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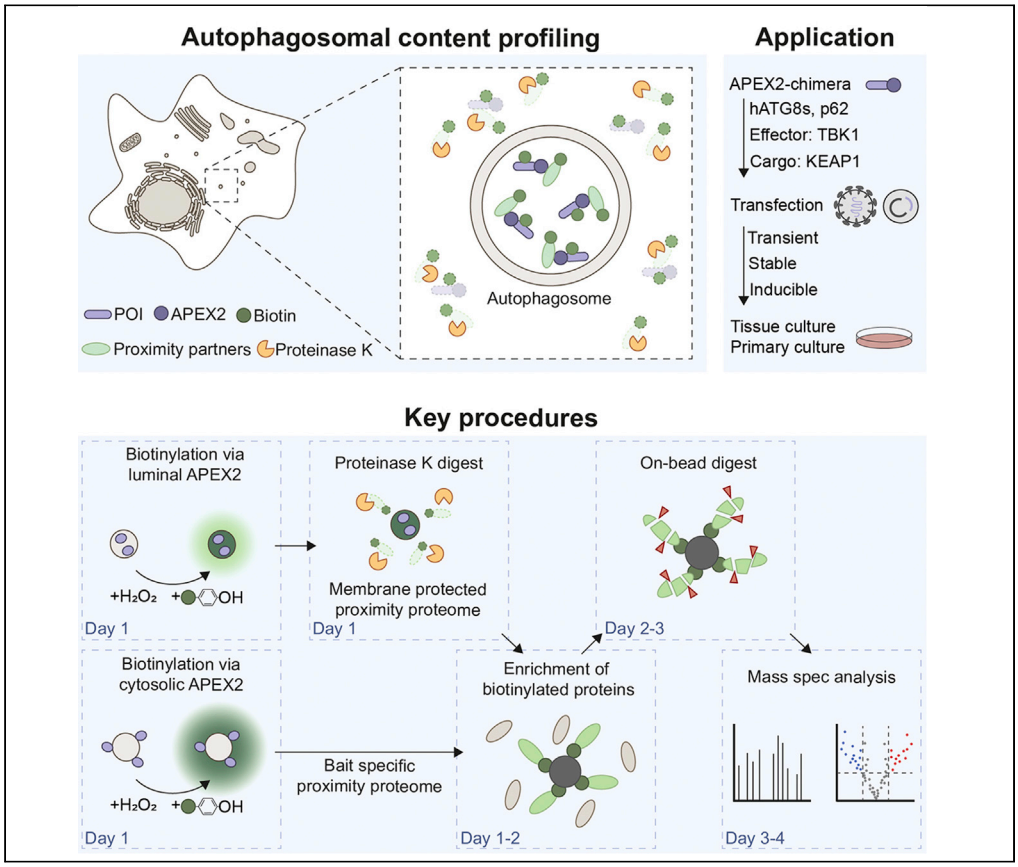
Autophagosome content profiling using proximity biotinylation proteomics coupled to protease digestion in mammalian cells



The ascorbate peroxidase APEX2 is commonly used to study the neighborhood of a protein of interest by proximity-dependent biotinylation. Here, we describe a protocol for sample processing compatible with immunoblotting and mass spectrometry, suitable to specifically map the content of autophagosomes and potentially other short-lived endomembrane transport vesicles without the need of subcellular fractionation. By combining live-cell biotinylation with proteinase K digestion of cell homogenates, proteins enriched in membrane-protected compartments can be readily enriched and identified.

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Highlights

Directing APEX2 into autophagosomes

Coupling of proximity biotinylation and limited proteolysis

Enrichment of membrane-protected autophagy cargo

Compatible with small-scale validation and explorative proteomics approaches

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June 18, 2021
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Autophagosome content profiling using proximity biotinylation proteomics coupled to protease digestion in mammalian cells

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SUMMARY

The ascorbate peroxidase APEX2 is commonly used to study the neighborhood of a protein of interest by proximity-dependent biotinylation. Here, we describe a protocol for sample processing compatible with immunoblotting and mass spectrometry, suitable to specifically map the content of autophagosomes and potentially other short-lived endomembrane transport vesicles without the need of subcellular fractionation. By combining live-cell biotinylation with proteinase K digestion of cell homogenates, proteins enriched in membrane-protected compartments can be readily enriched and identified.

For complete details on the use and execution of this protocol, please refer to Zellner et al. (2021).

BEFORE YOU BEGIN

In this protocol, we describe the use of enzyme-based proximity biotinylation combined with proteinase K digestion and streptavidin enrichment to profile the content of autophagosomes using mass spectrometry (mass spec). For this purpose, the ascorbate peroxidase APEX2 is coupled to a bait protein that is known to be engulfed by autophagosomes (Figure 1). This method builds on previous approaches to map the autophagy degradome in a manner dependent on LC3 and GABARAP proteins (Le Guerroue et al., 2017). However, to increase the robustness and throughput of our method we implemented several improvements from other established protocols such as on-bead digestion (Lobingier et al., 2017). While we selected APEX2 due to its minute-scale labeling and versatile use for electron microscopy, other proximity biotinylation enzymes such as BioID, TurboID or MiniTurbo may be equally well suited for this organellar proteome profiling strategy. Moreover, we demonstrate that the use of label-free quantification works well with our protocol. Alternatively, SILAC-labeling or other labeling methods might also be compatible with this protocol and could be chosen before starting with the experiment. By omitting the proteinase K digestion step, this protocol also allows to map the protein neighborhood of given baits. The protocol was tested in HEK293T, HeLa and MEF cells and should in principle be applicable to a wide range of eukaryotic cells. The topology and abundance of the bait will be of importance and might need adjustments before starting with the experiments for which we give advice in this protocol. We here describe a fast method of three days starting from sample collection to obtain mass spec samples that are very likely to give intriguing new insights into the lumen of autophagosomes. Before starting, all reagents should be prepared and aliquoted as described below. When working with mass spec samples, working in a clean environment and with MS-compatible reagents is of importance.

