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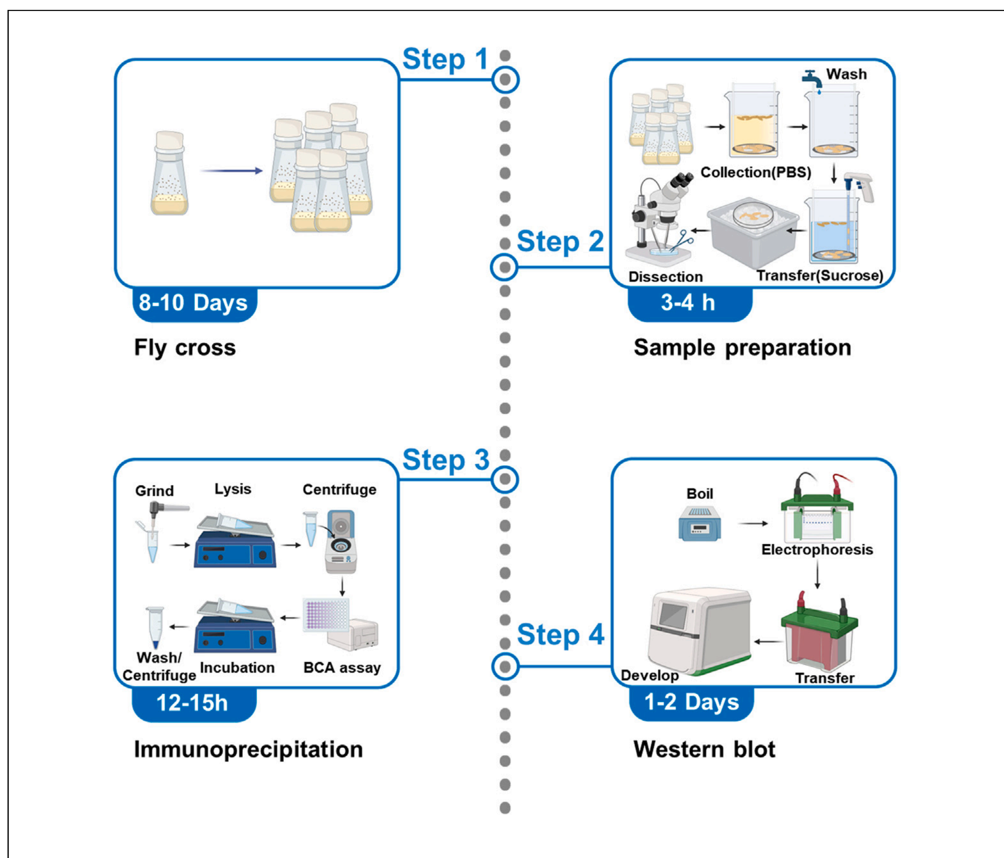
Protocol

Mass isolation of staged *Drosophila* pupal intestines for analysis of protein ubiquitylation



Ruoxi Wang, Panagiotis D. Velentzas, Eric H. Baehrecke

ruoxi.wang@umassmed.edu (R.W.)
panagiotis.velentzas@umassmed.edu (P.D.V.)
eric.baehrecke@umassmed.edu (E.H.B.)

Highlights

This protocol enables mass isolation of *Drosophila* pupae for biochemistry experiments

Pupae are staged based on buoyancy in sucrose and morphology

Staged *Drosophila* intestines are isolated for analysis of protein ubiquitylation

This protocol can be adapted for isolation of other pupal tissues

Large quantities of developmentally synchronized pupal intestines are required for biochemistry experiments. Here, we present a protocol for the mass isolation of staged pupal intestines during *Drosophila melanogaster* development based on buoyancy in sucrose for biochemical evaluation of protein ubiquitylation. We describe steps for crossing flies, preparation of samples, immunoprecipitation of proteins from staged isolated tissues, and analysis of samples by western blot. This protocol can be extended to investigate biochemical changes in other tissues.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Mass isolation of staged *Drosophila* pupal intestines for analysis of protein ubiquitylation

Ruoxi Wang,[1,2,*] Panagiotis D. Velentzas,[1,2,*] and Eric H. Baehrecke[1,3,*]

[1]Department of Molecular, Cell and Cancer Biology, University of Massachusetts Chan Medical School, Worcester, MA 01605, USA

[2]Technical contact

[3]Lead contact

*Correspondence: ruoxi.wang@umassmed.edu (R.W.), panagiotis.velentzas@umassmed.edu (P.D.V.), eric.baehrecke@umassmed.edu (E.H.B.)
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SUMMARY

