Chemicals and supply:

- 100X Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts (Sigma-Aldrich, P8340; "PiM" aliquots at -20°C)
- 100X Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich, P2850; "PP" aliquots at 4°C, "Antibodies, large vials" box); 100X Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich, P5726; at 4°C, "Antibodies, large vials" box)
- Thickwall Polycarbonate Tubes (Beckman Coulter, 343775)
- 10X Glycoprotein Denaturing Buffer (NEB, re-order B1704S)
- 10X G5 Reaction Buffer (NEB, re-order B1702S)
- Endo H (NEB, P0702L)
- Novex® 10% Tris-Glycine Mini Gels, 1.5 mm, 10 wells (Life Technologies, EC6078BOX)
- Novex® 10% Tris-Glycine Midi Gels, 1 mm, 12+2 wells (Life Technologies, WT0101BOX)
- Novex® 10% Tris-Glycine Midi Gels, 1 mm, 20 wells (Life Technologies, WT0102BOX)
- 10X TRIS-Glycine-SDS Running Buffer (Boston BioProducts, BP-150)
- Precision Plus Protein™ Dual Color Standards (Bio-Rad, 161-0374; -20°C)
- 10X TRIS-Glycine Transfer Buffer (Boston BioProducts)
- Rb anti-Nicastrin (Cell Signaling Technology, 3632S; -20°C)
- Rb anti-GCase (Sigma-Aldrich, G4171; 4°C, "Antibodies, large vials" box)
- Ms anti-GAPDH (EMD Millipore, MAB374; 4°C)
- Gt anti-rb IgG, DyLight™ 800 conjugated (Rockland, 611-145-003; Lauren's fridge)
- Gt anti-ms IgG, DyLight™ 680 conjugated (Rockland, 610-144-003; Lauren's fridge)

Buffers:

Table 1| Mazzulli lysis buffer. Preparation of the lysis buffer: first add glycerol, NaCl and HEPES into a measuring cylinder, top up with diH_2O to 195 ml in total. Add EGTA, $MgCl_2$ and finally Triton X-100. Be very careful when adding the viscous Triton X-100 to make sure that exactly 2 ml go into the solution. Stir for ~ 20 min, adjust the pH to 7.4 and top up with diH_2O to 200 ml in total using a measuring cylinder. Filter with a SteriCup to ensure long-term storage – in this way the lysis buffer can be kept at 4°C for up to 6 months or aliquoted at -20°C (40 ml aliquots).

Ingredient	200 ml	Stock concentration	Final concentration
HEPES	4 ml	1 M	20 mM
NaCl	6 ml	5 M	150 mM
Glycerol	40 ml	50% (v/v)	10% (v/v)
EGTA	200 µl	1 M	1 mM
$MgCl_2$	300 µl	1 M	1.5 mM
Triton X-100	2 ml		1% (v/v)
diH_2O	147.5 ml		

Recipe according to Mazzulli et al. (2006), PMID: 17005870.

Table 2| 5X Sample loading buffer (SB). Aliquot and store at -20°C.

Ingredient	10 ml	Stock concentration	Final concentration
Tris-Cl, pH 6.8	1 ml	1 M	100 mM
Glycerol	4 ml	50% (v/v)	20% (v/v)
β-ME	630 µl		900 mM
Bromophenol blue	1.5 mg		0.015% (w/v)
SDS*	1 g		10% (w/v)
diH_2O	~ 4 ml		

*Work under the fume hood when adding SDS! Powder is toxic! Recipe according to Mazzulli et al. (2006), PMID: 17005870.

Work flow:

1. **PREPARE**:
   - Make sure the lysis buffer (**Table 1**) is chilled.
   - Cool down ultracentrifuge to 4°C. Set parameters: TLA-100 rotor, 100,000 x g, 30 min, 4°C; apply vacuum.
   - Prepare lysis buffer MM: Add 100X protease inhibitor cocktail ("PiM") as well as 100X phosphatase inhibitor cocktail I ("PP") and II to the lysis buffer in a 1:100 ratio. Keep the lysis buffer MM on ice from this point onwards.

