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Assays to monitor aggrephagy in Drosophila brain

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Assays to monitor aggrephagy in *Drosophila* brain

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Running head: Aggrephagy in *Drosophila*

Summary

Accumulation of ubiquitinated protein aggregates is a hallmark of most ageing-related neurodegenerative disorders. Autophagy has been found to be involved in the selective clearance of these protein aggregates, and this process is called aggrephagy. Here we provide two protocols for the investigation of protein aggregation and their removal by autophagy using western blotting and immunofluorescence techniques in *Drosophila* brain. Investigating the role of aggrephagy at the cellular and organismal level is important for the development of therapeutic interventions against ageing-related diseases.

Keywords: *Drosophila*, aggregate, aggrephagy, protein extraction, western blot, immunofluorescence, Ref(2)P, ubiquitin, brain

1. Introduction

Autophagy is a dynamic catabolic pathway based on the formation of double-membraned vesicles (autophagosomes) that eventually fuse with the lysosomes for the degradation of their content [1]. This pathway has been related to many physiological processes that are involved in the maintenance of cellular homeostasis and quality control of the cell by the removal of redundant or damaged proteins and organelles [2].

Damaged proteins often form large aggregates that are ubiquitinated. Selective removal of these aggregates by autophagy is called aggrephagy [3]. Ubiquitination may facilitate the uptake of aggregates into autophagosomes. Indeed, aggrephagy receptors such as p62/SQSTM1, NBR1, NDP52/CALCOCO2, optineurin and Tollip recognize ubiquitin via their ubiquitin-binding domains [3-6].

To date, only one selective autophagy receptor has been described in *Drosophila*; the protein Ref(2)P is homolog to mammalian p62/SQSTM1. Like its mammalian counterpart, Ref(2)P contains multiple interaction domains, including a ubiquitin-binding domain UBA and a LIR motif crucial for its connection to autophagy through the interaction with Atg8a (homolog to LC3/GABARAP mammalian proteins) [7,8].

Autophagy is essential for long-lived cells such as neurons. Indeed, neurons are post-mitotic cells and cannot dilute their cytosol by cell division, thus relying on autophagy for the elimination of aggregates. Dysfunctional autophagy has been associated with premature aging and neurodegeneration [9]. In that aspect,

Drosophila constitutes a model of choice due to its short life cycle, rapid aging, and easy and low-cost maintenance.

In this chapter, we describe two protocols for the investigation of aggregate formation in adult *Drosophila* brain. The first technique consists in the global analysis of protein aggregation by differential detergent protein extraction and western blotting. Indeed, unlike their non-aggregation prone counterpart, aggregated proteins are often characterized by being insoluble in lysis buffers containing mild detergents. Also, it is possible to investigate the turnover of aggregated protein by extraction of soluble proteins using a mild detergent-containing lysis buffer, followed by solubilizing the pelleted material under harsher conditions with a lysis buffer containing 2% SDS [10].

The second protocol described the dissection and immunostaining of *Drosophila* brain. Fluorescence microscopy is another widely used technique for the study of aggregated proteins. The advantage in fluorescence microscopy resides in the *in situ* staining and observation of the aggregate proteins in tissues, organs, and cells.

2. Materials

2.1. Drosophila stocks and maintenance

Flies are raised on a yeast/cornmeal diet and are kept at 25ºC and 70% humidity with a 12-12 hours' light-dark cycle. The fly stocks used in this chapter are available from

the Bloomington Drosophila Stock Center (Indiana University): w^{1118} (#3605, referred to as 'WT') and $Atg8a^{KG07569}$ (#14639, referred to as 'Atg8a')

Fly food: 1 L H_2O, 42 g inactive dry yeast, 60 g yellow cornmeal, 130 g sucrose, 5.5 g agar and 15 mL Nipagin 10% (*see* **Note 1**).