Large quantities of developmentally synchronized pupal intestines are required for biochemistry experiments. Here, we present a protocol for the mass isolation of staged pupal intestines during *Drosophila melanogaster* development based on buoyancy in sucrose for biochemical evaluation of protein ubiquitylation. We describe steps for crossing flies, preparation of samples, immunoprecipitation of proteins from staged isolated tissues, and analysis of samples by western blot. This protocol can be extended to investigate biochemical changes in other tissues. For complete details on the use and execution of this protocol, please refer to Wang et al. (2023).[1]

BEFORE YOU BEGIN

Collecting large amounts of tissues from staged *Drosophila melanogaster* during development for biochemistry experiments is challenging. Here, we describe a protocol to isolate pupae 2–3 h after puparium formation (APF) based on cuticle color, intestine length (Figure 1) and the buoyancy of pupae (Figure 2) for the study of protein ubiquitylation in intestines during the transition from larva to adult. This protocol can also be used to conduct biochemistry experiments using different tissues from pupae at the same stage. The researcher should have a good knowledge of *Drosophila* morphology[2] and genetics.[3] This protocol is modified from the study by Reis et al.[4]

Fly crosses

Timing: 8–10 days

1. Collect 200–300 virgin female animals of the appropriate genotype.

   Note: In this study, we use a strain of PTEN Induced Kinase 1 (*PINK1*) loss-of-function mutant allele (*PINK1*^{B9})[5] (*PINK1*^{B9}/FM7, Act-GFP) and a *white* allele *w*^{1118} as control. Our genetic studies indicate that the Keap1/Cullin3 ubiquitin ligase functions downstream of PINK1 to influence Rtnl1,[1] and therefore we tested the influence of *PINK1* loss on Rtnl1 ubiquitylation.

2. In a vial containing *Drosophila* food (Table 1), add 200–300 virgin females and ∼50 male adult animals of the appropriate genotype.

   Note: In this study, we use males from a strain of reticulon-like protein (Rtnl1) tagged with 3× FLAG-V5 (Rtnl1-3×FLAG-V5).[1]

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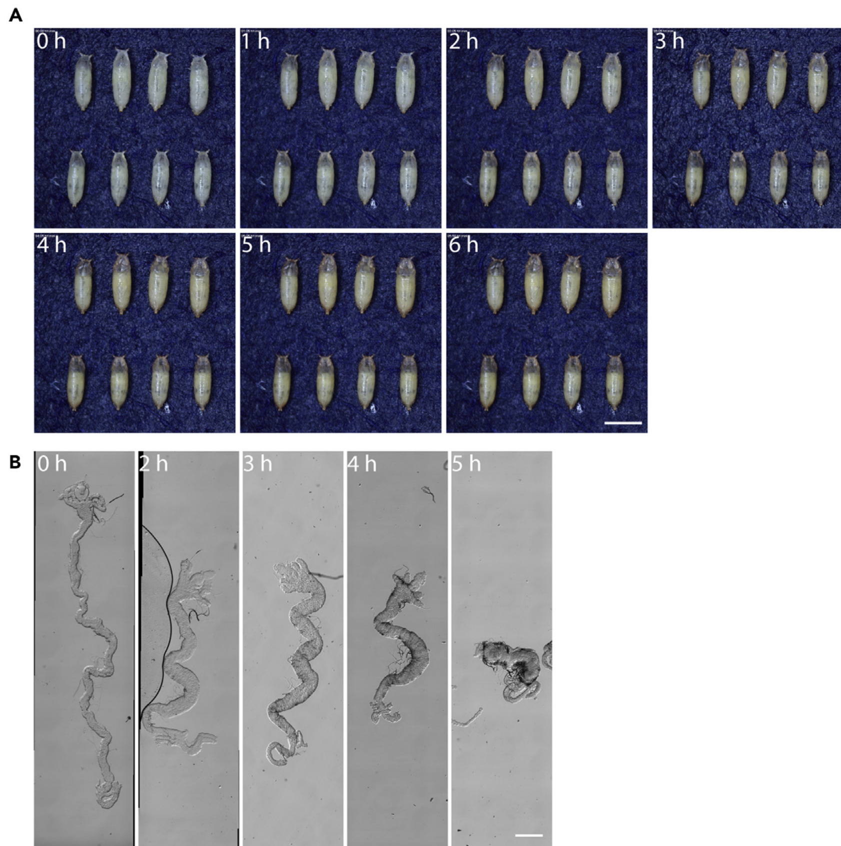

Figure 1. Staging pupal intestines

Pupal cuticle color changes (A) and intestine size and morphology changes (B) during development. Scale bar in (A) represents 2 mm, and scale bar in (B) represents 500 μm.

