APPENDIX

Autophagosome-Lysosome Fusion in Neurons Requires INPP5E, A Protein Associated with Joubert Syndrome

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Appendix Figure Legends

Appendix Figure S1. siRNA screening to identify phosphoinositide phosphatases involved in autophagy.

HeLa cells treated with siRNAs against the indicated phosphatases for 48 h were cultured in Earle's Balanced Salt Solution (EBSS) for 2 h. Cells were fixed and stained with anti-LC3 and anti-p62 antibodies, and then analyzed by immunofluorescence microscopy. Bar, 10 μm. Red text indicates phosphatases that affect autophagy.

Appendix Figure S2. INPP5E knockdown partially suppresses autophagic flux in MEFs.

A Levels of *INPP5E* mRNA 72 h after transfection of MEFs with siControl or siINPP5Es, as analyzed by RT-PCR.

B MEFs treated with siControl or siINPP5Es were cultured for 2 h in growth medium (nutrient) or EBSS (starved) with or without 125 nM Baf.A1, and then analyzed by immunoblot using anti-LC3 and anti-GAPDH antibodies.

C Quantitation of protein signal intensities from immunoblots in B showing difference

of LC3-II levels between the presence and absence of Baf.A1 following normalization to the control protein GAPDH. Results represent means ± s.d. of three independent experiments. **, $P < 0.01$.

Appendix Figure S3. The number of LC3 dots, as well as Atg5 dots, is elevated in INPP5E-depleted MEFs.

A MEFs stably expressing GFP-Atg5 treated with siControl or siINPP5Es were cultured for 2 h in growth medium (nutrient) or EBSS (starved). Cells were fixed and stained with anti-LC3 antibodies, and then analyzed by immunofluorescence microscopy. Bar, 10 μm.

B, C Quantitation of the number of LC3 puncta (B) or Atg5 puncta (C) per cell, as described in A (mean ± s.d.; $n > 40$ cells from three independent experiments). Starvation medium (S) with 100 nM wortmannin (W) was used as a negative control. **, $P < 0.01$.

Appendix Figure S4. INPP5E depletion decreases the ratio of autolysosomes to

autophagosomes.

A N1E-115 cells stably expressing tfLC3 treated with siControl or siINPP5Es were cultured in growth medium. Cells were fixed and analyzed by immunofluorescence microscopy. Bar, 10 μm.

B Percentages of colocalization are shown as RFP^+GFP^+ dots / total (RFP^+GFP^-) dots (means ± s.d.; n > 20 cells from three independent experiments). **, $P < 0.01$.

Appendix Figure S5. Autophagy is suppressed in Atg2a/2b-depleted N1E-115 cells.

A Levels of *Atg2a* and *Atg2b* mRNA 72 h after transfection of N1E-115 cells with siControl or siAtg2a/2b, as analyzed by RT-PCR.

B N1E-115 cells treated with siControl or siAtg2a/2b were cultured for 2 h in growth medium with or without 125 nM Baf.A1, and then analyzed by immunoblot using anti-p62, anti-LC3, and anti-GAPDH antibodies.

Appendix Figure S6. INPP5E knockdown does not affect transcriptional level of autophagy-related genes.

Transcriptional levels of the indicated genes 72 h after transfection of N1E-115 cells with siControl or siINPP5Es, as analyzed by quantitative real-time PCR. Data were normalized to β-actin and analyzed by the $2^{-\Delta\Delta Ct}$ method. *, $P < 0.05$; n.s., non significant.

Appendix Figure S7. Autophagy induction dose not affect INPP5E localization in N1E-115 cells and MEFs.

A NIE-115 cells stably expressing mSt-INPP5E (WT) were cultured in growth medium with or without 200 nM Torin1. Cells were fixed and stained with anti-LAMP1 antibodies, and then analyzed by immunofluorescence microscopy. Insets show the boxed areas at high magnification. Bar, 10 μm.

B MEFs stably expressing mSt-INPP5E (WT) were cultured for 2 h in growth medium (nutrient) or EBSS (starved). Cells were fixed and stained with anti-LAMP1 antibodies, and then analyzed by immunofluorescence microscopy. Insets show the boxed areas at high magnification. Arrowhead indicates the cilia formation and the localization of INPP5E at cilia. Bar, 10 μm.

Appendix Figure S8. INPP5E depletion does not decrease intralysosomal pH.

