Supporting Information

Development and Comparative Evaluation of Endolysosomal Proximity Labeling-based Proteomic Methods in Human iPSC-derived Neurons.

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Supplemental Methods: Development of LAMP1-APEX iPSC lines

For the endogenous KI-LAMP1-APEX line, iPSCs were engineered by CRISPR-mediated homologous recombination of the APEX2 transgene into the endogenous LAMP1 gene. APEX2 is the second generation of APEX with improved enzymatic activity.[1] We refer APEX2 as APEX in the paper for simplicity. Briefly, 1.5 million cells were seeded onto a 6-well dish for reverse transfection with Lipofectamine Stem (ThermoFisher). A ribonucleoprotein particle containing a crRNA targeting the 3' end of the LAMP1 ORF, tracrRNA, and recombinant Cas9 protein, was co-transfected with a custom DNA plasmid harboring 1-kb homology fragments flanking the APEX gene and a fluorescent selection cassette (Genewiz). The following day, the cells were dissociated onto a 10 cm dish, and maintained on Essential 8 medium for one week. Genomic DNA was collected from the unpurified cells using a Quick-DNA Microprep Kit (Zymo) and endogenous integration of APEX2 at the 3' end of a single LAMP1 ORF allele was confirmed by PCR. When the cultures reached an 80-90% confluency, a FACS Sony SH800S Cell Sorter was used to seed a 96-well plate with individual fluorescent cells, and scaled to 6-well dishes.

Two overexpression lines, KuD-LAMP1-APEX and KuB-LAMP1-APEX were generated to compare with the endogenous KI-LAMP1-APEX line. The HA line was generated by TALEN-mediated integration of a tetracycline-inducible KuD-LAMP1-APEX transgene at the CLYBL gene (UNIPROT: Q8N0X4). Since the high expression level of the TET-On promoter may drive partial mislocalization of LAMP1 to the cell membrane, we used a detuning strategy of upstream open reading frames (uORFs) to decrease transcriptional efficiency and enable more physiologic expression levels of LAMP1-APEX. Our previously developed LAMP1-APEX employed the moderate Kozak/uORF detuning strategy "KuB" (CAAATGGGTTGAACC-start).[2,3] Compared to the KuB line, KuD line employed the stringent Kozak/uORF "KuD" (GGGATGGGTTGATTT-start). KuB is predicted to reduce expression from the TET-ON promoter driving LAMP1-APEX to <15% of a consensus Kozak sequence (GCCACC-start), whereas KuD is predicted to reduce expression to <2% of the consensus sequence. The successful integration of APEX onto the LAMP1 locus was confirmed by PCR for each transgenic iPSC line.
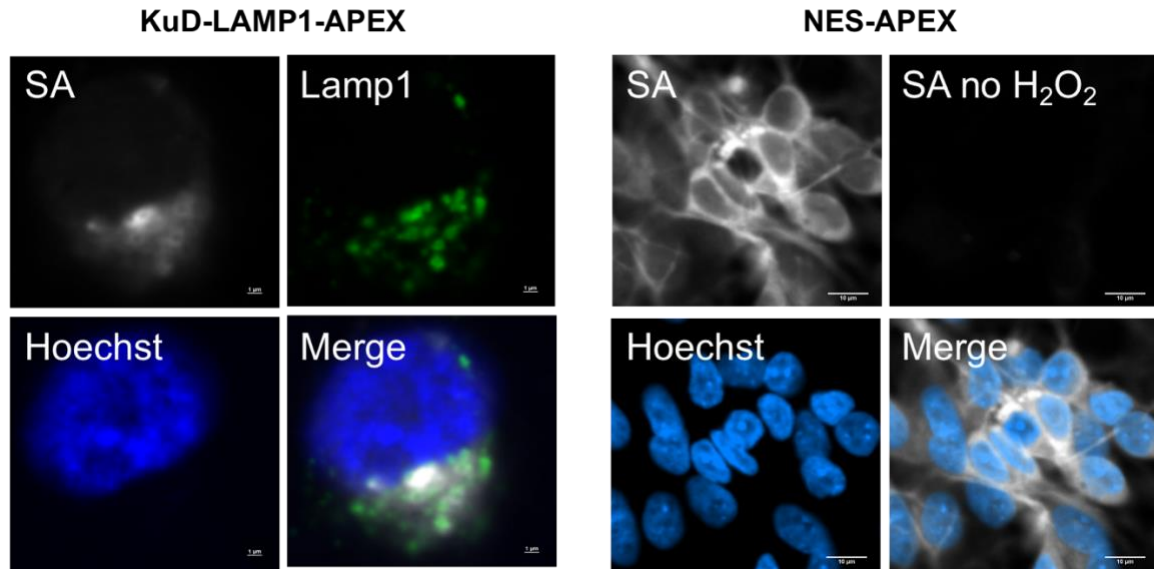
63 **Supplemental Figures:**