⚠ CRITICAL: Set-up the appropriate crosses for all of the genetic controls needed for each experiment. Delay 2–3 days to set-up the control crosses if the mutant cross grows slower than control. Add approximately 50 mg dry yeast powder on top of the food prior to adding flies.

3. Incubate the vial with the flies in a 25°C incubator with a 16 h day/8 h night cycle for 2 days.
4. Flip the mated flies onto bottles with *Drosophila* food and change the bottles every day so that females lay fresh eggs onto new food daily.

Note: White pre-pupae will start forming on the side of the bottle approximately five days after the eggs are laid (troubleshooting problem 1)

Preparation of 1× phosphate buffered saline (PBS)

⏲ Timing: approximately 5 min

5. In a 1 L beaker,
   a. Add 100 mL 10× PBS solution (Table 2).
   b. Add Milli-Q water to 1 L.

Preparation of 11% (w/v) sucrose solution

⏲ Timing: approximately 5 min

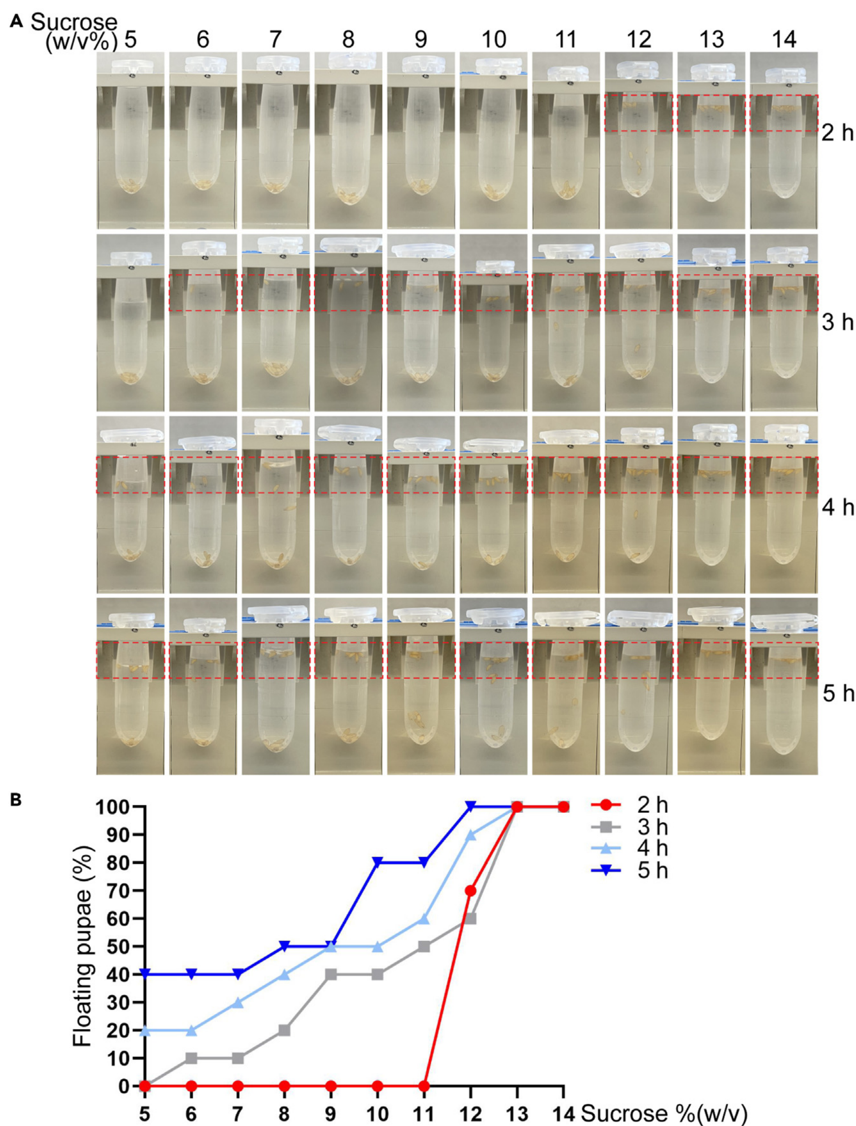

Figure 2. Buoyancy of staged pupae in sucrose solutions

(A) Photos of pupae at different developmental stages in different concentrations of sucrose. Red dotted boxes indicate floating pupae.

(B) Quantification of floating pupae at different developmental stages in different concentrations of sucrose solutions.