2.2. Buffers and solutions for protein extraction and western blotting

1. Triton lysis buffer: 1x PBS, 1% Triton X-100, 1mM Na_3VO_4, 50mM NaF, 5mM $Na_4P_2O_7$, 1x protease inhibitor cocktail

2. SDS lysis buffer: 50mM Tris pH 7.8, 2% (w/v) SDS, 1mM Na_3VO_4, 50mM NaF, 5mM $Na_4P_2O_7$, 1x protease inhibitor cocktail

3. 10x Gel Protein running buffer: 250mM Tris base, 2M glycine, 1% SDS, pH 8.3

4. Transfer buffer: 25mM Tris, 2mM glycine, 10% ethanol. Prepare a 10x Transfer buffer stock solution (250mM Tris base, 2M glycine, pH 8.3). Dilute 1 part of 10x stock solution with 8 part of water and 1 part of ethanol (*see* **Notes 2** and **3**)

5. 6x Laemmli buffer stock solution: 12% (w/v) SDS, 60% glycerol, 0.12% (w/v) Bromophenol blue, 0.375M Tris pH 6.8 (*see* **Note 4**)

6. 4x Laemmli buffer working solution: 6.7mL 6x Laemmli, 2mL 2-mercaptoethanol (20%), 1.3mL ddH_2O (*see* **Note 5**)

7. TBS (10x, 1L): 24.23g Trizma HCl, 80.06g NaCl, pH 7.6

8. TBST: 1x TBS, 0.1% Tween-20

9. Blocking buffer: 5% (w/v) non-fat milk or BSA in 1x TBST

10. Secondary antibody incubation buffer: 1% (w/v) non-fat milk or BSA in 1x TBST

11. Mild stripping buffer (1L): 15g glycine, 1g SDS, 10mL Tween-20, pH 2.2

2.3. Buffers and solution for immunofluorescence

1. Fixative: 4% methanol-free paraformaldehyde in 1x PBS (*see* **Note 6**)

2. Phosphate-buffered saline (PBS) (1× PBS pH 7.4).

3. PBS-T (wash buffer): 1x PBS, 0.1% Tween-20

4. Permeabilization buffer: 1x PBS, 0.1% Triton X-100, 0.3% BSA (*see* **Note 7**)

5. Hoechst dissolved in distilled water to obtain a 1 mM (1000×) stock solution, which can be stored in a dark vial at 4 °C

6. Mounting medium: 70% glycerol, 2% w/v propyl gallate, 1× PBS. Can be stored for 6 months in a dark vial at 4 °C

2.4. Specific equipment

2.4.1. Protein extraction and western blotting

1. Cryo-tubes

2. Funnels, chilled at –80°C

3. Tea strainers with two different mesh size, chilled at –80°C

4. Pestle motor and pestles

5. Sonicator

2.4.2. Immunofluorescence

1. Fine tweezers and sharpening stone
2. Deep-well glass slide
3. 48-wells plate and Terasaki plate
4. Basket makes by cutting a cap from cell strainer tube
5. Microscope slides and coverslips (22x22mm)

3. Method

3.1. Preparation of the flies

1. Flies from the appropriate genotype are collected within 24 hours from hatching and packed in cohorts of maximum 25 flies.
2. Flies are transferred into new tubes every 2 to 3 days until they reach the desired age (5- to 7-day old for young flies, 30- to 50-day old for old flies) (*see* **Note 8**).

3.2. Differential detergent protein extraction analysis (Fig. 1)

3.2.1. Differential detergent protein extraction

1. Flash freeze the flies in liquid nitrogen (*see* **Notes 9** and **10**).
2. Decapitate the flies by vortexing the tubes twice (*see* **Note 11**).

3. Separate the heads from the bodies and other small body parts such as legs and antenna using two tea strainers with different mesh size, starting with the smaller size to remove the small parts. Using the larger mesh and a funnel, collect the heads in a new tube.