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66 **Figure S1. Fluorescence imaging of APEX activity in overexpression KuD-LAMP1-APEX neurons**

67 **(left) and cytosolic NES-APEX neurons (right).** Biotinylation is visualized by staining against

68 streptavidin (SA) Fluor 680 (far red). Hoechst is a nuclear marker (blue). LAMP1 (green) is used as an

69 endolysosome marker. Control neurons without H_2O_2 treatment exhibit no biotinylation signals. The APEX

70 activity of the KuB-LAMP1-APEX probe was shown in our previous publication.[3]
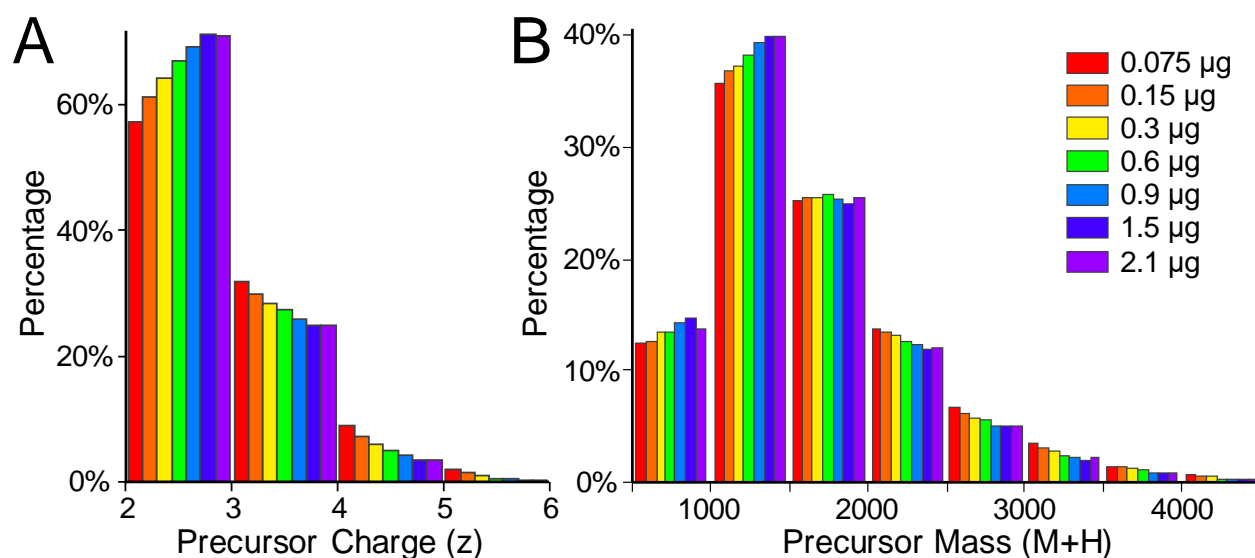