6. In a 500 mL beaker,
   a. Add 55 g sucrose.
   b. Add 1× PBS buffer to make up the volume to 500 mL.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
V5 (1:1,000)	Invitrogen	Cat#R960-25, RRID: AB_2556564
Ubiquitin (P4D1) (1:1,000)	Cell Signaling Technology	Cat#3936, RRID: AB_331292

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Actin (1:1,000)	Developmental Studies Hybridoma Bank	Cat#JLA20, RRID: AB_528068
Goat anti-mouse IgG (H + L) secondary antibody, HRP (1:10,000)	Invitrogen	Cat#31430, RRID: AB_228307
Chemicals, peptides, and recombinant proteins		
ChromoTek V5-Trap agarose	Proteintech	V5ta-20
Agar	MoorAgar	41004
Brewer's yeast	Fisher Scientific	ICN90331225
Cornmeal	Fisher Scientific	ICN90141125
Molasses	Thomsen Food Services	02625
Propionic acid	Fisher Scientific	A258
Phosphoric acid	Fisher Scientific	A260
Tegosept	Genesee Scientific	20-259
D(+)-sucrose	Fisher Scientific	BP220-1
Halt protease and phosphatase inhibitor single-use cocktail, EDTA-free (100×)	Thermo Scientific	78445
NuPAGE LDS sample buffer (4×)	Invitrogen	NP0007
Novex WedgeWell 4%–20%, Tris-glycine, 1.0 mm, mini protein gel, 12-well	Invitrogen	XP04202BOX
β-Mercaptoethanol	Sigma-Aldrich	63689
SuperSignal West Pico PLUS chemiluminescent substrate	Thermo Scientific	34580
SuperSignal West Atto ultimate sensitivity substrate	Thermo Scientific	A38555
EveryBlot blocking buffer	Bio-Rad	12010020
Pierce BCA protein assay kits	Thermo Scientific	23225
Blotto, non-fat dry milk	Santa Cruz Biotechnology	sc-2324
10× Tris/glycine/SDS	Bio-Rad	1610732
Triton X-100	Sigma-Aldrich	T8787
NaCl	Fisher Scientific	BP358-212
KCl	Fisher Scientific	BP366-500
Na_2HPO_4	Fisher Scientific	BP322-500
KH_2PO_4	Fisher Scientific	BP-362-500
HEPES	Sigma-Aldrich	H3375
$MgCl_2$	Invitrogen	AM9530G
Glycerol	Fisher Scientific	G33-1
Tris base	Fisher Scientific	BP152-1
Glycine	Fisher Scientific	G46-1
Sodium dodecyl sulfate (SDS)	Fisher Scientific	PI28364
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	E9884
Trizma hydrochloride	Sigma-Aldrich	T3253
Methanol	Fisher Scientific	A452-4
Hydrophobic PVDF transfer membrane, 0.45 μm pore size	Millipore	IPVH00010
Chromatography paper	Fisherbrand	05-714-4
Non-pyrogenic serological pipet	Fisherbrand	13-678-11E
Experimental models: Organisms/strains		
w^{1118}	Bloomington Drosophila Stock Center	3605
Rtnl1-3×FLAG-V5	Eric Baehrecke Lab[1]	N/A
$PINK1^{B9}$	Jongkyeong Chung	N/A
Software and algorithms		
ImageJ 2.14.0	Fiji	https://fiji.sc/
Image Studio Lite v.5.2	LI-COR	https://www.licor.com/bio/image-studio-lite/
GraphPad Prism 9.0	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Other		
Scissors	Fine Science Tools	15000-02

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dumont #5 forceps	Fine Science Tools	11251-30
BD Clay Adams nutator mixer	BD Diagnostics	421105
Petri dish	Fisherbrand	FB0875713
Drummond Scientific original Pipet-Aid	Drummond Scientific	D43791
Incubator	VWR	1510E
Incubator with day/night cycle	Percival Scientific	I-36VL
Micro-grinder, pestle mixer	RPI Research Products International	299220
Mini gel tank	Invitrogen	A25977
Mini Trans-Blot electrophoretic transfer cell	Bio-Rad	1703930
PowerPac HC high-current power supply	Bio-Rad	1645052
Nutator	Fisher Scientific	260100F
Shaker	Corning	6780-FP
Graduated cylinders	Fisher Scientific	N/A
Refrigerator	Kenmore	N/A
ChemiDoc MP imaging system	Bio-Rad	N/A
Centrifuge	Eppendorf	5418
Refrigerated centrifuge	Beckman Coulter	C63124

MATERIALS AND EQUIPMENT

- 20% (v/v) Triton X-100 solution: Add 10 mL Triton X-100 to Milli-Q water to 50 mL final volume. Store at 22°C.
- 0.1% PBST solution: Add 5 mL 20% Triton X-100 solution to 1× PBS make 1 L final volume. Store at 22°C.
- 10× transfer buffer: Dissolve 90 g Glycine, 19.3 g Tris-Base in Milli-Q water and make up the volume to 1 L. Store at 22°C.
- 1× running buffer: Add 100 mL 10× Tris/Glycine/SDS Buffer and make up the volume to 1 L with Milli-Q water. Store at 22°C.
- 1× transfer buffer: Add 100 mL 10× transfer buffer, 200 mL methanol and make up the volume to 1 L with Milli-Q water. Store in refrigerator at 4°C.