N1E-115 cells treated with siControl or siINPP5Es were cultured for 1 h in growth medium with 1 μM Lysosensor Yellow/Blue DND-160. Cells were fixed and stained with anti-LAMP1 antibodies and analyzed by immunofluorescence microscopy. Quantitation of signal intensity ratio (yellow / blue) (mean ± s.d.; n > 20 cells from three independent experiments). n.s., non significant.

Appendix Figure S9. Lysosomal glycosidase activity is unchanged in INPP5E-depleted N1E-115 cells.

Activities of lysosomal enzymes were measured in N1E-115 cells treated with siControl or siINPP5Es. Measurements of the activities of the indicated glycosidases represent means ± s.d. of three independent experiments. **, $P < 0.01$; *, $P < 0.05$; n.s., non significant.

Appendix Figure S10. ML1N probe binds specifically to PI(3,5)P_2 on lysosomes.

A NIE-115 cells stably expressing mSt-2xML1N were cultured in growth medium treated with siControl or siPIKfyve, or with 1 μM YM201636 for 2 h. Cells were fixed and stained with anti-LAMP1 antibodies, and then analyzed by immunofluorescence microscopy. Insets show the boxed areas at high magnification. Bar, 10 μm.

B Quantitation of signal intensities in A showing mSt-2xML1N colocalizing with LAMP1 (means ± s.d.; n > 30 cells from three independent experiments). **, $P < 0.01$.

Appendix Figure S11. INPP5E acts as 5-phosphatase against $PI(3,5)P_2$ and $PI(4,5)P_2$ *in vitro*.

Activities of INPP5E 5-phosphatase were measured in HEK293A cells transfected with empty vector or FLAG-INPP5E (WT, D477N). Measurements of the activities against the indicated phosphoinositides represent means ± s.d. of three independent experiments. **, $P < 0.01$; *, $P < 0.05$.

Appendix Figure S12. Lysosomal PI(4)P level is unchanged in INPP5E-depleted N1E-115 cells.

A NIE-115 cells stably expressing OSBP-PH-mCherry treated with siControl or siINPP5Es were cultured in growth medium. Cells were fixed and stained with anti-LAMP1 antibodies, and then analyzed by immunofluorescence microscopy. Insets show the boxed areas at high magnification. Bar, 10 μm.

B Quantitation of signal intensities in A showing OSBP-PH-mCherry colocalizing with LAMP1 (means ± s.d.; n > 100 cells from three independent experiments). n.s., non significant.

Appendix Figure S13. Double knockdown of PIKfyve and INPP5E abolished lysosomal PI(3,5)P_2 staining and showed accumulation of LC3 dots.

A NIE-115 cells stably expressing mSt-2xML1N were cultured in growth medium treated with siControl or siINPP5E plus siPIKfyve. Cells were fixed and stained with anti-LAMP1 antibodies, and then analyzed by immunofluorescence microscopy. Insets show the boxed areas at high magnification. Bar, 10 μm.

B N1E-115 cells treated with siControl, siINPP5E, siPIKfyve, or siINPP5E plus siPIKfyve were cultured in growth medium. Cells were fixed and stained with anti-LC3

antibodies, and then analyzed by immunofluorescence microscopy. Insets show the boxed areas at high magnification. Bar, 10 μm.