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Figure S2. Distribution of peptide charges (A) and precursor masses (B) with different amount of proteases for on-beads protein digestion. Increased amount of protease (Trypsin/LysC mix) shifted the peptides towards lower charges and smaller precursor masses.
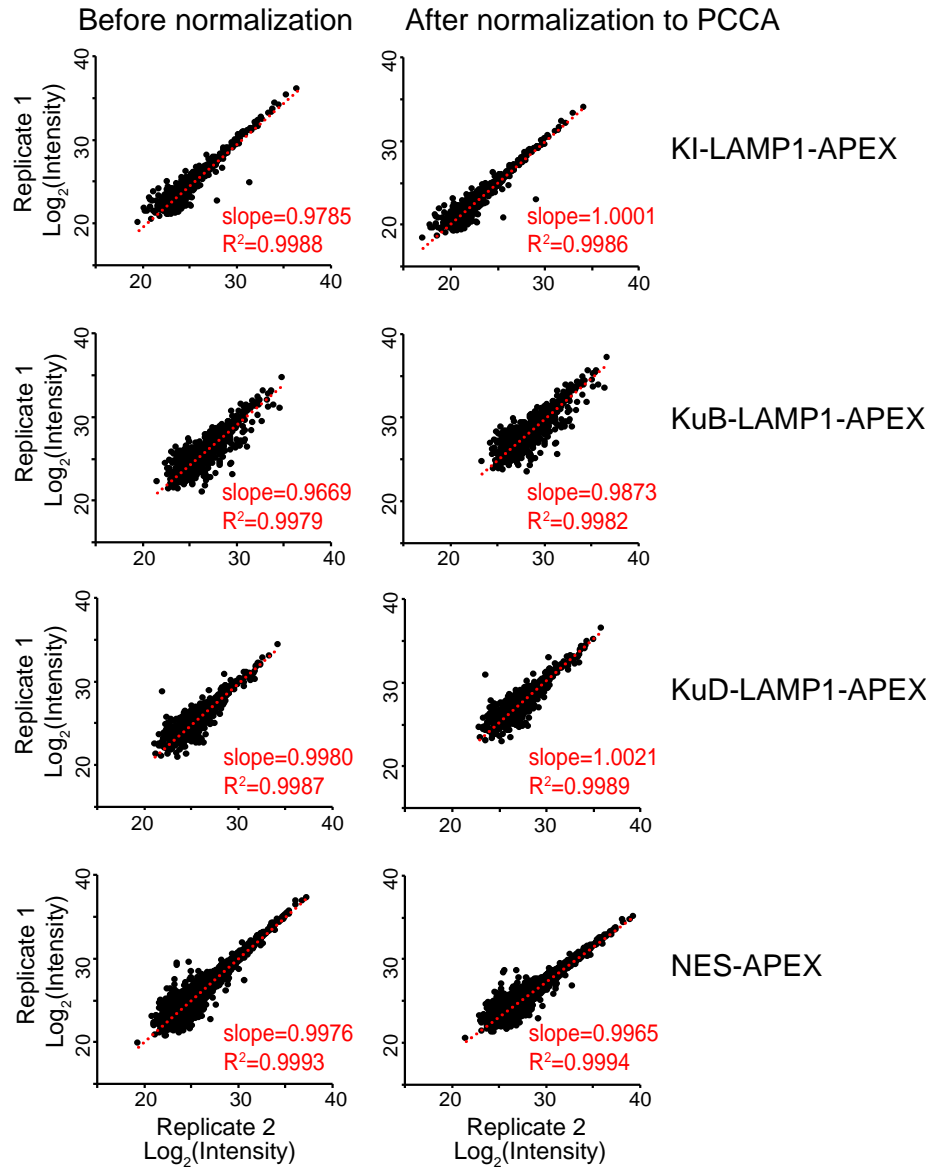
Figure S3: Scatter plots showing reproducibility between biological replicates in the same batch of APEX labeling experiment before and after normalization to PCCA.
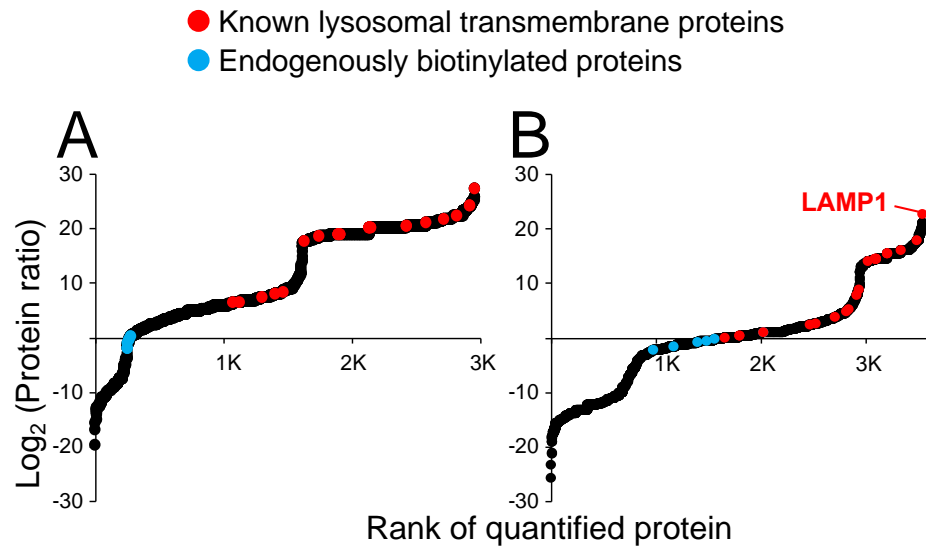
Known lysosomal transmembrane proteins
Endogenously biotinylated proteins