4. Lyse the heads in chilled Triton lysis buffer for protein extraction from the soluble fraction. Homogenize with a pestle until heads can no longer be discerned (about 20 seconds when using a motorized pestle) (*see* **Note 12**).

5. Centrifuge lysate at 17,000 g for 15 min at 4ºC in a pre-chilled centrifuge.

6. Save the supernatant; this fraction contains the soluble proteins (Fig. 1A).

7. Wash the pellet once with 200µL of Triton lysis buffer.

8. Centrifuge at 17,000 g for 2 min at 4˚C in a pre-chilled centrifuge and discard the supernatant

9. Add 100µL of SDS lysis buffer onto the pellet for protein extraction from the insoluble fraction (*see* **Note 13**).

10. Sonicate on ice until the pellet appears to be lysed.

11. Centrifuge lysate at 13,000 g for 10 min.

12. Save the supernatant; this fraction contains the insoluble proteins originally contain within aggregates (Fig. 1A).

13. Quantitate amount of protein using a kit or assay of choice.

14. Prepare the loading samples in 2x or 4x Laemmli buffer as for convenience.

3.2.2. Gel electrophoresis and western blotting

1. Separate 10-20 μg of protein by SDS–PAGE at constant voltage per gel (*see* **Notes 14, 15, 16** and **17**).

2. Transfer proteins onto nitrocellulose or PVDF membranes in a pre-chilled transfer buffer that contains 10% ethanol (*see* **Notes 18, 19** and **20**).

3. Block the membrane in blocking buffer for 1 h at room temperature with gentle rocking to reduce nonspecific antibody binding.

4. Incubate in primary antibody in TBST overnight at 4ºC with gentle rocking (*see* **Notes 21** and **22**).

5. Wash the membrane four times (5 min each) in 1x TBST with gentle rocking at room temperature.

6. Dilute horseradish peroxidase-conjugated secondary antibody in TBST + 1% non-fat milk or BSA. Incubate with secondary antibody for 45 minutes at room temperature with gentle rocking (*see* **Note 23**).

7. Wash the membrane three times (5 to 10 min each) with TBST with gentle rocking.

8. Rinse once with TBS (*see* **Note 24**).

9. Remove the excess of buffer and cover the membrane with chemiluminescence reagent, in accordance with manufacturer's recommendations.

10. Document the western blotting results (X-ray film or digital imaging) (Fig. 1B).

3.2.3. Membrane stripping and reprobing

1. After imaging, place the membrane back into TBS and wash twice in TBS to remove the chemiluminescence reagent.

2. Cover the membrane with mild stripping buffer and incubate at room temperature for 5 minutes with gentle agitation.

3. Discard the stripping buffer and repeat incubation with fresh mild stripping buffer for 10 minutes.

4. Wash twice the membrane in TBS (at least 10 min each) to ensure all the stripping buffer is washed away.

5. Wash twice the membrane in TBST (5-10 min each).

The membrane is now ready for blocking and immunostaining as described in Subheading 3.2.2.

3.2.4. Analysis using ImageJ/Fiji

Several methods exist for protein quantitation by densitometric analysis using ImageJ/Fiji, which give similar results. One of these methods, using the 'Gel analysis' tool embedded in the software, is detailed hereafter.

Before proceeding to densitometric analysis, the X-ray films must be scanned in grayscale mode at a DPI of 300 or greater, and saved in TIFF format.

1. Open the scan file and using the LUT option in the main toolbar, make sure that none of the band to quantify is oversaturated (*see* **Note 25**).

2. If necessary, the background noise can be reduced; go to the menu under Process>Subtract Background, start with a rolling ball radius of 50. This may need to be adjusted for each densitometric analysis.

3. On the toolbar, select the rectangle selection tool and draw a rectangle around the first band. From the Analyze>Gels submenu, choose the 'Select First Lane' command, or type the "1" key on the keyboard, to designate the first lane. Move the rectangle to the next lane and go to Analyze>Gels>Select Next Lane or press the "2" key and continue to do this for each subsequent lane.