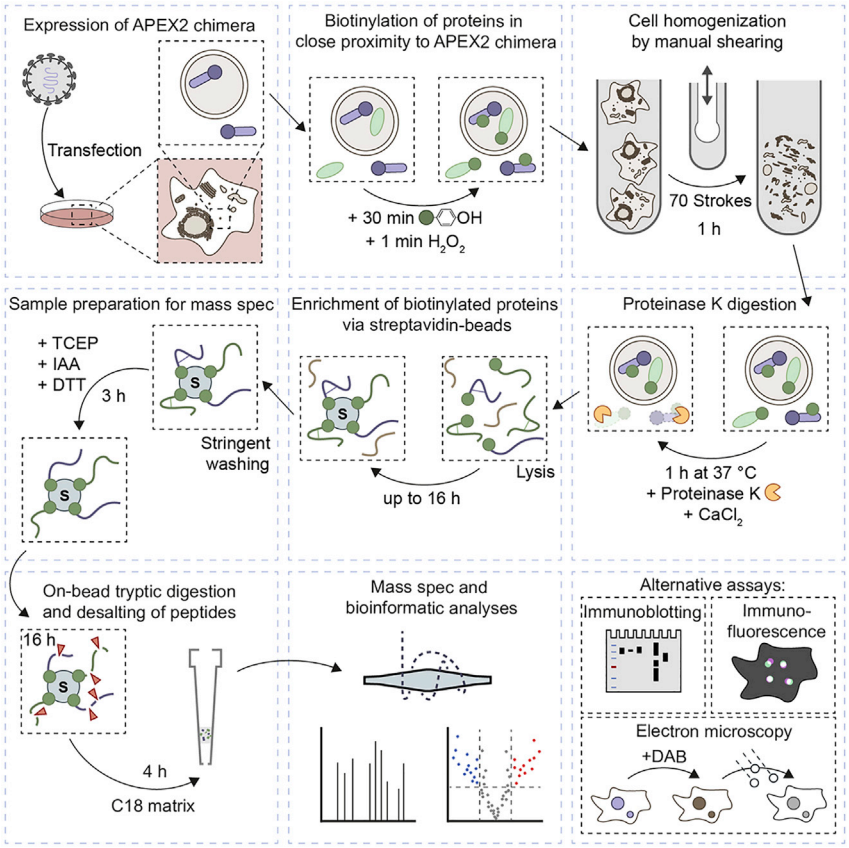

Figure 1. Overview of the stepwise protocol procedure

Cloning and expression of the APEX2 bait chimera

Cloning of ORFs into the APEX2 expression vectors described in this protocol is easily achieved by using the Gateway cloning system (Thermo Fisher Scientific) but conventional cloning methods might work as well. For Gateway cloning follow the manufacturer's guidelines (http://tools.thermofisher.com/content/sfs/manuals/gatewayman.pdf). It is advisable to consider how tagging of the ORF might affect its cellular localization or function and whether tagging of the N- or C-terminus makes more sense. Please note that pNEWS myc-APEX2 is used for generating stable but inducible Flp-In T-REx cells (e.g., HeLa or HEK293T). For detailed information on the generation of Flp-In T-Rex cell lines expressing APEX2 fused to a protein of interest, please refer to the manufacturer's manual (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/flpintrexcells_man.pdf). Conversely, pHAGE myc-APEX2 drives stable and constitutive expression in a wide range of cell types following lentiviral transduction. Alternatively, this latter plasmid is also suitable for transient overexpression approaches. Flp-In T-Rex cells are inducible with doxycycline (4 μg/mL). The appropriate time of induction or transient overexpression should be carefully determined for each APEX2-ORF chimera using standard immunoblotting and immunofluorescence experiments (Figures 2A–2C). 24–48 h before harvesting is probably a good starting point but this certainly differs depending on the respective ORF and the biological question. Additionally, it is advisable to examine the biotinylation activity of each APEX2-ORF construct as described in the first section. The resulting biotinylation pattern can then be examined in lysed or fixed cells using immunoblotting or -staining for biotin, respectively (Figure 2D). In case of aberrant APEX2 bait chimera expression or localization, or in case of absent or low biotinylation induction, please refer to troubleshooting remarks, problems 1 and 2.

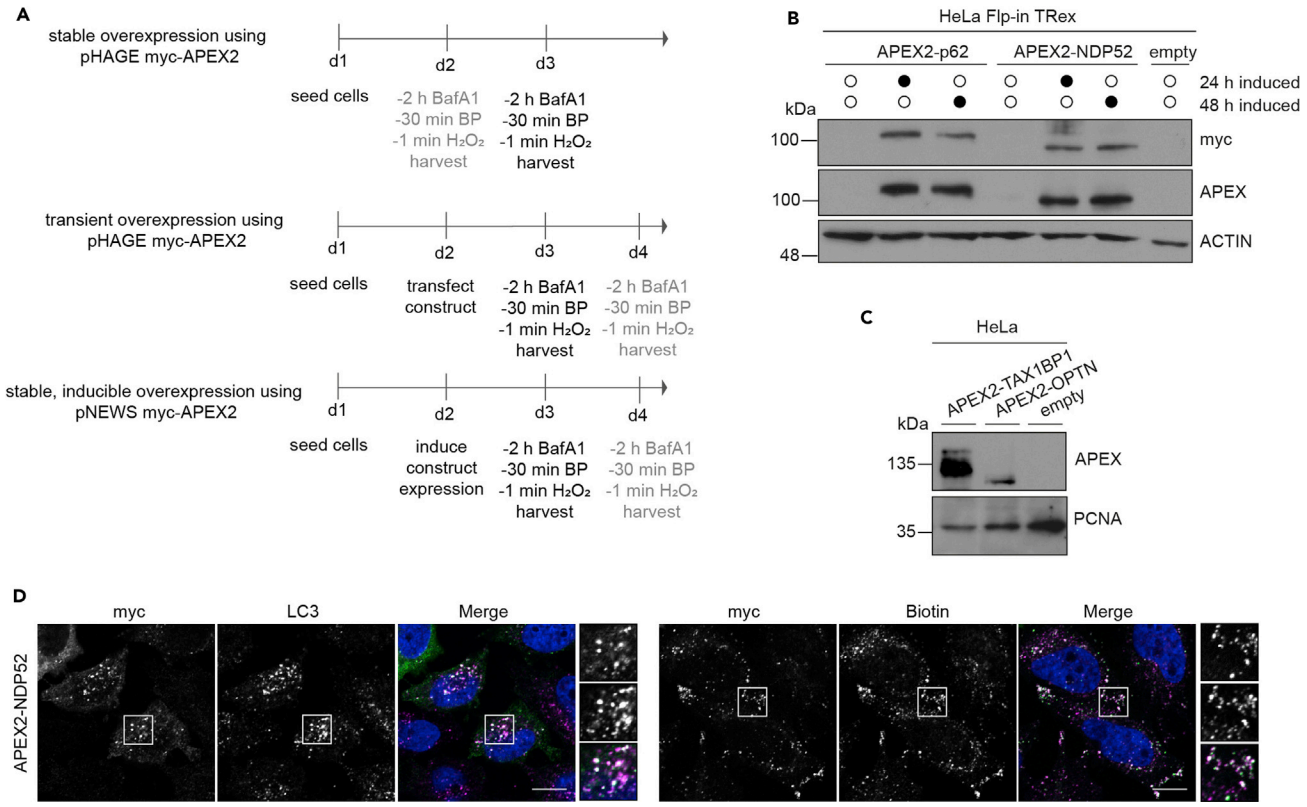

Figure 2. APEX2 construct overexpression

(A) Time course schemes of proximity biotinylation experiments with transient or stable APEX2 bait expression.

(B) APEX2-p62 or APEX2-NDP52 HeLa Flp-in TRex cells were left untreated or stimulated 24 h or 48 h with doxycycline to induce bait expression followed by lysis and immunoblotting.

(C) Immunoblotting of cell lysates from HeLa cells expressing APEX2-TAX1BP1 or APEX2-OPTN. Control cells not expressing APEX2 are designated as "empty".

(D) HeLa Flp-in TRex cells overexpressing APEX2-NDP52 were treated with BafA1, biotin-phenol and H_2O_2 as indicated in (A) before fixation and immunofluorescence labeling. Insets show magnifications of boxed areas. Scale bars, 10 μm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APEX (IgG2A) (1:200)	Custom made	N/A
ATG9A (1:500)	Abcam	ab108338, RRID: AB_10863880
Biotin (1:500)	Pierce	31852, RRID: AB_228243
Biotin-FITC (1:600)	Abcam	ab106219, RRID: AB_10901938
c-myc (1:500)	Bethyl	A190-104A, RRID: AB_66864
LC3 (1:500/1:300)	Cell Signaling Technology/MBL	2775, RRID: AB_915950 /PM036, RRID: AB_2274121
myc 9E10 (1:250)	Custom made	N/A
p62 (1:500)	BD/MBL	610832, RRID: AB_39815 / PM045, RRID: AB_1279301
PCNA (1:500)	Santa Cruz	sc-7907, RRID: AB_2160375
ß-Actin (1:5,000)	Sigma	A5316, RRID: AB_476743
Anti-goat-HRP (1:10,000)	Promega	V8051, RRID: AB_430838
Anti-mouse-HRP (1:10,000)	Promega	W402B