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83
84 **Figure S4. Evaluation of false discoveries in KuB-LAMP1-APEX using different control dataset.**

85 (A) KuB-LAMP1-APEX Proteomics with no-APEX line as control; (B) KuB-LAMP1-APEX with NES-

86 APEX as control. Protein intensities were normalized to the most abundant endogenously biotinylated
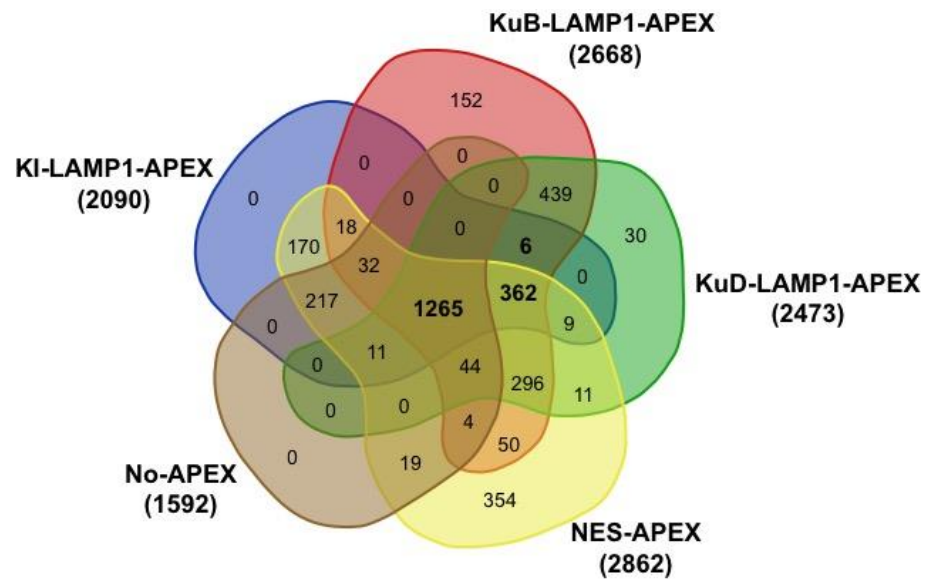
87 protein, PCCA.