4. Once all the lanes are highlighted, select the command 'plot lanes' from the Analyze>Gels submenu. This will open a new window displaying the densitometry histogram for each lane.

5. Use the line tool to draw two vertical lines, one on each side of the peak, to enclose the peak area.

6. Select the magic wand from the toolbar and click on each peak. This will open a new result window gathering the area for each selected peak. This data can be copy and paste into an Excel spreadsheet.

7. Proceed the same way for all the proteins you need to quantify as well as the loading control.

8. In Excel, calculate the ratio between the peak area of a protein of interest and its corresponding loading control. For ease of interpretation, normalize the ratio to a control condition to get a 'fold increased protein quantity' for the test condition ((Fig. 1C).

3.3. Immunofluorescence of aggregates in adult Drosophila brain using Ref(2)P and ubiquitin staining (Fig. 2)

3.3.1. Adult brain dissection

1. Moisten a piece of tissue with water. This will be used to wipe the tweezers and remove debris during the dissection (*see* **Note 26**).

2. Anesthetize the flies on a CO_2 pad. Transfer a fly into a drop of PBS in a deep-well and orient it ventral side up. With one of the tweezer, grab the thorax just below the head. Using the second tweezer, grab the extended proboscis to remove the head. Discard the body and submerge the head (*see* **Notes 27** and **28**) (Fig. 2A (a, b)).

3. With the free forceps, obtain a grip between the proboscis and the eye. Alternating left and right, gently make use of both of your forceps to tear apart the retina, working toward the back of the head to remove the cuticle.

4. After isolating the brain, carefully remove the trachea (*see* **Note 29**).

3.3.2. Immunostaining and mounting

1. Transfer the isolated brains into a basket and immerse it in a well of the 48-wells plate containing the fixative (Fig. 2A (c, e)). Incubate for 30 min at room temperature.

2. Rinse three times with 1x PBS (5 min each) by transferring the basket from one well another.

3. Block in permeabilization buffer for 1h at room temperature

4. Carefully transfer the brains into a well of a Terasaki plate filled with 10-15µL of antibody solution in the permeabilization buffer. Incubate overnight at 4ºC with a piece of tissue soaked in water (*see* **Note 30**) (Fig. 2A (d)).

5. Transfer back the brains into the basket and wash 3 times in PBS-T at room temperature with gentle agitation (10 min each).

6. Dilute the secondary antibody in the permeabilization buffer and incubate for 2 hours at room temperature (*see* **Notes 31** and **32**).

7. Wash 3 times in PBS-T at room temperature with gentle agitation (10 min each).

8. Transfer the basket into a well containing the Hoechst solution (1x dilution in PBS) and incubate for 15 minutes at room temperature with gentle agitation.

9. Rinse once in PBS.

10. On a microscope slide, put a drop of mounting medium (*see* **Note 33**).

11. Carefully transfer the brains onto the slide and orientate them with their antennal lobes facing upward.

12. Gently cover with a coverslip to avoid the formation of bubbles.

13. Once the mounting medium has completely spread beneath the coverslip, seal the edges with nail polish.

14. Store the slides in a dark box at 4ºC.

15. Visualized using a confocal microscope. We preconize using first a low magnification lens (10x) to get global micrographs of the brains before moving to higher magnification lenses (typically 63x) (Fig. 2B, C). As much as possible, view

multiple confocal sections within a tissue. When comparing samples, examine similar regions within the brains.

4. Notes

1. The quantity of agar needs to be adjusted in-house depending on the provider.

2. Ethanol proportion may vary from 5 (large proteins) to 20% (small proteins) depending on the size of the main protein of interest.