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-rabbit-HRP (1:10,000)	Promega	W401B
Anti-rat-HRP (1:10,000)	Sigma	A-9037, RRID: AB_258429
Donkey anti-rb-555 (1:1,000)	Life Technologies	A31572, RRID: AB_162543
Goat anti-mouse-488 (1:1,000)	Life Technologies	A11001, RRID: AB_2534069
Chemicals, peptides, and recombinant proteins		
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX)	Sigma-Aldrich	238813
Acetonitrile (ACN)	Roth	AE70.1
Ammonium bicarbonate (ABC)	Sigma-Aldrich	09830
Bafilomycin A1	Biomol	Cay11038-1
Biotin-phenol	Iris Biotech	41994-02-9/LS-3500.5000
Blasticidin	InvivoGen (CAYLA)	ANT-BL-1
Bortezomib	LC Labs	B-1408
Dithiothreitol (DTT)	Sigma	43815-5G
DMEM	Gibco	61965-026
DMSO	AppliChem	A3672, 0100
Doxycyline	Sigma	D981-10g
Formic acid (FA)	Merck	1.00264.1000
H_2O_2	Sigma	H1009
Hygromycin B	Invitrogen	10687-010
Iodoacetamide (IAA)	Sigma-Aldrich	I1149-5g
Isopropanol (ISO)	Merck	109634
LC-MS grade Water	Roth	AE72.1
Lipofectamine 2000	Invitrogen	11668-019
Methanol (MeOH)	Roth	AE71.1
NP-40	Sigma-Aldrich	I3021-500ML
PEI	Sigma	P3143
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	P7626-1G
Phosphate buffered saline (PBS)	Thermo Fisher Scientific	14190169
PhosSTOP	Roche	4906837001
Protease Inhibitor Cocktail, EDTA-free	Roche	4693132001
Proteinase K	Roche	3115801001
Puromycin dihydrochloride	Sigma	P8833-100mg
RapiGest SF	Waters	186001861
*Rapi*Gest™ SF	Waters	186001861
Sodium azide	Merck	6688-1000
Sodium L-ascorbate	Sigma	A7631
Streptavidin-agarose	Sigma	S1638-5ML
Trifluoroacetic acid (TFA)	Honeywell Fluka	302031-100ML
Tris-(2-carboxyethyl)-phosphin Hydrochlorid (TCEP)	Roth	HN95.2
Triton X-100	Merck	1.08603.1000
Trypsin, sequencing grade	Promega	V5113
C18 reversed-phase resin	Dr. Maisch	ReproSil-Pur 120 C18-AQ
Deposited data		
MS data	ProteomeXchange via PRIDE partner repository	PXD024335
Immunofluorescence, electron microscopy, and western blot data	Mendeley Data	https://data.mendeley.com/datasets/33dx4p8d4n/1
Experimental models: cell lines		
Human: HeLa	ATCC	Cat#CCL-2
Human: HeLa Flp-In T-REx	(McEwan et al., 2015)	https://doi.org/10.1016/j.molcel.2014.11.006
Recombinant DNA		
pHAGE myc-APEX2 (N-terminal)	(Zellner et al., 2021)	N/A

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
pHAGE myc-APEX2 (C-terminal)	(Zellner et al., 2021)	N/A
NEWS myc-APEX2 (N-terminal)	(Le Guerroue et al., 2017)	N/A
Software and algorithms		
MaxQuant	(Cox and Mann, 2008)	version 1.6.0.1
Perseus	(Tyanova et al., 2016)	version 1.6.5.0
DAVID	LHRI	version 6.8
Other		
Easy-nLC1200	Thermo Scientific	N/A
Q Exactive^{HF} mass spectrometer	Thermo Scientific	N/A
Dounce homogenizer, 1 mL	VWR	432-1270

MATERIALS AND EQUIPMENT

LC-MS setup

Analysis of samples was carried out using an Easy-nLC1200 liquid chromatograph (Thermo Fisher Scientific) coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Samples were loaded on a 75 μm × 15 cm custom-made fused silica capillary packed with C18 reversed phase resin (ReproSil-Pur 120 C18-AQ, 1.9 μm, Dr. Maisch HPLC). Other capillaries, e.g., from New Objective (FS360-75-8-N20-C15) have proven to work as well. Peptide mixtures were separated at a 400 nL/min flow rate using a 35 min ACN (Acetonitrile; in 0.5% acetic acid) gradient. Dynamic exclusion was enabled for 20 s and charge states above 8, species without a detectable charge or singly charged species were excluded.

Preparation of stock solutions

Homogenization buffer I [pH 7.5]		
Reagent	Final concentration	Amount
KCl (1 M)	10 mM	5 mL
$MgCl_2$ (1 M)	1.5 mM	750 μL
HEPES-KOH (1 M, pH 7.5)	10 mM	5 mL
DTT (100 mM)	1 mM	5 mL
ddH_2O	N/A	484.25 mL
Total	**N/A**	**500 mL**

Note: Prepare and store at 4°C. It is advisable to aliquot the stock to avoid contaminations. The buffer is stable for up to one year.

Homogenization buffer II [pH 7.5]		
Reagent	Final concentration	Amount
KCl (1 M)	375 mM	37.5 mL
$MgCl_2$ (1 M)	22.5 mM	2.25 mL
HEPES-KOH (1 M, pH 7.5)	220 mM	22 mL
DTT (100 mM)	0.5 mM	500 μL
ddH_2O	N/A	37.75 mL
Total	**N/A**	**100 mL**

Note: Prepare and store at 4°C. It is advisable to aliquot the stock to avoid contaminations.

The buffer is stable for up to one year.

Quenching solution		
Reagent	Final concentration	Amount
Sodium azide (1 M)	1 mM	50 µL
Sodium ascorbate (1 M)	10 mM	500 µL
Trolox (700 mM)	5 mM	375 µL
PBS	N/A	49.08 mL
Total	**N/A**	**50 mL**

Note: Prepare fresh and keep at 4°C.

⚠ CRITICAL: Sodium azide is toxic already at small dosage. Avoid skin contact and swallowing. Always wear gloves when handling this substance and be careful not to spill it.

qRIPA buffer		
Reagent	Final concentration	Amount
Tris (1M, pH 7.4)	50 mM	2.5 mL
NaCl (1 M)	150 mM	7.5 mL
SDS (10%)	0.1%	500 µL
Sodium deoxycholate (5%)	0.5%	5 mL
Triton X-100 (10%)	1%	5 mL
Protease-inhibitor (25×)	1×	2 mL
PhosSTOP (10×)	1×	5 mL
Sodium ascorbate (1 M)	10 mM	500 µL
Sodium azide (1 M)	1 mM	50 µL
Trolox (700 mM)	1 mM	71.4 µL
ddH_2O	N/A	21.88 mL
Total	**N/A**	**50 mL**

Note: Prepare fresh and keep at 4°C.

⚠ CRITICAL: Sodium azide is toxic already at small dosage. Avoid skin contact and swallowing. Always wear gloves when handling this substance and be careful not to spill it.

Alternatives: Alternative RIPA recipes may also be used (e.g., Triton X-100 can be substituted by other detergents like NP-40) and supplemented with the quenching components.

Stage tipping buffer A		
Reagent	Final concentration	Amount
Formic Acid	0.1%	100 µL
MS-grade H_2O	N/A	99.9 mL
Total	**N/A**	**100 mL**

Note: Buffer can be stored at room temperature (20°C–24°C) for 10 weeks.

Stage tipping buffer B		
Reagent	Final concentration	Amount
Formic Acid	0.1%	100 µL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Acetonitrile	80%	80 mL
MS-grade H_2O	N/A	20 mL
Total	**N/A**	**100 mL**

Note: Buffer can be stored at room temperature (20°C–24°C) for 10 weeks.

Stage tipping buffer C		
Reagent	Final concentration	Amount
TFA	1%	1 mL
Acetonitrile	5%	5 mL
MS-grade H_2O	N/A	94 mL
Total	**N/A**	**100 mL**

Note: Buffer can be stored at room temperature (20°C–24°C) for 10 weeks.

We recommend to prepare and aliquot stocks of the following reagents or solutions at the suggested concentrations and store them as indicated. All reagents can be stored at least for two years.