STEP-BY-STEP METHOD DETAILS

Isolation of pupae

Timing: 1–2 h

The steps below describe how to isolate a large amount of *Drosophila* at 2–3 h after puparium formation (APF) by differential buoyancy on sucrose solution to conduct biochemistry experiments.

Table 1. *Drosophila* food

Reagent	Final concentration (%)	Amount
Agar	0.65%	6.5 g
Brewer's Yeast	2.35%	23.5 g
Cornmeal	6%	60 g
Molasses	6%	60 mL
Propionic Acid	0.2%	2 mL
Phosphoric Acid	0.2%	2 mL
Tegosept	0.13%	1.3 g
Water	N/A	to 1 L

Store at 4°C for up to 1 week.

Table 2. 10× PBS (pH 7.4)

Reagent	Final concentration	Amount
NaCl	1.37 M	80 g
KCl	27 mM	2 g
Na_2HPO_4	100 mM	14.4 g
KH_2PO_4	18 mM	2.4 g
Water	N/A	to 1 L

Storage: 1 month, 22°C.

1. Use a wet paint brush (Figure 3A) to gently move all pupae on the side of bottles (10 bottles or more for each genotype) to a 50 mL beaker with 1× PBS (Figure 4A).

   Note: Use a moist brush to wet all pupae with water and wait for 2–3 min. This step helps to detach the pupae from the bottle walls without damage.

2. Gently stir the floating pupae with a brush.

   Note: This step helps dissociation of attached pupae.

3. Use a plastic spoon to remove and discard all floating pupae (Figure 4B).

   Note: Keep the pupae that sink to the bottom of the beaker with PBS for the next step of isolation.

4. Transfer the PBS with the remaining pupae into an inverted embryo collection cage in a beaker of similar size (Figures 3B and 3B′; Figure 4C).
5. Remove the collection cage and discard the PBS solution (Figure 4D).
6. Gently shake the cage in a beaker with Milli-Q water 2–3 times to wash the pupae (Figure 4E).

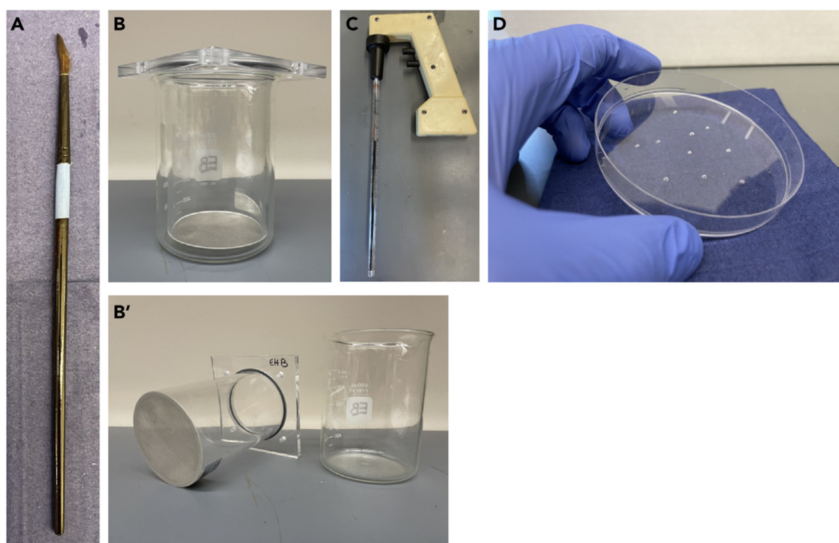

Figure 3. Tools used
(A) Brush to remove pupae.
(B and B′) Embryo collection cage and beaker.
(C) Pipette with the tip cut to be larger than pupae (15 mL).
(D) Petri dish with holes for liquid absorbance.