3. We do not use methanol for our transfer but prefer ethanol as it is less toxic and can be put down the sink safely.

4. Because the high concentration of SDS, the 6x Laemmli buffer can become viscous due to the precipitation of the SDS. The buffer can be warmed up in a water bath at 60ºC before use.

5. For ease of use, 4x Laemmli buffer with 2-mercaptoethanol can be aliquoted and stored at -20ºC for a few months.

6. The 4% fixative solution can be stored at −20 °C for a few months.

7. The concentration of Triton X-100 can vary from 0.1 to 0.3% and needs to be optimized for each antibody.

8. Flies with a defective autophagy have a much shorter lifespan than their isogenic wild-type counterpart. So, for working with old autophagy-deficient flies, both wild-type and autophagy-deficient flies are frozen at 30 days from hatching.

9. Use SafeLock 1.5mL Eppendorf tubes or cryotubes for freezing in liquid nitrogen safely.

10. If the flies are not processed immediately, store at -80ºC.

11. Vortex for a few seconds and keep the tubes on dry ice between rounds of vortexing to avoid thawing the flies.

12. The number of head determines the amount of lysis buffer to add. We suggest starting with 20-30 heads in 200µL of lysis buffer.

13. Add the SDS right before use as it will precipitate if kept on ice.

14. To get a good separation of Ref(2)P and tubulin, we use 8% acrylamide gels.

15. We usually run the SDS-PAGE at 50-60V until the proteins cross the stacking gel, and then increase to the desired voltage (up to 200V).

16. When probing for ubiquitinated protein, we recommend running the SDS-PAGE at low voltage (max 100V).

17. SDS-PAGE are run at a constant voltage to minimize the heating of the gels chamber.

18. The usual transfer is done at 100V for 1 hour; except for ubiquitinated proteins: 25V for 2 hours.

19. When transferring onto PVDF membrane, it is crucial to incubate them for 5-10 minutes in 100% ethanol before assembling the transfer sandwich.

20. The use of a constant voltage ensures that field strength remains constant, thus providing a most efficient wet transfer.

21. The incubation time for the primary antibody may vary and must be optimized depending on the antibody and protein concentration.

22. Primary antibody buffer can contain 1% non-fat milk or BSA to reduce the nonspecific binding. Note that some antibodies behave better in BSA than milk.

23. We do not recommend to incubate the secondary antibody for more than 1 hour to avoid nonspecific binding.

24. It is important to rinse the membranes once in TBS without Tween-20 as it can interfere with the chemiluminescence reagent.

25. It is crucial that the bands are not oversaturated as it will result in an underestimation of the densitometry.

26. Sharp forceps are essential for live dissections. Using a sharpening block gently pass the forceps back and forth on each side until they are sharp enough.

27. It is imperative to always hold the head firmly with tweezers; otherwise, it will float and is difficult to retrieve.

28. If the head moves out of focus, move it back into the focal plane without adjusting the microscope.

29. It is crucial to remove as much as possible of the trachea as those air-filled sacs can cause brains to float and affect the staining and imaging.