Reagent	Stock concentration	Aliquot size	Storage
Biotin-Phenol	500 mM	100 µL	−80°C
Trolox in DMSO	700 mM	500 µL, protect from light	−20°C
Proteinase K in ddH_2O	20 mg/mL	20 µL	−20°C
$CaCl_2$ in ddH_2O	100 mM	1–10 mL, no aliquots needed	(20°C–24°C)
Triton X-100 in ddH_2O	10 %	50 mL, no aliquots needed	(20°C–24°C)
PMSF in Methanol	200 mM	2 mL	−20°C
TCEP in 50 mM ABC	1 mM	10 µL	−20°C
IAA in 50 mM ABC	550 mM	50 µL	−20°C
DTT in 50 mM ABC	1 M	50 µL	−20°C
Ammonium bicarbonate (ABC) in MS-grade H_2O	100 mM	100 mL, no aliquots needed	(20°C–24°C)

STEP-BY-STEP METHOD DETAILS

Biotinylation of live cells

Timing: 45–90 min

This section covers APEX2-mediated proximity biotinylation, radical quenching and cell harvest exemplified for 1× 15 cm dish as start material per replicate. Dependent on the APEX2 chimera and the biotinylation levels, 1× 10 cm dish might be sufficient. Buffer volumes should be adjusted accordingly.

1. Cool the centrifuge (4°C), get a bucket of ice, prepare the quenching solution and thaw a biotin-phenol aliquot on ice
2. Grow APEX2-expressing cells in DMEM with 10% FBS until they reach confluency of ~90%
   a. For enrichment of autophagosomes, treatment with Bafilomycin A1 (BafA1) at a final concentration of 200 nM for 2 h, which is a vATPase inhibitor that blocks the fusion of autophagosomes and lysosomes, is advisable

b. Depending on the biological question, cells can be treated as required

3. Adapt the medium volume to 15 mL
4. Pre-dilute biotin-phenol in 100 µL DMSO to avoid its precipitation in DMEM
5. Add 500 µM final concentration (500 mM stock) biotin-phenol to the medium, gently stir the dish and place the cells back in the incubator for 30 min
6. Transfer the cells to a workbench for further steps
7. Add 1 mM hydrogen peroxide (H_2O_2) at room temperature (20°C–24°C) for exactly one minute
8. Immediately pour off the medium in a waste flask and overlay cells with quenching solution, discard the medium and wash 2× more in quenching solution
9. Wash the cells 3× with PBS, discard the supernatant and add 2 mL of PBS
10. Collect the cells using a cell scraper
11. Centrifuge the cells at 1,000 × *g* for 3 min and discard the supernatant
12. Wash once more with 1 mL PBS, repeat centrifugation and discard the supernatant

Note: APEX2 labeling is possible over a broad range of passage numbers but this strongly depends on the cell type that is being used.

Note: It is advisable to intentionally vary the time of biotin-phenol incubation (step 5) and H_2O_2 pulsing (step 7) to probe the biotinylation capacity of a given APEX2 chimera. Guidelines for this can be found in (Hung et al., 2016). Once optimal labeling conditions are determined make sure to stick to them for every dish when labeling multiple dishes at once to minimize variations between samples. This is in particular important for the stimulation with H_2O_2.

Note: When working with loosely adherent cells (e.g., HEK293T), these might easily get flushed away when rinsed too many times (step 8). Here, it can make sense to rinse once with the quenching solution and then collect cells in this solution and use centrifugation to get rid of the quencher. Then wash the pellets in PBS.

Note: Use of trypsin for harvesting cells has a negative impact on the outcome of probing proteinase K digestion by immunoblotting, so we cannot recommend this (step 10). An example is provided in Figure 6A in the troubleshooting section.

Optional: When working with SILAC-labeling, a 1:1 protein concentration ratio between different labeling conditions (e.g., heavy/light) should be adjusted before continuation or freezing.

Note: Suggested controls are testing the proteinase K efficiency (see Figure 5A) and checking for indigestible proteins by performing proteinase K digests in the presence of a membrane lysing detergent (e.g., RAPIGest) as described in the "Expected Outcome" / "Statistical Analysis" sections.

Note: If no biotinylation can be detected, please refer to remarks on troubleshooting, problem 2.

Pause point: Dried cell pellets can be stored a few weeks at −80°C until further use.

Proteinase K protection

Timing: 2–3 h

This section covers homogenization of cells by douncing and proteinase K digestion to obtain only membrane-protected proteins.

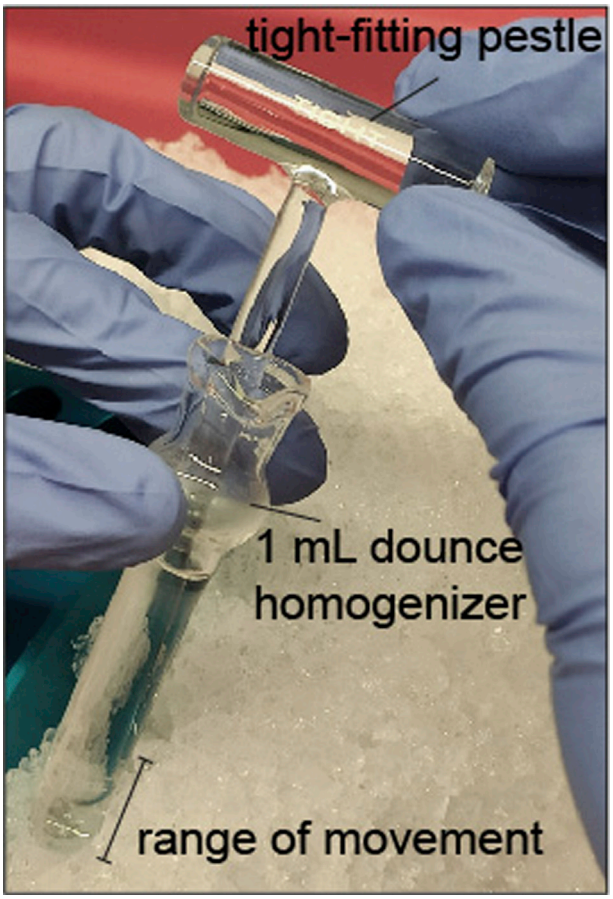

Figure 3. Dounce homogenization step

The cells are transferred to a glass dounce homogenizer and subjected to 70 manual strokes with a tight-fitting pestle. The picture also shows the scale of the movement in between the dounces.

13. Cool the centrifuge (4°C) and pre-heat an appropriate device for incubation of 1.5 mL tubes to 37°C
14. Thaw cell pellets on ice or continue directly with cells from step 12
15. Suspend cell pellets in 1.5 mL of homogenization buffer I and transfer suspension (if necessary) to a 1.5 mL centrifuge tube
    a. Centrifuge at 500 × *g* for 3 min at 4°C
    b. Discard the supernatant
16. Suspend cells in 1 mL of homogenization buffer I
17. Apply gentle agitation on an overhead shaker at 4°C for 20 min
    a. Centrifuge at 500 × *g* for 3 min at 4°C to collect the whole sample
18. Suspend the cell pellet in the supernatant and transfer it to a glass 1 mL dounce homogenizer with a tight-fitting pestle (Figure 3)
    a. Apply 70 manual strokes in order to achieve efficient rupture of cells which should be verified by light microscopy (fewer strokes might not be sufficient to achieve complete cell rupture)
    b. Collect the sample in a fresh 1.5 mL centrifuge tube
    c. Before douncing the next sample, rinse the douncer and the pestle with ddH_2O and remove all liquid
19. Add 200 µL of homogenization buffer II, mix by inverting the tube 3×
    a. Centrifuge at 600 × *g* for 10 min at 4°C
    b. Transfer supernatant to a fresh 1.5 mL centrifuge tube

20. Add 100 μg/mL (6 μL of 20 mg/mL stock) proteinase K and 1 mM $CaCl_2$ (12 μL of 100 mM stock)
    a. Incubate for 1 h at 37°C
21. Stop proteinase K digestion by adding of 10 mM (61 μL of 200 mM stock) phenylmethylsulphonyl fluoride (PMSF)
    a. Mix by inverting the tube 3×
    b. Centrifuge at 17,000 × *g* for 15 min at 4°C
    c. Discard the supernatant carefully which contains cleaved cytosolic material

Note: To profile the protein neighborhood of an APEX2 bait, the proteinase K digestion can be left out (steps 13–21). Instead, the thawed pellet can directly be lysed in qRIPA and subjected to streptavidin pulldown and on-bead digest as described from step 22 onwards. For this purpose, 1× 10 cm dish can be enough as start material per replicate.