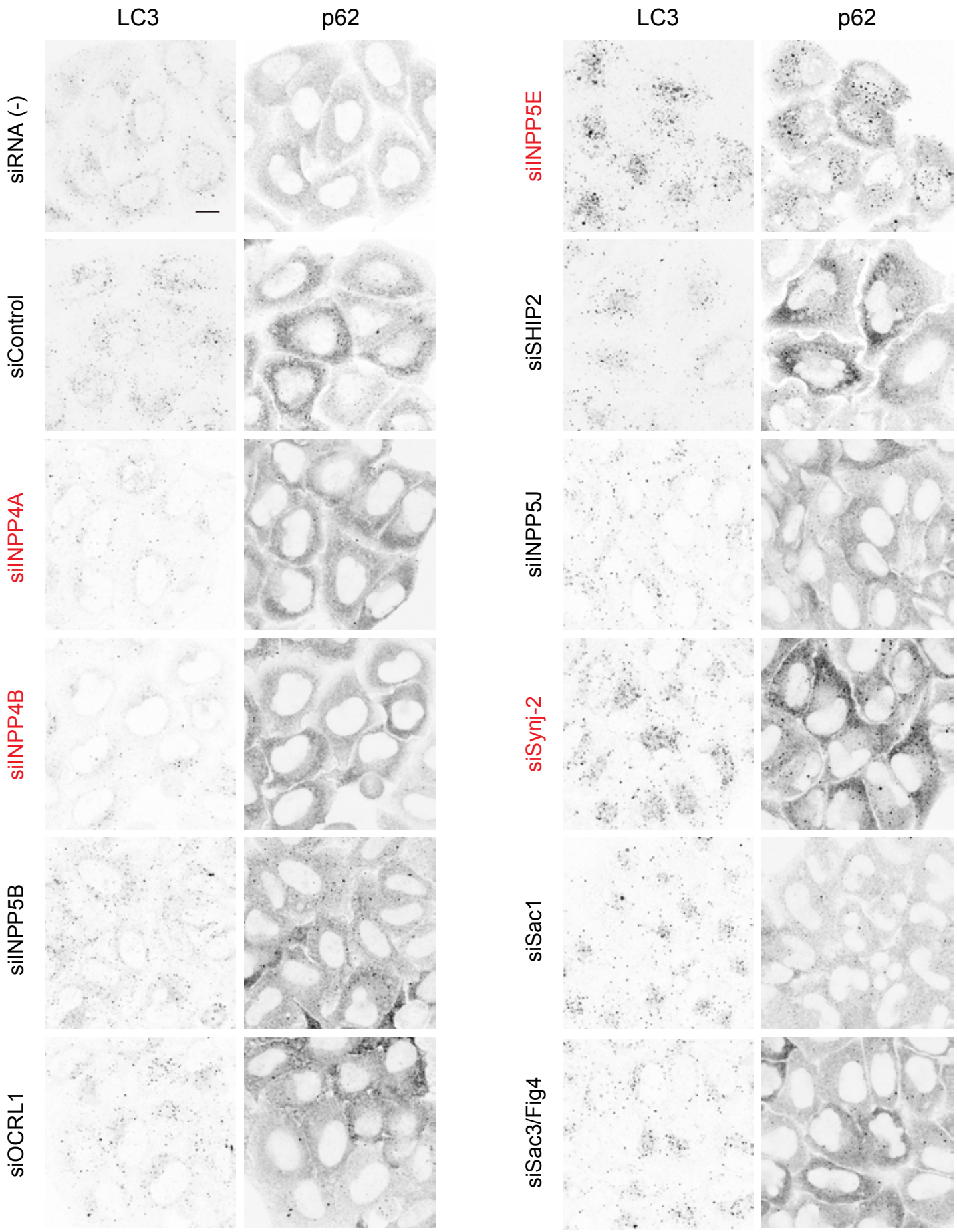
Appendix Figure S14. Localization of cortacin on lysosomes is reduced in INPP5E-depleted N1E-115 cells.

A N1E-115 cells treated with siControl or siINPP5Es were cultured in growth medium. Cells were fixed and stained with anti-LAMP1, anti-cortactin antibodies and Phalloidin, and then analyzed by immunofluorescence microscopy. Insets show the boxed areas at high magnification. Bar, 10 μm. Quantitation of signal intensities showing cortactin colocalizing with LAMP1 (means ± s.d.; n > 40 cells from three independent experiments). **, $P < 0.01$; *, $P < 0.05$.

B N1E-115 cells treated with siControl or siINPP5Es were cultured in growth medium. Cells were fixed and stained with anti-phospho-Y466 cortactin and anti-LAMP1 antibodies, and then analyzed by immunofluorescence microscopy. Insets show the boxed areas at high magnification. Bar, 10 μm. Quantitation of signal intensities

showing cortactin (pY466) colocalizing with LAMP1 (means ± s.d.; n > 40 cells from three independent experiments). **, $P < 0.01$; *, $P < 0.05$.