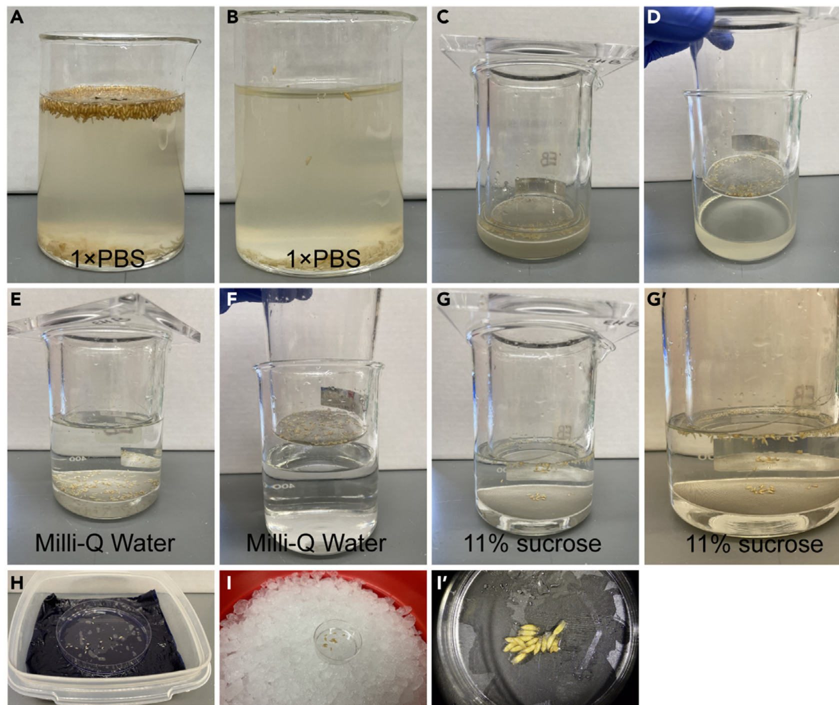

Figure 4. Photos of each key step in the isolation of pupae at different developmental stages

7. Remove the collection cage and discard the water (Figure 4F).
8. Add 11% sucrose solution to the collection cage in a beaker, stir the solution and wait 2–3 min for separation of pupae based on buoyancy (Figures 4G and 4G′).

   Note: Stirring the sucrose solution can also help to move the pupae to the center.

9. Use a pipette with the tip cut to be larger than the pupae (Figure 3C) to collect all pupae from the bottom of the sucrose solution, and move them to a 10 cm Petri dish with holes (Figure 3D).

   Note: For the purpose of this protocol approximately 80 pupae are required for each genotype.

   Note: Place the Petri dish in a container lined with napkins or tissue paper to soak up the excess sucrose solution.

10. Use a brush to move all pupae to a 5 cm Petri dish, and place the dish on ice (Figures 4I and 4I′).

   Note: Fly development is significantly delayed on ice, and pupae can be kept on ice 40–60 min.

Tissue dissection and lysate preparation

Timing: 3–4 h

The steps below describe tissue dissection and lysate preparation that are needed for immunoprecipitation in the following steps.

11. Use two forceps and a pair of scissors to dissect fly intestines and place them in a tube with chilled 1× PBS.

Note: Dissect in batches of 20 pupae to minimize proteolysis of the samples. Intestines from approximately 80 pupae will be required for each genotype/sample.

12. Remove the PBS solution and add 40 μL Co-IP buffer (Table 3) supplemented with Halt Protease and Phosphatase Inhibitor (1:100). Homogenize the intestines using a Micro-Grinder and a pestle. Place the sample on ice and repeat for the next batches.
13. Combine the homogenates from all of the batches to create a single sample, add Co-IP buffer to 500 μL and lyse for 1 h at 4°C with rotation (troubleshooting problem 2).
14. Remove 15 μl of lysate from each genotype and mix the lysate with 15 μl 2×SDS sample buffer (Table 4) which will be used as the input positive control. Centrifuge the remainder of the samples at 20,412 g for 10 min at 4°C. Move the supernatant to a new tube.
15. Measure protein concentration of each sample and the associated inputs using bicinchoninic acid (BCA) protein assay kit according to the manufacturer's protocol.

Note: Intestines from 80 pupae will yield approximately 1 mg of protein (2 mg/mL).

16. Input positive control:
    a. Remove 20 μg of protein from each input sample.
    b. Add 2×SDS sample buffer to a final volume of 30 μL.
    c. Add 10 μL of 4× NuPAGE LDS sample buffer supplemented with β-mercaptoethanol (10%) and mix.

Note: These samples can be stored at −80°C

Immunoprecipitation of proteins from staged isolated tissues

Timing: approximately 12–15 h

The steps below describe immunoprecipitation of proteins from staged isolated intestine lysates.

17. Briefly wash 15 μL V5 trap agarose bead slurry with 1× PBS and centrifuge for 2 min at 1500 g to pellet beads. Remove the supernatant and repeat the wash 2 more times.

Note: This step can be conducted simultaneously with step 16.

18. Mix equal amounts of protein (800–1000 μg) from each sample with Co-IP buffer to a final volume of 1 mL and incubate the samples with V5 Trap agarose beads for 12–14 h with rotation at 4°C.