Note: To control for cytosolic copurifying proteins that are insensitive to proteinase K digestion, an extra set of proteinase K treated samples can be incubated with 0.1% RAPIGest™ (1% stock in 50 mM ABC) (step 20). Proteins in this set might represent potential background proteins and could potentially be excluded from your data.

Note: The pellet can be quite fluffy after addition of PMSF and is easily removed (step 21c). Try taking up as much supernatant as possible with a pipette rather than a vacuum pump. However, if there is some residual liquid left covering the pellet, this is not problematic as it is dissolved in excess qRIPA and will be centrifuged again later.

Note: Glass dounce homogenizers from different companies can be used but the volume is of importance. It should not be too large, otherwise the samples will not be dounced properly. If the starting material is decreased to 1× 10 cm dish, the volumes of buffers can be decreased and also using a smaller douncer might be helpful.

Note: To resolve potential failures in homogenization or proteinase K digestion, please refer to notes on troubleshooting, problem 3.

Lysis of protective endomembranes and pulldown of biotinylated proteins

Timing: 30–80 min → 16 h incubation

This section describes the lysis of endomembranes and the enrichment of biotinylated proteins with a streptavidin pulldown.

22. Suspend the cell pellet in 500 μL cold qRIPA buffer.
    a. Briefly sonify each sample for 2× 1 s or alternatively incubate the sample for 40 min on ice
    b. Centrifuge at minimum 10,000 × *g* for 10 min at 4°C
23. In the meantime, prepare the streptavidin-agarose beads
    a. Add 75 μL of slurry beads per sample to a fresh centrifuge tube and wash 3× in 500 μL of qRIPA buffer
    b. In between spin samples down at 1,000 × *g* for 1 min at 4°C
    c. Discard the supernatant of each wash
    d. After the last wash remove the supernatant completely with the help of capillary tips that do not allow beads passing through
24. Apply the supernatant from step 22b to the pre-equilibrated beads and incubate 16 h at 4°C with overhead rotation

Note: In order to wash the beads, it is helpful to leave a small liquid layer on top of the beads for intermediate washes; after the last wash use capillary tips to get rid of all liquid without taking up the beads (step 23d).

Note: The incubation time with Streptavidin-agarose beads can be decreased from 16 h to only a few hours if non-specific binding of proteins to the resign is problematic. However, the pulldown efficiency at shorter incubation times should be monitored with appropriate controls before continuation (step 24).

Note: Alternative beads like Streptavidin-sepharose or magnetic Streptavidin-agarose beads can also be used.

On-bead tryptic digest

Timing: 2–3 h → 16 h incubation

This section covers in-solution tryptic digestion of biotinylated proteins.

25. Cool the centrifuge (4°C) and pre-heat a shaker to 55°C
26. Prepare qRIPA, 3 M (in 50 mM ABC) and 2 M Urea (in 50 mM ABC) solutions and store them at 4°C until use
27. Take the tubes from the overhead shaker and place them on ice
    a. Centrifuge at 1,000 × *g* for 1 min at 4°C, discard supernatant. Wash the beads 3× in 500 µL of qRIPA buffer with repeated centrifugation in between the washes, discard the supernatant each time
    b. Wash the beads 3× in 3 M Urea with repeated centrifugation in between the washes, discard the supernatant each time
    c. After the last wash remove all liquid using capillary tips and add 500 µL of 3 M Urea buffer
28. Add TCEP to a final concentration of 5 mM (2.5 µL of 1 M stock) and incubate for 30 min at 55°C with orbital shaking at around 400 rpm
    a. Take the samples of the shaker and let them cool down for 10 min
29. Further steps are carried out at room temperature (20°C–24°C)
30. Add IAA to alkylate the sample to a final concentration of 10 mM (9.5 µL of 550 mM stock) and incubate for 20 min with orbital shaking at around 400 rpm
31. Quench the reaction by addition of DTT to a final concentration of 20 mM (10.7 µL of 1 M stock), invert the tube 3×
    a. Centrifuge for 1 min at 1,000 × *g*, discard the supernatant
    b. Wash 2× with 2 M Urea buffer and repeat the centrifugations
    c. After the 2nd wash carefully remove all supernatant using capillary tips and add 200 µL of 2 M Urea buffer
32. Measure the pH of each sample. It should have a pH of 8. If necessary adjust the pH
33. Add 8 µL (1 µg/ 20 µL bead volume) Trypsin (0.5 mg/mL stock), vortex the samples briefly and incubate them at 37°C for 16 h

Note: For adjusting the pH in step 32 addition of a few (3–10) µL of 3M Tris-HCl has proven useful but similar reagents will most likely work as well.

Elution and desalting of peptides

Timing: 3–6 h

This section covers the elution of peptides from streptavidin beads following tryptic on-bead digest and the purification of eluted peptides using reversed-phase extraction disks.

34. Prepare a fresh 2 M Urea solution (in 50 mM ABC)
35. Centrifuge the samples at 1,000 × *g* and collect the supernatant which now contains digested peptides in a fresh microcentrifuge tube

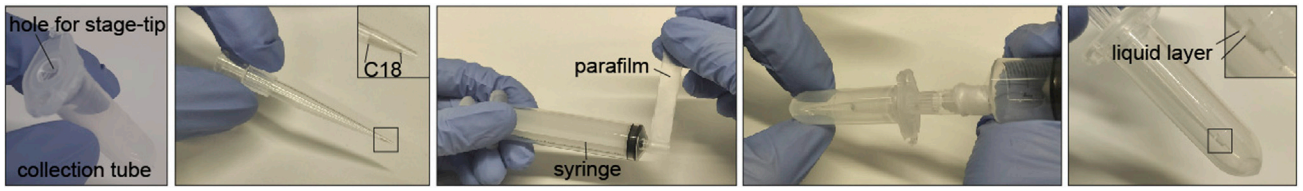

Figure 4. Preparation and handling of custom-made stage-tips

In order to generate custom-made stage-tips, a hole was inserted into a collection tube using a scalpel. Two layers of C18 disks were stacked in a 200 μL pipette tip. A syringe wrapped with parafilm is a helpful tool to manually push the buffer through the C18 disks. It is important that a small liquid layer is left over the C18 disks so that they don't dry out.

a. Wash the beads 2× in 100 μL of 2 M Urea solution, collect the supernatant and pool it with the first supernatant
b. After the 2nd wash be careful to take up all the liquid on the beads by using capillary tips. Make sure no beads stick to the outer wall of the tips in order to avoid transferring them to your sample

36. Acidify the samples with TFA at a final concentration of 1% (e.g., from a 10% stock) and invert the tube 3×
37. Dry down collected supernatants (~ 440 μL) by vacuum centrifugation at 30°C to a volume of around 80–100 μL
38. Mix the samples 1:1 with stage tipping buffer C

Pause point: Acidified samples (after step 36) can be stored at −80°C. However, it is advisable to decrease sample volume first.

Note: Stage-tips can already be equilibrated (steps 39–43) during vacuum centrifugation (step 37). Leave the 2nd round of Buffer A on the stage-tips and centrifuge it immediately before addition of the samples.

