Lysosomal flux assay
(as reported by Giacomo Monzio Compagnoni)

PURPOSE
To assess the autophagic flux in cells, by evaluating LC3II and p62 amount before and after bafilomycin treatment

MATERIALS AND REAGENTS
- Western blot running tank
- iBlot transfer system
- LI-COR Clx scanner
- bench centrifuge
- 4-12 % Bis-Tris gel
- iBlot nitrocellulose stacks
- LDS 4X buffer
- Protease/phosphatase inhibitors
- Bafilomycin
- PBS
- Odyssey blocking buffer
- tween20
- LC3 antibody (Cell Signaling 2775)
- p62 antibody (Millipore MABN130)
- Actin antibody (Sigma A2066)
- Secondary antibodies (800 anti Rb, 800 anti Ms, 680 anti Rb)

PROTOCOL

Bafilomycin treatment:
1. Change medium to complete medium + bafilomycin (eg 50nM, 100 nM, 200nM)
2. Treat control wells with complete medium + DMSO (eg 500X, to match bafilomycin dilution) (bafilomycin aliquots are resuspended in DMSO)
3. Keep at 37° until designated collection timepoint(s) (e.g. 12 h)
4. Collect cell pellet

Protein extraction:
5. Keep samples on ice throughout extraction
6. Dilute 4X blue LDS buffer (Cat. no. B0007, Life Tech) in water plus protease inhibitors to get 1X LDS buffer
7. Resuspend each pellet in 1X LDS buffer(100μL, but reduce or increase the volume according to the pellet size)
8. Sonicate twice for 15 seconds at 50% power, keeping sample on ice
9. Boil for 5 minutes at 100°C; return directly to ice
10. Centrifuge for 10 minutes at 850x*g*

11. Retain the supernatant
12. Perform BCA assay on protein samples; dilute the standards and the blank in 1X LDS buffer
13. Store lysates at -80°C

Western blot:

14. Calculate the volume of each sample containing 30 μg of proteins; add 1X LDS to bring volume to 22.5 μL; add 2.5 μL Thermo Fisher reducing reagent per sample (if using a 10-wells gel)
15. Boil samples at 100°C for 5min
16. Load 25 μL per well into 10-well mini Bis-Tris 4-12% gel(s)
17. Dilute protein ladder in 1X LDS (e.g. 4 μl protein ladder + 16 μl 1X LDS buffer)
18. Run in MES buffer (1X), 30 minutes, at 200V
19. Cut off wells and bottom of gel; move gel directly to transfer stack
20. Transfer using iBlot P0 program to nitrocellulose membrane (Cat. no. IB23002, Thermo)
21. Do not touch membrane with gloves—use forceps and razor
22. Cut off edges (perimeter of gel)
23. Cut across sample section between 2nd and 3rd (100kDa) ladder bands from top (or somewhere else above 75 KDa)
24. Cut across samples at 25kDa (lower red) band (middle of the band)
25. Rehydrate membrane in PBS for 5min on orbital shaker
26. Block 1 hour at room temp in Licor Odyssey Buffer PBS (Cat. no. 927-40000, Licor)
27. Prepare primary antibody solutions in Odyssey plus 0.1% Tween:
    - HMW: rabbit-anti-actin 1:1200 (-20°C) + mouse-anti-p62 1:1000 (labeled "SQSTM1", at 4°C)
    - LMW: rabbit-anti-LC3 1:1000
28. Recover blocking solution to be used for secondary antibodies—keep on ice or at 4°C
29. Incubate in primary antibody solutions for 2 hours at room temp on orbital shaker or at 4°C overnight
30. Wash 4x 5 minutes with PBS-T (0.05% Tween)
31. Incubate secondary antibodies at 1:10,000 dilution in Licor Odyssey plus 0.1% Tween, 1 hour at room temp in black box or aluminium foil:
    - HMW: 680-anti-rabbit, 800-anti-mouse
    - LMW: 800-anti-rabbit
32. Wash 4x 5 minutes with PBS-T (0.05%), in black box or aluminium foil
33. Wash 1x 5 minutes with PBS
34. Change to fresh PBS
35. Immediately before acquisition, dry membranes on kimwipe
36. Reassemble membrane and scan with Licor Clx scanner
    - Flip membranes so that lower left corner is in upper left

37. Analysis: LC3 II normalized over Actin (or LC3 I), then divided by baseline (DMSO condition)
    - LC3 II is larger than LC3 I, but charge makes it run faster: ratio is lower band divided by upper band (or Actin)
    - Normalize p62 over Actin to corroborate LC3

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