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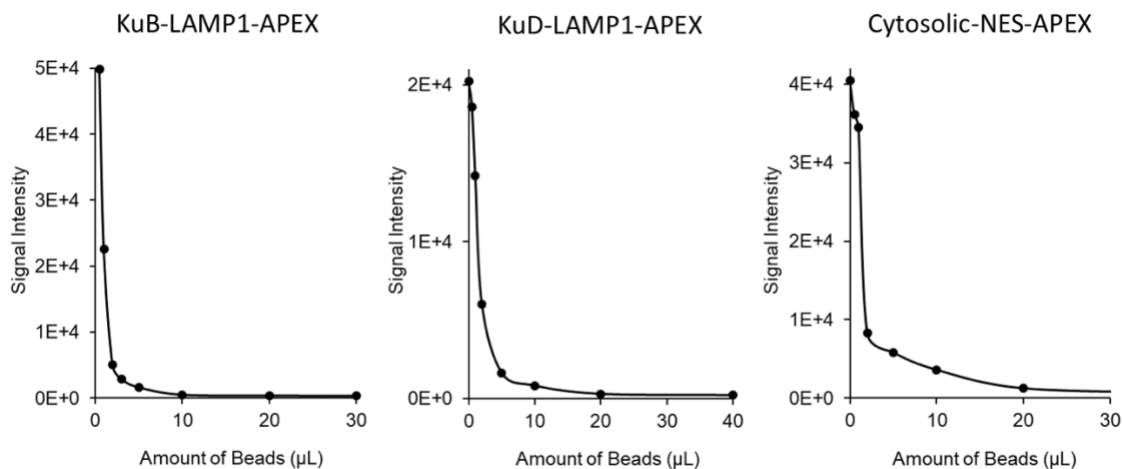
Figure S5. Beads titration assay for overexpression APEX probes: KuB-LAMP1-APEX, KuD-LAMP1-APEX, and cytosolic NES-APEX. Increasing amount of beads were incubated with 20 µg of input protein lysate in different tubes, followed by dot-blot assay against streptavidin staining.

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99   **Figure S6. Venn diagram of all identified proteins from all APEX probes and controls.** Three

100   LAMP1-APEX proteomics, cytosolic NES-APEX, and No-APEX control groups.

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Reference for Supporting Information

(1) Lam, S. S.; Martell, J. D.; Kamer, K. J.; Deerinck, T. J.; Ellisman, M. H.; Mootha, V. K.; Ting, A. Y. Directed Evolution of APEX2 for Electron Microscopy and Proximity Labeling. *Nat. Methods* **2014**. https://doi.org/10.1038/nmeth.3179.

(2) Liao, Y. C.; Fernandopulle, M. S.; Wang, G.; Choi, H.; Hao, L.; Drerup, C. M.; Patel, R.; Qamar, S.; Nixon-Abell, J.; Shen, Y.; Meadows, W.; Vendruscolo, M.; Knowles, T. P. J.; Nelson, M.; Czekalska, M. A.; Musteikyte, G.; Gachechiladze, M. A.; Stephens, C. A.; Pasolli, H. A.; Forrest, L. R.; St George-Hyslop, P.; Lippincott-Schwartz, J.; Ward, M. E. RNA Granules Hitchhike on Lysosomes for Long-Distance Transport, Using Annexin A11 as a Molecular Tether. *Cell* **2019**, *179* (1), 147-164.e20. https://doi.org/10.1016/j.cell.2019.08.050.

(3) Fernandopulle, M. S.; Prestil, R.; Grunseich, C.; Wang, C.; Gan, L.; Ward, M. E. Transcription Factor–Mediated Differentiation of Human IPSCs into Neurons. *Curr. Protoc. Cell Biol.* **2018**, *79* (1), 1–48. https://doi.org/10.1002/cpcb.51.