Table 3. Co-immunoprecipitation buffer (Co-IP buffer)

Reagent	Final concentration	Amount
Triton X-100	1% (v/v)	0.5 mL
HEPES (pH 7.5)	10 mM	119 mg
KCl	142.5 mM	53 mg
$MgCl_2$	5 mM	250 μL, 1 M $MgCl_2$ (stock solution)
EDTA	1 mM	1.5 mg
Glycerol	10% (w/v)	5 mL
Water	N/A	to 50 mL

Storage: freshly made, 4°C.

Table 4. 2× SDS sample buffer

Reagent	Final concentration	Amount
SDS	2% (w/v)	1 g
Tris-HCl (pH 6.8)	63 mM	50 mg
Glycerol	10% (w/v)	5 mL
Water	N/A	to 50 mL

Storage: 1 month, 22°C.

Note: 70–80 intestines will typically yield >800 μg protein. The number of intestines used for immunoprecipitation depends on both bait protein expression levels in the tissue and the antibody used for immunoprecipitation. The amount can be optimized accordingly for each experiment.

19. Spin the beads at 1500 g for 2 min at 4°C.

⚠ CRITICAL: The centrifuge speed must be below 2000 g to avoid breaking of the beads.

20. Remove the supernatant and add 1 mL chilled Co-IP buffer. Wash for 20 min with rotation at 4°C.
21. Repeat steps 19–20 5 times.
22. Remove the supernatant and add 50 μL 1× NuPAGE LDS Sample Buffer supplemented with β-mercaptoethanol (10%) to the beads. Boil the beads and the input samples (Step 16) for 10 min at 100°C.
23. Spin down the beads at 1500 g for 5 min at 22°C and move the supernatant from the beads to a new tube.
24. Prepare to load samples onto the SDS-PAGE gels for electrophoresis (troubleshooting problems 3 and 4).

Western blot

⏲ Timing: 1–2 days

The steps below describe analysis of immunoprecipitation samples by western blot. Western blot was conducted according to standard protocol.[6,7]

25. Prepare a Novex Mini Protein Gel (12-well) for protein separation by electrophoresis. Add 1× SDS running buffer into the assembled gel tank.
26. Load 20 μg of each input sample and IP lysates into the wells of a gel.
27. Run at 80 V until all lysates are stacked in the gel and then change the voltage to 140 V. Run the samples until the phenol red line reaches the bottom of the gel.
28. Transfer the proteins to PVDF membrane in prechilled 1× transfer buffer for 90 min at 290 mA.
29. Block the membrane with 5% non-fat milk in 0.1% PBST for 1 h at 22°C.
30. Wash the membrane briefly and incubate with antibody against ubiquitin (1:1000) in EveryBlot blocking buffer 12–14 h at 4°C with shaking.
31. Remove the primary antibody and wash the membrane with 0.1% PBST for 15 min with shaking at 22°C.
32. Repeat step 31 twice.
33. Incubate the membrane with mouse HRP secondary antibody in 5% non-fat milk in 0.1% PBST for 1 h at 22°C with shaking.
34. Wash the membrane with 0.1% PBST for 15 min at 22°C.
35. Repeat step 34 twice.

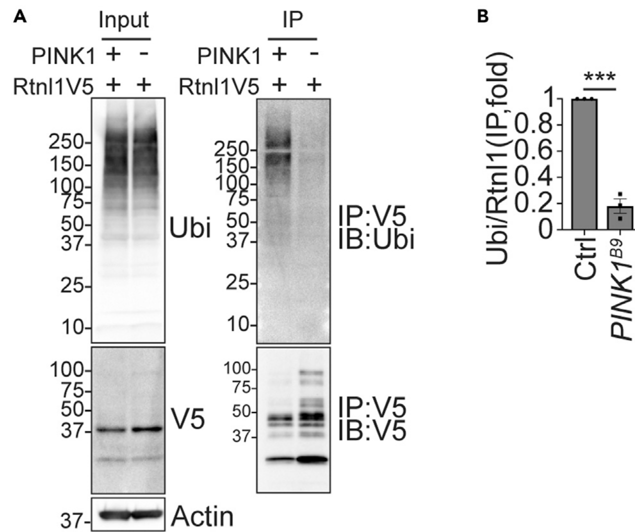

Figure 5. PINK1 regulates Rtnl1 ubiquitination

(A) Rtnl1-3×FLAG-V5 was immunoprecipitated from either control or $PINK1^{B9}$ mutant intestines. Whole lysates (Input) and immunoprecipitated proteins (IP) were immunoblotted with antibodies against ubiquitin (P4D1), V5, and Actin.
(B) Quantification the ratio of ubiquitin (IP)/total Rtnl1-V5 (IP) in $PINK1^{B9}$ mutant intestines normalized to control intestines. n = 3 independent experiments. ***. $p < 0.001$.