39. The next steps describe the pre-equilibration of the custom-made stage-tips (Figure 4). All centrifugations are performed at 400 × *g* for 1 min or less with visual inspection after each spinning step. After addition of each buffer make sure to get rid of potential bubbles by snapping the collection tube.
40. Wash the stage tips by addition of 30 μL Methanol
    a. Centrifuge until only a small liquid layer is left on the C18 disk
    b. Empty the collection tube
41. Wash the stage tips by addition of 30 μL stage-tipping buffer B
    a. Centrifuge until only a small liquid layer is left on the C18 disk
    b. Empty the collection tube
42. Wash the stage tips by addition of 30 μL stage-tipping buffer A
    a. Centrifuge until only a small liquid layer is left on the C18 disk
    b. Empty the collection tube
43. Repeat step 42
44. Label the stage-tips with your sample ID and load your sample on it
    a. Centrifuge for 3,500 × *g* for 6 min
    b. Make sure all sample went through, if not repeat centrifugation
    c. Collect the flow-through and store it at −20°C. In case something went wrong you can add this to new stage-tips.
45. Wash the stage-tips by addition of 30 μL stage-tipping buffer A
    a. Centrifuge at 3,500 × *g* for 2 min
    b. Discard the flow-through and dry the stage-tips with a syringe (push all residual liquid through)

⚠ CRITICAL: Make sure there is always some liquid left over the C18 disks until sample loading so that they do not dry out. Inspect every stage-tip after every centrifugation round and decrease centrifugation time if necessary. In case a stage-tip has dried, restart the whole washing / pre-equilibration beginning with addition of Methanol.

Note: A syringe wrapped with parafilm can be helpful to push the buffer through in order to only obtain a small liquid layer over the C18 disks.

Pause point: Dried stage-tips can be stored at 4°C up to half a year.

46. For elution of the sample from the stage-tip, place the latter in a new collection tube
47. Add 30 µL Buffer B to the stage-tip
    a. Use a syringe to push a bit of the buffer through until the C18 material is wet
    b. Incubate for 30 min at room temperature (20°C–24°C)
48. Push all liquid through and discard the stage-tip
49. Dry down the liquid by vacuum centrifugation at 30°C to a volume of 2–3 µL
50. Re-substitute the liquid with stage-tipping buffer A to a total volume of 10 µL
51. Transfer the sample to a MS-compatible tube in order to store it at −20°C (short-term) or −80°C (long-term) or directly proceed with injecting the MS sample.

Pause point: Eluted samples can be stored at −80°C for a year.

52. Load 5 µL of your sample into a 96-well plate for injecting it on your LC-MS system. Make sure not to make any bubbles. The rest of the sample can be stored as a backup.

EXPECTED OUTCOMES

Coupling proximity biotinylation with proteinase K digestion and mass spectrometry is a useful tool to study the content of a transient membrane compartment such as autophagosomes. In the first step, it is important to check for expression, subcellular localization and biotinylation activity of the bait chimera (Figures 2B–2D). Antibodies against biotin, epitope tags (e.g., myc) or fusion partners (e.g., APEX2) can be used for detection by standard immunoblotting and immunofluorescence approaches. In the next step, it is advisable to test the efficiency of cell homogenization and proteinase K digestion by immunoblotting before conducting the actual MS experiment. For this purpose, follow the instructions given in the sections "Biotinylation of live cells" and "Proteinase K protection". The starting material can be decreased to one 10 cm dish per condition but is dependent on the abundance of the bait. Importantly, before conducting the proteinase K assay in step 20 equally split the cell homogenate in 4 aliquots and incubate them with either 30 µg/mL proteinase K, 0.2% Triton X-100, 30 µg/mL proteinase K and 0.2% Triton X-100 or leave the sample untreated (Mock) (Figure 5A). Immunoblotting should reveal a partial protection of the autophagosome targeted bait, known autophagy substrates (e.g., p62) and biotinylated proteins in the sample incubated with proteinase K compared to the Mock and Triton X-100 samples. Conversely, a cytosolic control (e.g., the cytosolic tail of the multi-pass membrane protein ATG9A) should not be protected from proteinase K treatment. However, all marker and biotinylated proteins should be degraded when proteinase K is applied together with a membrane lysing detergent such as Triton X-100 or the mass spec compatible detergent *RAPI*Gest™ as the protease can then access the lumen of autophagosomes and other membranes compartments (Figure 5A).

For setting up the proteomics experiment, consider carefully how many conditions and replicates (minimum triplicates) are needed and whether additional control samples should be processed in parallel. The workflow for the label-free quantification (LFQ) mass spec analysis is exemplarily shown for APEX2-NDP52 expressing HeLa cells differentially treated with DMSO or BafA1. The filtering method, the program used for this filter and the total number of identified or valid proteins are indicated for each processing step (Figure 5B). To get a first overview of your data, it is recommended to

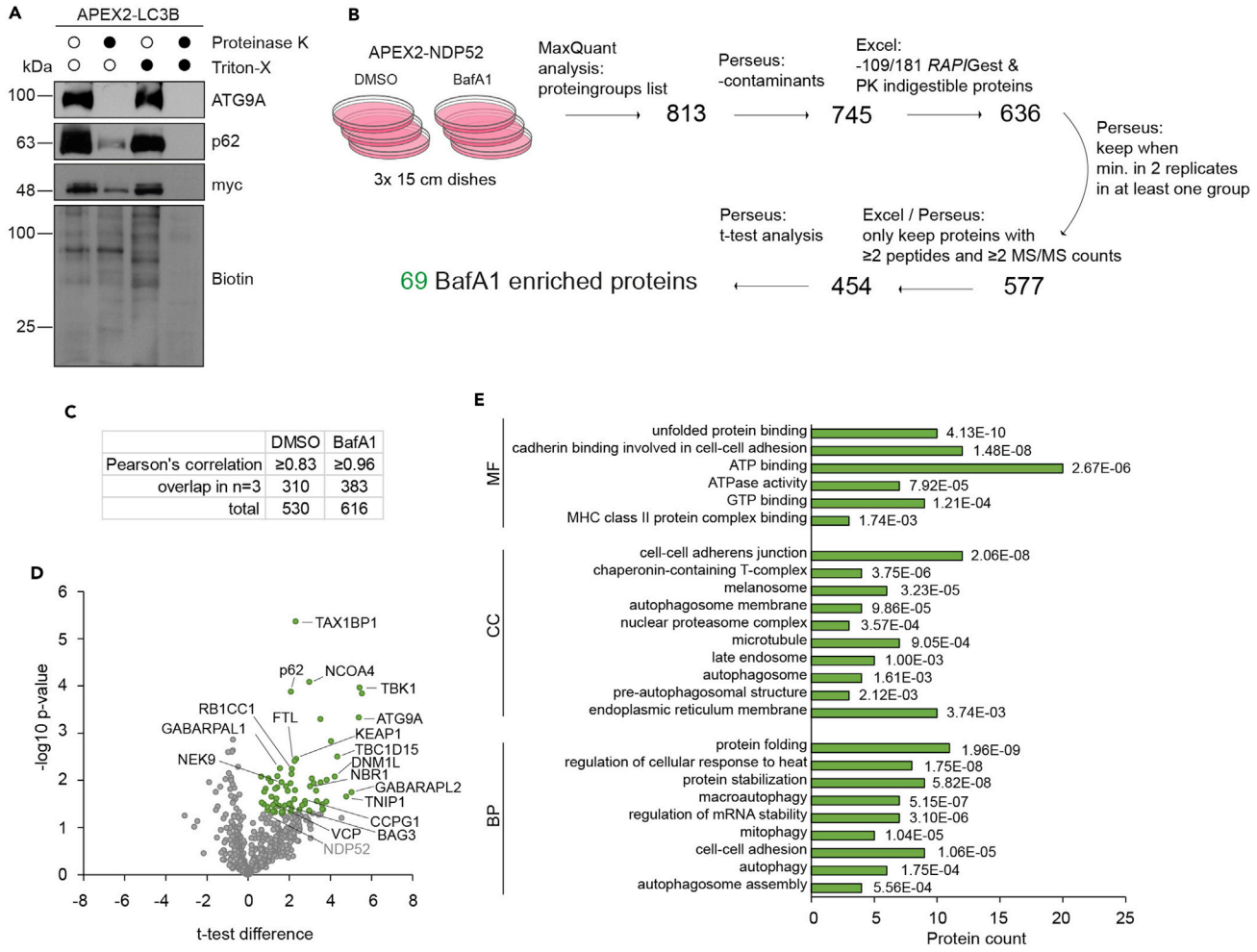

Figure 5. Biotinylated cargo candidates upon autophagosomal content profiling

(A) Protease protection assay of homogenates derived from APEX2-LC3B overexpressing HeLa Flp-In cells grown in the presence of BafA1. Homogenates were left untreated or were incubated with proteinase K, Triton X-100 or both before immunoblotting with the indicated antibodies.