     The protease inhibitor cocktail can be briefly thawed using the 37°C heat block. Keep at RT before re-freezing.
   - Get a large box and fill to approximately 2/3 with wet ice for the ice/water slurry (see below).
2. Get samples and keep on dry ice.
3. When ready, thaw samples on wet ice and add an appropriate volume of the lysis buffer MM as soon as the cell pellet is almost thawed.

   Can also be thawed briefly at RT but make sure that the cell pellet is not completely thawed when lysis buffer MM is added.

   Lysis buffer MM volume: approximately 50-150 µl; around 3x the volume of the cell pellet; better too little than too much.
4. Resuspend the cell pellet to a homogenous (!) solution by trituration and keep on wet ice; make sure the top of the Eppis is carefully labeled since side labels will likely disappear during a later incubation in the dry ice/EtOH slurry!
5. Prepare the ice/water slurry: add cold water to approximately half of the height of the ice layer.
6. Incubate samples for 20 min in the ice/water slurry.
7. In the meantime **PREPARE** the following:
   - Label ultracentrifuge tubes on the side.

     Use P200 tip holder as a rack for the ultracentrifuge tubes.
   - Prepare dry ice/EtOH slurry approximately 10 min before the 20 min incubation on the ice/water slurry is completed:
     - Fill small box to 2/3 with EtOH (190 proof, 95%).
     - Add dry ice until saturation (dry ice doesn't resolve quickly anymore; temperature of -80°C has stabilized).
8. Put Eppis into a floating rack and immediately place them in the dry ice/EtOH slurry in order to snap freeze the samples; incubate for 1 min.

   Make sure that the lid does not touch the dry ice/EtOH slurry to prevent EtOH leaking into the Eppis; only cell lysate needs to be exposed to the dry ice/EtOH slurry.

   Incubation time of 1 min is minimum; can incubate longer.
9. Directly transfer Eppis onto a heat block and incubate at 37°C for exactly (!) 1 min (no longer!).
10. Repeat the described freeze/thaw cycle once → two freeze/thaw cycles (-80°C/37°C) in total.
11. Keep samples on wet ice afterwards.
12. Get TLA-100 rotor (Beckman Coulter) and keep on wet ice, including the lid.

    TLA-100 rotor usually kept in the 4°C cold room.

13. Transfer the cell lysate from the Eppis into the labeled ultracentrifuge tubes (thickwall polycarbonate tubes) and place them in the TLA-100 rotor on ice; keep the empty Eppis for a later step in case the labeling is sufficient and hasn't been removed by the EtOH.
14. Place TLA-100 rotor into the ultracentrifuge; make sure it's correctly placed (!); re-apply vacuum, make sure that the parameters are set correctly (100,000 x g for 30 min at 4°C) and start.

    The ultracentrifuge might take a while to start because it will have to re-establish the vacuum; make sure that the ultracentrifuge gets back to 4°C.
15. During the ultracentrifugation **PREPARE** the following:
    - Prepare a plate for the BCA test; each condition in triplicate.
    - Label Eppis and chill them on wet ice.
    - Get dry ice.
16. Get TLA-100 rotor and keep on wet ice.
17. Remove ultracentrifuge tubes and transfer the supernatant to a new, correctly labeled and chilled Eppi; keep Eppis on wet ice.
18. A small cell pellet should be visible; transfer to the appropriate old, correctly labeled Eppi and keep on dry ice.

    This cell pellet can be re-used, e.g., to check for αSyn levels by Western blotting; in this case store cell pellet at -80°C and later resuspend in approximately 10 µl of the appropriate lysis buffer for Western blotting.
19. For the BCA test either add 1 µl of the cell lysate to 24 µl water or dilute 2 µl of the cell lysate in a 1:1 ratio with 2 µl water before adding 1 µl of the 1:2 dilution to 24 µl water; in the latter case don't forget to change the dilution factor on the Endo H spreadsheet to 50!