36. Develop the membrane with SuperSignal West Pico PLUS Chemiluminescent Substrate or SuperSignal West Atto Ultimate Sensitivity Substrate and image using ChemiDoc MP imaging system.
37. Briefly wash the membrane with 0.1% PBST and strip the membrane with Restore PLUS Western Blot Stripping Buffer for 20 min at 22°C with shaking.
38. Wash the membrane with 0.1% PBST for 15 min at 22°C with shaking.
39. Repeat step 38 twice and block the membrane with 5% non-fat milk in 0.1% PBST for 1 h at 22°C.
40. Incubate the membrane with antibody against V5 in EveryBlot blocking buffer (1:1,000) 12–14 h at 4°C with shaking.
41. Repeat steps 31–36.
42. Repeat steps 37–41 with antibody against Actin (1:1000) (troubleshooting problem 5).

EXPECTED OUTCOMES

Autophagy is activated in *Drosophila* enterocytes during development.[8] Mitochondria and endoplasmic reticulum (ER) are selectively removed by developmentally programmed autophagy in fly intestine enterocytes.[1,9,10] PINK1 kinase and the E3 ubiquitin ligase complex component Keap1 function in the same pathway to regulate ER removal. ER-phagy receptor Rtnl1 ubiquitylation is decreased in $PINK1^{B9}$ loss-of-function mutant intestines compared to control intestines 2–3 h APF (Figure 5). Using the protocol described above, we discovered that the E3 ubiquitin ligase complex component Keap1 plays a similar role in ubiquitylation of the ER-phagy receptor Rtnl1.[1]

QUANTIFICATION AND STATISTICAL ANALYSIS

ImageJ and Image Studio Lite were used to quantify protein levels by western blot analyses. p values were calculated using a two-tailed unpaired t-test from GraphPad Prism 5 (https://www.graphpad.com/scientific-software/prism/). No animals were excluded from statistical analyses, the experiments were not randomized, and the investigators were not blinded. All error bars are SEM.

LIMITATIONS

Two major limitations of this protocol are the availability of antibodies that are suitable for immunoprecipitation of *Drosophila* proteins, and that the generation of endogenously tagged proteins by gene editing is labor intensive. Thus, these issues limit the proteins that can be studied. Additionally, fly intestines, like those in mammals, contain several different cell types. Investigation of mutant gene function may influence the composition of intestine stem and other cell types, and this should be taken into consideration as stem cell proliferation and differentiation changes will affect enterocyte numbers. Since only enterocytes exhibit robust developmental programmed autophagy at this stage, the indirect impact of other cell types on enterocytes needs to be evaluated with rigorous cell biological analysis to complement whole intestine lysate biochemistry.

TROUBLESHOOTING

Problem 1

Fail to collect enough pupae for biochemistry experiments (before you begin).

Potential solution

- Keep flipping the original crosses onto new food every day until enough material is collected.
- Some mutant flies grow slower and are less healthy than control strains. Set up the mutant cross before control cross. Set up replicate crosses (2–3 replicates) and flip all replicates of crosses onto new food every day.
- If the mutant genetic strain is very unhealthy, use tissue-specific RNAi as an alternative approach to decrease gene function. For the enterocytes of the intestine, UAS-RNAi can be specifically expressed by crossing to the NP1-GAL4 strain for RNAi expression in enterocytes.[11]

Problem 2

Dissection speed is slow (Step 13).

Potential solution

- Practice dissection and prepare all required materials before dissection.

Problem 3

Fail to yield sufficient amounts of bait protein to be detected (Step 24).

Potential solution

- Pretest target protein levels by western blot.
- Adjust the number of intestines for immunoprecipitation.
- If using a protein tagged by gene editing, use homozygous target protein for experiments.
- If using epitope tag conjugated agarose beads, test if a different company source performs better in experiments.
- Boil the beads in LDS sample buffer before freezing to avoid sample degradation.
- Utilize a dual-tag approach when designing knock-in strategy for the target protein to enable a broader range of choices for immunoprecipitation.

Problem 4

The change of target protein is not obvious between samples (Step 24).

Potential solution

- Increase the duration and frequency of bead washes.
- Rotate the tubes while washing the beads.

Problem 5

Antibodies against fly proteins are not available for immunoblot analysis (Step 42).

Potential solution

- Test antibodies against other species to determine if the target protein is conserved in other species by western blot.
- Tag the target protein by gene editing.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Eric Baehrecke (eric.baehrecke@umassmed.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets/code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

R.W. and P.D.V. performed the experiments and R.W., P.D.V., and E.H.B. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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