(B) Analysis workflow for autophagosome content profiling of APEX2-NDP52 overexpressing HeLa cells treated with BafA1 or DMSO. Filtering of data is shown with the resulting changes in protein numbers including *RAPI*Gest™ background protein subtraction.

(C) Total numbers, overlap of n=3 replicates and Pearsons' correlations are given for the autophagosome content profiling experiment described in B.

(D) Volcano plot of proteinase K protected proteins identified in streptavidin pulldowns (student's t-test, p-value ≤ 0.05). BafA1 enriched cargo candidates are colored in green.

(E) GO term enrichment analysis of BafA1 enriched cargo candidates. BP, biological process; CC, cellular component; MF, molecular function.

assess the correlation between replicate samples and the overall number of identified proteins (Figure 5C). With our experimental and instrumental setting, we routinely identify between 500 and 900 proteinase k protected proteins in our streptavidin pulldowns. However, depending on instrumentation, processing and analysis platforms, quantification approach, number of conditions and replicates as well as the choice of cell type and bait this yield may vary. Following statistical analysis using student's t-test, proteinase K protected proteins can be visualized by plotting their p-values and t-test differences against each other in a volcano plot (Figure 5D). Autophagy cargo candidates should increase in their abundance upon BafA1 treatment since degradation by the autophagosomal-lysosomal system is blocked under this condition. Therefore, cargo candidates can be defined with t-test differences ≥ 0 and p-values ≤ 0.05. Depending on the dataset, more stringent additional cutoffs can help to further narrow down the list of candidates. As quality control step and initial characterization cargo candidates can be subjected to GO analysis by the DAVID functional annotation

database. The results of this analysis can be easily displayed as bar graphs (Figure 5E) and are helpful to verify the enrichment of autophagy-related proteins. In our autophagosome content profiling example with the selective autophagy receptor NDP52 (alias CALCOCO2) fused to APEX2 we identified known autophagy machinery components (ATG9A, RB1CC1, TBK1), selective receptors (NCOA4, p62, TAX1BP1, CCPG1, NBR1), human ATG8 family members (GABARAPL1, GABARAPL2) and their binding partners (TBC1D15, NEK9) in addition to a number of new cargo candidates which can be prioritized according to their GO terms. In our example, the associations "ATP/GTP binding" represents a potentially new category of autophagy substrates. Notably, if you do not detect any known cargo or components of the autophagy system with your approach, this might be due to technical issues or your choice of bait, cell type or growth condition. It is therefore advisable to include positive control samples for which published data is available.

Note: In case there are any issues with proper identification of expected proximity partners, high background of unspecific identifications, or general data quality, please refer to notes on troubleshooting, problems 4, 5, and 6.

QUANTIFICATION AND STATISTICAL ANALYSIS

Raw data files were analyzed using MaxQuant (version 1.6.0.1) (Cox and Mann, 2008) and referenced with a human Uniprot FASTA reference proteome (UP000005640) and label-free quantification was selected. The protein group output file was then further analyzed in Perseus (version 1.6.5.0) (Tyanova et al., 2016). Matches to common contaminants, reverse identifications or identifications based on side-specific modifications were removed, as well as peptides with <2 peptides or <2 MS/MS counts. Proteins matching hits identified as proteinase K indigestible proteins were removed. A background list was generated in Zellner et al. 2021, using a control set of APEX2-LC3B cell homogenates treated with proteinase K and *RAPI*Gest^{TM} in 6 replicates that yielded a total of 181 non-redundant proteins. For further analysis, only proteins with LFQ intensities in 2 out of 3 replicates were kept, $\log_2$ transformed and imputed following a normal distribution. Statistical significance of abundance alterations for BafA1 vs. DMSO treatment was determined using student's t-test (p-value $\leq$ 0.05). These BafA1-enriched proteins are highlighted in green in the Volcano-plot (Figure 5D) and were subjected to functional annotation enrichment analysis using the DAVID database (version 6.8). Exemplary, only gene ontology (GO) terms are displayed (Figure 5E).

LIMITATIONS

By combining APEX2-mediated proximity biotinylation with proteinase K digestion and mass spectrometry, the presented protocol was used to systematically identify selective autophagy receptor-specific cargo. Although it might be a powerful tool to assess the content of various transient membrane-bound compartments, certain inherent limitations should be considered.

APEX2-mediated biotinylation depends on a short H_2O_2 pulse in the cellular presence of biotin-phenol. Due to general low membrane permeability of biotin phenol, sufficient biotinylation might only be easily applicable in mammalian cells. Other organisms such as yeast may need adjustments to improve the delivery of biotin-phenol. Additionally, H_2O_2 is toxic and thus only applicable in tissue and primary culture but not in animal *in vivo* studies. Hence, other proximity biotinylation enzymes such as TurboID or MiniTurbo (Branon et al., 2018) might be better suited for these applications. Biotinylation occurs at electron rich amino acids such as tyrosines, posing the potential risk of under-representation of proteins with limited numbers of accessible tyrosines. The same applies to proteins that are buried in macromolecular or membrane-rich complexes.

As for every overexpression study, artificially expressed APEX2 chimera might be prone to some caveats, including altered cellular localization and function or identification of false-positive proximity partners. Employing endogenous tagging can overcome this limitation, however also requires longer preparation procedures and might be difficult to establish. Due to the size of the

APEX2-tag, it may interfere with proper bait expression, localization or function. Potential disturbances need to be excluded in pilot experiments.

Some proteins might not be responsive to proteinase K digestion, leading to both, a higher background of unspecific cytoplasmic proteins, and falsely removed proximity interactors due to stringent statistical exclusion. Few specific proximity candidates may not be identified due to limited expression in particular cell lines or inadequate treatments and stimuli.

TROUBLESHOOTING

Problem 1

The APEX2 chimera is not properly expressed or shows an altered cellular localization or function. This could interfere with downstream mass spec analysis as it may lead to the identification of false-positive interactors (See section "cloning and expression of the APEX2 bait chimera").

Potential solution

Verify proper expression and localization of the APEX2 chimera using immunoblotting, immunofluorescence and electron microscopy before proceeding with the mass spec experiment. In case the bait protein is known to be crucial for organelle morphology or to be involved in specific pathways, further assess its functionality. If the APEX2 chimera is mislocalized, move the APEX2 tag to the opposite terminus of your bait or use only a fragment of your bait. In case the expression is too strong or weak, switch the promotor, cell line or expression method (stable, transient, inducible).

Problem 2

The APEX2 chimera is expressed and correctly localized within the cell but no biotinylation is detectable by immunoblotting as biotin smear or immunofluorescence at the compartment of interest (See protocol steps 1–12).