    I got more accurate results with a dilution of 1:25.
20. **PAUSE POINT**

    If the Endo H is proceeded with on another day, transfer the Eppis with the cell lysate directly onto dry ice and immediately store them at -80°C (to reuse, thaw cell lysates on wet ice); if the Endo H is to be proceeded with on the same day, place Eppis on wet ice and store intermediately in the 4°C cold room.
21. During the incubation for the BCA test at 37°C for 26 min roughly label 2 Eppis for each condition (→ +/- Endo H).
22. Thaw 10X glycoprotein denaturing buffer and 10X G5 buffer at RT (don't vortex denaturing buffer as this will create a lot of small bubbles!).

    Fine to briefly keep them in the heat block if they are not thawed when needed in the steps below; make sure that these buffers are very well mixed before adding them to the reaction volume!
23. After BCA test use Chee-Yeun's Endo H spreadsheet to calculate the protein concentration.
24. **PREPARE**: thaw Endo H (stored at -20°C) on wet ice.
25. From this point onwards all in duplicate (→ +/- Endo H):
    - Refer to the spreadsheet for mixing an appropriate volume of the cell lysate (30-40 µg protein) with diH_2O for a total volume of 15.3 µl; from this point onwards samples can be kept at RT.
    - Add 1.7 µl of the glycoprotein denaturing buffer for a total reaction volume of 17 µl.
    - Briefly vortex and spin down.
    - Boil at 100°C for 10 min. In the meantime transfer the cell lysate stock to -80°C.

Make sure that the lids of the Eppis are tightly closed!

- In the meantime **PREPARE** the +/- Endo MMs:
  - MM-1 (+ Endo H): (1 µl **Endo H** + 2 µl 10X G5 buffer)/rxn; can be kept at RT.
  - MM-2 (- Endo H): (1 µl **diH_2O** + 2 µl 10X G5 buffer)/rxn.
- Spin down samples briefly; add 3 µl of MM-1 or -2, briefly vortex and spin down.
- Incubate at 37°C for 2 h (minimum incubation time).
- Spin down briefly.
- **PAUSE POINT**

  Samples can be stored at -80°C or -20°C if the gel is to be run on another day.

26. Thaw sample buffer ("5X SB", kept at -20°C; **Table 2**) and protein ladder at RT.
27. In case +/- Endo H samples have been frozen down, thaw at RT and briefly spin down.
28. Add 5 µl 5X SB to each 20 µl rxn; briefly vortex and spin down; denature proteins at 100°C for 10 min; briefly spin down.
29. **PREPARE** Novex® 10% Tris-glycine gel with adapter. For 1 gel prepare 500 ml running buffer: 50 ml 10X Tris-glycine-SDS running buffer (Boston BioProducts) + 450 ml diH_2O.

    No need to recycle running buffer.
30. Load 8 µl of the protein ladder and the entire reaction volume of the +/- Endo H samples – first all -Endo H samples followed by all +Endo H samples
31. Run the gel:
    - Run at 100 V first for 20 min – until the running front is completely condensed by means of the stacking gel.
    - Then increase voltage to 120 V and set timer to 2h.
      - Run the gel until the 37 kDa protein ladder band is approximately 1-1.5 cm above the lower end of the gel cassette; check gel regularly after 60 min; don't run too far!!
32. Wet electrophoretic transfer onto a PVDF membrane (Bio-Rad) with 10X Tris-glycine transfer buffer (Boston BioProducts): 700 ml diH_2O + 100 ml 10X Tris-glycine transfer buffer + 200 ml **MeOH** [20% (v/v)].
    - Prepare 2 L transfer buffer and keep in 4°C cold room; can be recycled 3-5x.
    - Transfer for 120 min at 60 V and 4°C or for 150 min at 65 V in the case of two simultaneous transfers.
33. Continue with immunoblotting using the Odyssey infrared imaging system (LI-COR Biosciences).
34. Antibodies:

Primary antibody		Secondary antibody	
Rb anti-GCase	1:500	Gt anti-rb IgG, DyL 800	1:5,000
Rb anti-nicastrin	1:500		
Ms anti-GAPDH	1:3,000	Gt anti-ms IgG, DyL 680	1:10,000