Appendix Figure S1



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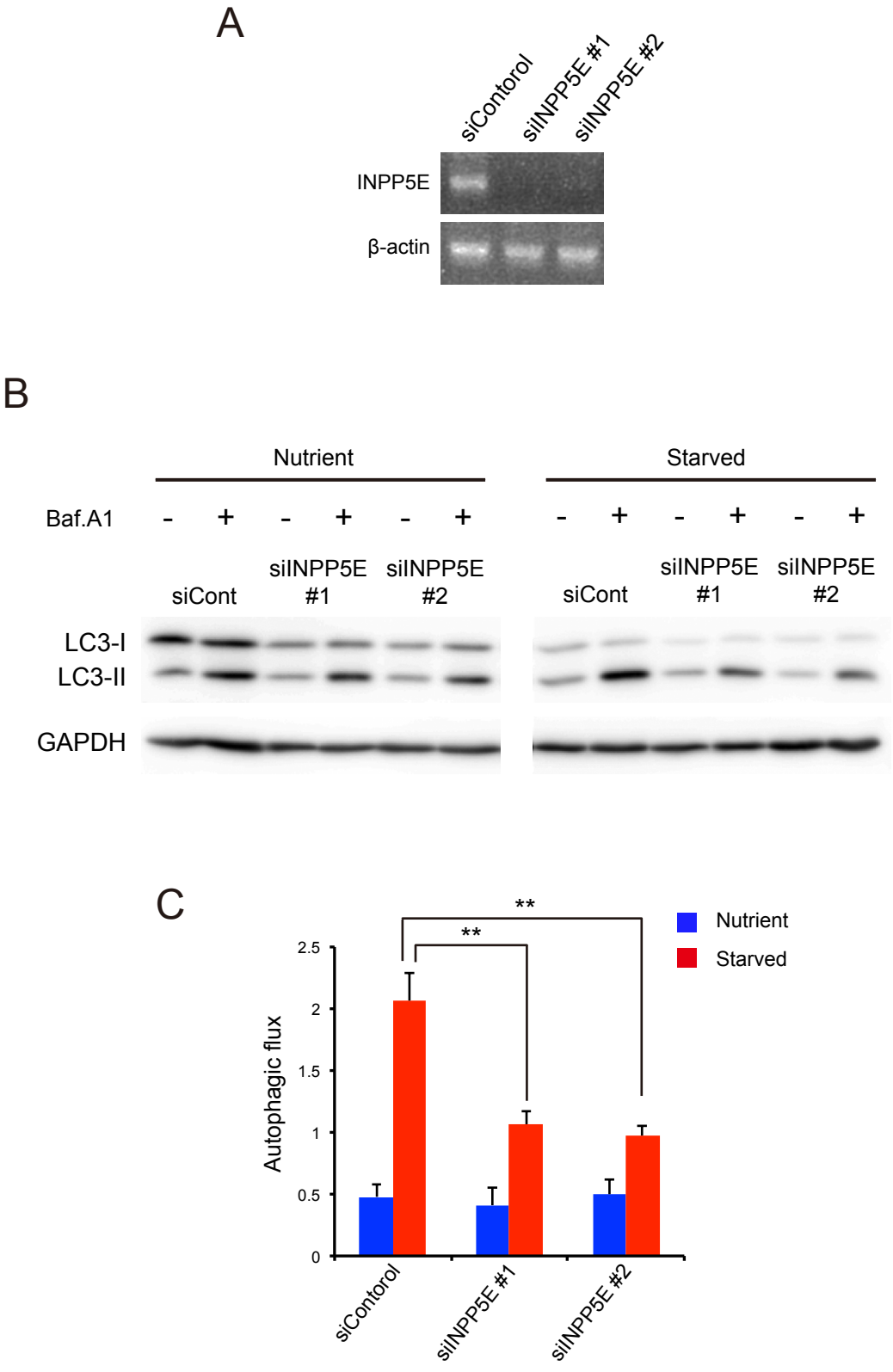