Potential solution

The APEX2 tag might not be functional. First of all, verify the correct in-frame cloning of your construct. Secondly, a longer linker region between APEX2 and the bait might be needed. If necessary, switch protein terminus, promotor or expression method. Biotin-phenol might not be adequately delivered to the compartment of interest. Verify that biotin-phenol is fully dissolved in (pre-warmed) medium before adding it to the cells and that your cells are suitable for biotin-phenol delivery. White precipitates indicate insufficient dissolution. If necessary, additional steps can be performed to increase biotin-phenol permeability. These may include compromising the integrity of the cell wall in yeast or increasing biotin-phenol concentration and incubation time in multilayered tissue cultures. Prolonged H_2O_2 pulses are not recommended due to increased toxicity and reduced labeling specificity. Low level expression of the APEX2 chimera may only result in low levels of biotinylation. In this instance, switching the blocking reagent for the immunoblots or sampling other antibodies might be necessary to improve signal quality. In case the APEX2 chimera is expressed, use a different APEX2 tagged protein as a positive control to exclude technical sample handling errors. Additionally, APEX2 activity can be probed by utilizing peroxidase sensitive agents such as Amplex™ Red (Thermo Fisher Scientific).

Problem 3

Immunoblotting reveals that the proteinase K treatment is not sufficient to digest the majority of proteins (Figure 6B) or that there is no bait detectable in the protected fraction. This may compromise the mass spec analysis and lead to reduced proximity interactor identification or high levels of cytoplasmic background proteins (See protocol steps 13–21).

Potential solution

No visible differences between the different conditions of the proteinase K assay may indicate incomplete cell homogenization. Efficient cell shearing can be verified by light microscopy. In case you observe many unbroken cells, increase the number of strokes per sample. Depending on your bait, digestion

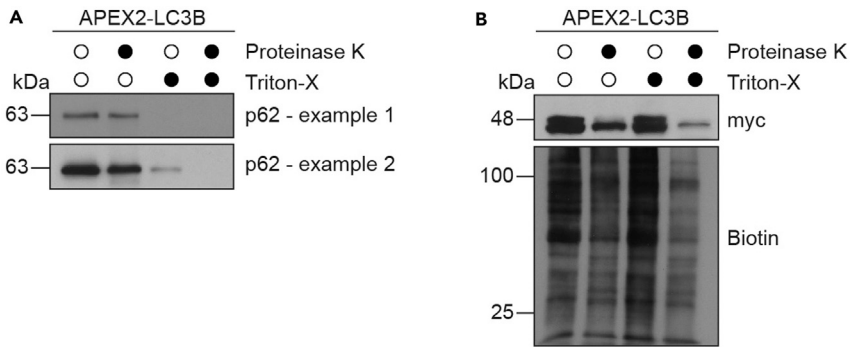

Figure 6. Troubleshooting examples

(A) Harvesting the cells with trypsin instead of scraping affects the outcome of the proteinase K protection assay. As exemplarily shown for these two independent experiments, incubation of homogenates with Triton-X yielded dramatically reduced levels of p62 which should not be affected by this condition.

(B) No overt proteinase K digestion of biotinylated proteins is detectable in these homogenates. In addition, APEX2-LC3B is still partially protected despite the combined incubation of homogenates with proteinase K and Triton-X.

conditions may need adjustments including changing temperature (4°C vs. 37°C), incubation time (30 min vs. 60 min), supplementation with $CaCl_2$ and enzyme concentration (30 μg/mL vs. 100 μg/mL) or the use of another enzyme such as trypsin. In case the bait or control protein is not detectable in the protected fraction, membranes might be ruptured. Try milder homogenization and reduce the number of strokes per sample. Notably, low abundance of the bait in the proteinase K treated fraction is reasonable if only a small proportion of it is present inside of membrane compartments at a given time.

Problem 4

Poor enrichment of biotinylated proteins by streptavidin pulldown although proper bait expression and APEX2 activity was verified (in case of doubt see problems 1 and 2). This error might lead to a loss of potential proximity interactors and high unspecific background identification in mass spec analyses (See protocol steps 22–27).

Potential solution

While the streptavidin-biotin affinity is very high and therefore in general quite robust, suboptimal homogenization, lysis and binding conditions might diminish protein enrichment. Ensure that cells were extensively washed after the biotinylation reaction to reduce potential biotin-phenol contaminations that might compete with biotinylated proteins for binding to the streptavidin resin. Avoid possible biotin containing components in lysis and binding buffers. Even though they are usually not a limiting factor, increasing the amount of streptavidin beads might help enriching biotinylated proteins. Absent biotin or protein detection by immunoblotting may arise from suboptimal elution, which can be increased by prolonged heating at 95°C (up to 10 min) in SDS-containing gel loading buffer supplemented with free biotin (up to 50 mM). For mass spec sample procession, biotinylated proteins are cleaved off the streptavidin beads via tryptic on-bead digest. Inadequate digestion conditions might compromise peptide recovery. Ensure optimal digestion conditions (pH 8.0, 37°C and 16 h incubation) and sufficient concentration of trypsin. In the case of low expression of the APEX2 chimera or its activity in a low abundant, transient subcellular compartment, increasing the amount of input material might support protein enrichment and interactor identification. If membrane protection is expected, technical errors in performing homogenization and proteinase K digestion may lead to organelle rupture and subsequent digestion of the total proteome (Problem 3).

Problem 5

In addition to the APEX2 chimera and some specific interactors or compartment-specific markers, a high number of seemingly random proteins are identified, compromising data quality (See protocol steps 22–52 as well as sections "quantification and statistical analysis" and "expected outcomes").

Potential solution

In case these proteins are not expected to be present in the compartment to which the bait is directed but instead represent cytoplasmic contamination, the proteinase K digestion step might be compromised. Please refer to problem 3. Ensure that the APEX2 tag is not cleaved off the protein of interest, as this is likely to result in unspecific biotinylation of cytosolic proteins. As some proteins function in different intracellular compartments, apparently unspecific proteins could actually represent differing functions of these prey proteins. Endogenously biotinylated proteins may contribute to background noise but can be easily excluded in the statistical analysis by including negative controls. Combining SILAC labeling with an additional cytosolic APEX2-tagged control protein might simplify the identification of high-confident proximity interactors. Unspecific binding of proteins to the beads might be a consequence of insufficient washing or usage of too many beads per sample. In this case, increase the number and volume of washing steps, prolong the incubation time between washes, reduce the amount of streptavidin beads, or use more stringent wash conditions such as slightly higher urea concentrations to ensure full denaturation. Unspecific binding may also be reduced by shortened incubation times with streptavidin-agarose beads to a few hours or switching the type of streptavidin-coupled beads.

Problem 6

Statistical analysis shows high variances between replicates and in general low correlations between samples. The overall data quality is poor with high differences in peptide counts or LFQ intensities, thereby limiting biological conclusions (See sections "quantification and statistical analysis" and "expected outcomes").

Potential solution

Even though this protocol describes a fast and relatively simple method for proximity proteomics in membrane protected compartments, unexperienced or inconsistent experimental handling might compromise data outcome. In general, it is not advisable to switch operators during an experiment as this may introduce unnecessary variations. It is crucial to keep all steps consistent between replicates, including the number of strokes used for homogenization or proteinase K digestion conditions. All controls should be performed in parallel. Identical growth conditions are also highly critical for good data quality, as varying cell density and passage number or nutrient availability heavily affect cellular systems. After biotinylation, ensure proper and consistent storage of cell pellets, processing of lysates, and immunoprecipitation of proteins.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christian Behrends (christian.behrends@mail03.med.uni-muenchen.de).

Materials availability

All unique reagents used in this protocol are available from the lead contact.

Data and code availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD024335 (http://www.ebi.ac.uk/pride/archive/projects/PXD024335). The original data for confocal microscopy images and immunoblots in the paper have been deposited to Mendeley Data: https://data.mendeley.com/datasets/33dx4p8d4n/1.

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AUTHOR CONTRIBUTIONS

S.Z. acquired all data and prepared data-related figures; K.N. prepared schematic figures. All authors wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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