30. Adding a piece of tissue soaked in water will contribute to reducing the evaporation of the antibody solution.

31. The incubation with the secondary antibodies can also be conducted overnight at 4ºC.

32. From that point, it is crucial to perform all the incubation in a dark box to avoid bleaching of the staining.

33. For a coverslip 22x22mm, a 15μL drop of mounting medium is sufficient.

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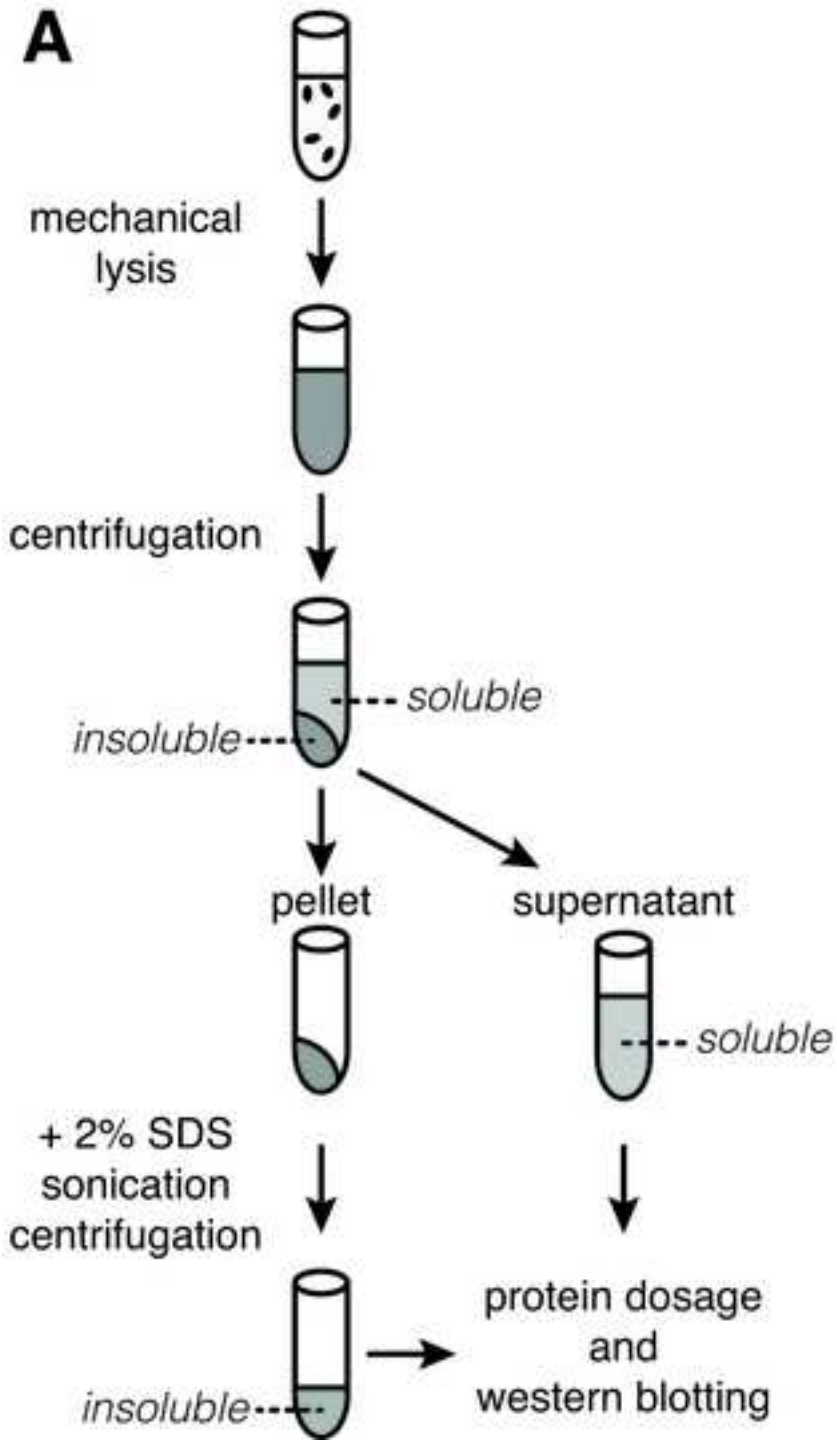
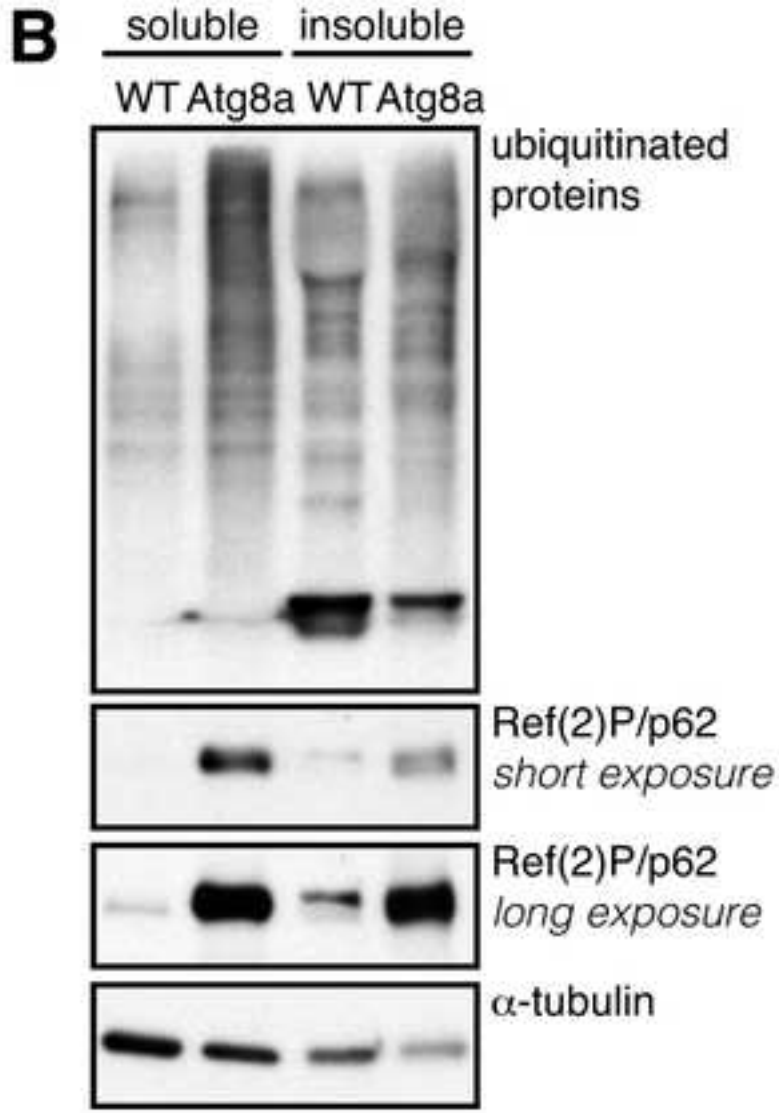
Figure legends

