (provided by the kit) and then incubated with equal volume of luciferase. Luminescence was immediately measured using a FlexStation 3 (Molecular Devices). A minimum of 7 replicates were performed for each genotype. Values were normalized to total protein, as measured by the BCA assay.

Sample preparation for imaging and data analysis: Stereoscopic images of adults and larvae were taken using a DEM130 Digital Eyepiece (2M pixels CMOS) or a Leica S9i (Leica Microsystems). Fluorescence patterns in *Drs-GFP* flies were assessed using a SMT1-FL Fluorescence Stereo-Microscope System (Tritech Research). Adult flies were anesthetized using an ice pack during assessment. If fluorescence was observed in the inner tracheal tube of the leg, the fly was counted as having *Drs-GFP* expression in the trachea. For *Drs-GFP* examination of adults, females were assessed because males had reduced viability on this background. Flies were assessed at the same time of day to control for circadian variation in immune gene expression

(Neyen et al., 2014). *Drs-GFP* flies were imaged using a Leica M165 FC (Leica Microsystems). Flies were anesthetized using dry ice during imaging. Confocal imaging was performed using a Leica SPE laser scanning confocal microscope (Leica Microsystems). For live-imaging of mito-GFP**,** 1st and 2nd instar larvae were collected using the methods described above, see "Quantification of tracheal air filling". Larvae were immobilized beneath a piece of clear tape and assessed for the presence of mito-GFP puncta within the dorsal trunk and tracheal branches. A posterior region of the dorsal trunk of the trachea was then selected for imaging. For *in vivo* ROS detection, *mito-roGFP2-Orp1* and *Grx1* were used to measure mitochondrial ROS levels in the trachea of intact larvae. *UAS-mito-roGFP2-Orp1* or *Grx1* were expressed in fly trachea using the *btl-GAL4* driver. 1st and 2nd instar larvae were collected and mounted using the methods described for mito-GFP. Larvae were imaged following the methods of Albrecht and colleagues (Albrecht et al., 2011, Barata and Dick, 2013). Specifically, excitation of roGFPs by the 405nm and 488nm laser was performed sequentially. Emission was detected at 500-530nm. This system was calibrated using dissected tracheal tissue treated with 10mM dithiothreitol or 1mM diamide, to produce fully reduced or oxidized samples, respectively. For processing, images were converted to a 32-bit format. Following background subtraction, the 488 nm image was thresholded and pixels outside of the threshold were converted to "NaN." Ratios were calculated by dividing the pixel intensity of the 405 nm image by the 488 nm image. For TUNEL staining, tracheae were dissected in PBS, fixed with 4% paraformaldehyde for 20 minutes at room temperature, and then washed with PBS. Tracheae were permeabilized for 20 minutes at room temperature with 0.6% (vol/vol) Triton-100 and 0.6% (wt/vol) sodium deoxycholate, and then washed with PBS. After permeabilization, the protocol outlined in the In Situ Cell Death Detection Kit (12156792910, Sigma/Roche) was followed. Tracheae were cover-slipped with ProLong™ Glass Antifade

Mountant with NucBlue™ Stain (Thermo Fisher, P36981) and cured at room temperature (22°C) for 48-60 hours, following the manufacturer's recommendations. All fluorescent images were adjusted for brightness.

Transmission electron microscopy: For TEM, 3rd instar male larvae were filleted in 1 × Ca^{2+} free saline (0.128 M NaCl, 2 mM KCl, 5 mM EGTA, 4 mM $MgCl_2$, 5 mM HEPES, 0.0355 M sucrose), and fixed in Karnovsky's fixative (0.1 M sodium cacodylate buffer pH 7.4, 2% glutaraldehyde, and 4% paraformaldehyde) at room temperature (22°C, RT) for 30 minutes and then kept at 4°C overnight. Specimens were post-fixed in cold/aqueous 1% osmium tetroxide for 1 hour, warmed to RT for 2 hours, and then rinsed 3 times in ultra-filtered H_2O. Specimens were subsequently stained en bloc with 1% uranyl acetate at RT for 2 hours, dehydrated in a graded ethanol series, immersed in propylene oxide (PO) for 15 minutes, infiltrated with a graded series of PO and Embed-812 resin, and then embedded in EMbed-812 resin at 65°C overnight. Specimens were sectioned transversely at segment A1/A2 or in the case of the *btl-GAL4 > UAS-dPTPMT1* RNAi larva, in a region where tracheal blackening was visible. Sections were cut to a thickness of 75-90 nm and laid on formvar/carbon-coated slot Cu grids. Sections were stained for 40 seconds with 3.5% uranyl acetate in 50% acetone followed by Sato's Lead Citrate for 2 minutes. Sections were observed using a JEM-1400 120kV (Joel, Japan) and images were taken using an Orius 832 4k X 2.6k digital camera with 9um pixel (Gatan, CA). Images were processed with Photoshop CS6. Tracheal tubes were identified by their unique structure (i.e. a simple epithelial monolayer, with a lumen and an internal tracheal cuticle).

Statistical analyses: Throughout this paper, the distribution of data points is expressed as mean ± SEM, unless otherwise stated. Individual data points are shown in bar graphs where $n \leq 6$. For roGFP experiments, data is displayed as box-and-whiskers, as is conventional for these experiments (Albrecht et al., 2011). Boxes show $25^{th}/75^{th}$ percentiles, whiskers are the minimum and maximum values, and x is the median marker. For comparisons between two groups, F-test was performed, followed by t-test. Chi-square test was used to determine statistical significance for groups of categorical data. The number of flies and experimental replicates (n) are in figure legends and methods. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Statistical experiments were performed in Excel or GraphPad Prism 8 Software.

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