Figure 1. Differential detergent proteins extraction. (A) Flow chart of the procedure described in the method section. (B) Differentially extracted proteins from young wild-type (WT) and autophagy-deficient fly heads (Atg8a) were subjected to immunoblotting using anti-ubiquitinated proteins and Ref(2)P antibodies. Tubulin was used as a loading control. (C) Quantification of Ref(2)P relative protein level by densitometry using Fiji/ImageJ as described in the method section.

Figure 2. Double staining of aggregates in adult tbrains from autophagy-deficient flies. (A) Tools for dissection and staining: fine tweezers (a), Deep-well glass slide (b), small baskets created by cutting in the cap of a cell strainer tube (c), Terasaki plate (d) and 48-wells plate (e). (B-C) Confocal micrographs of *Drosophila* autophagy Atg8a mutant adult brain showing the presence of protein aggregates due to defective autophagy. (B) low magnification of the brain and (C) a zoomed area of the optic lobe (C) after staining for ubiquitinated proteins (red), Ref(2)P (green) and nuclei (blue).

Figure 1





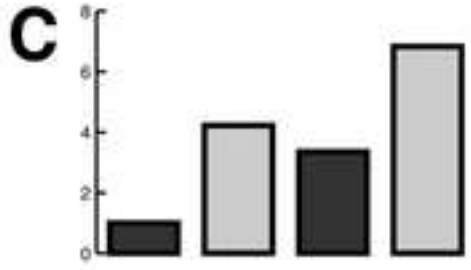


Figure 2

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