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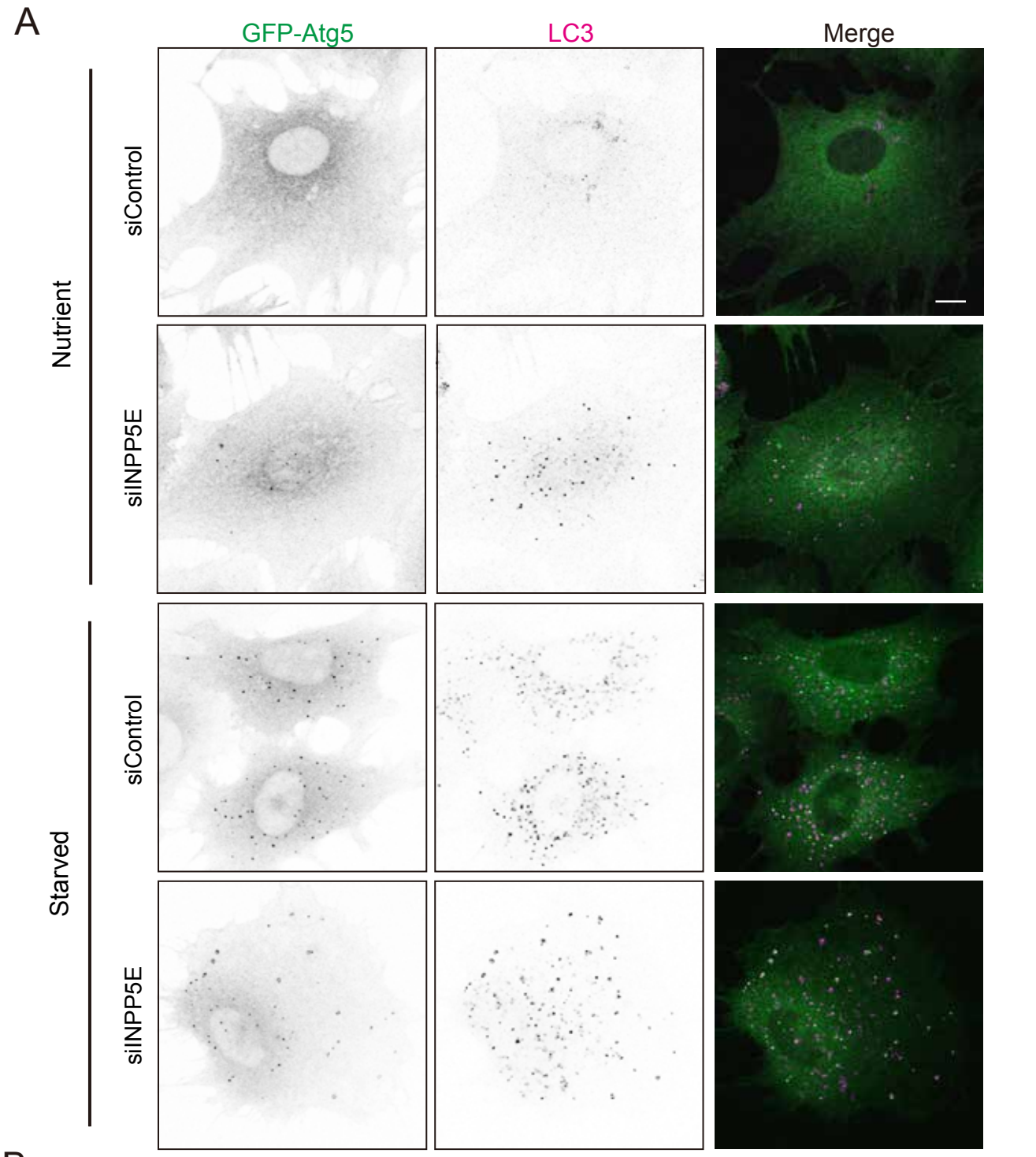

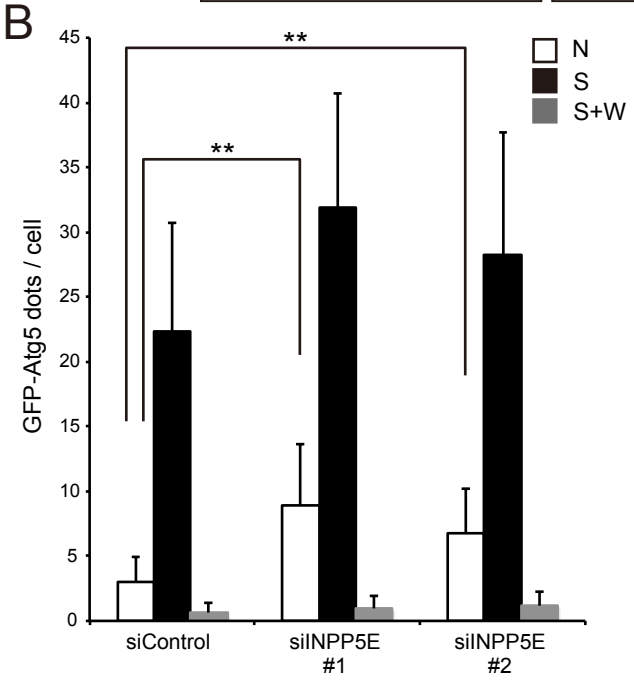

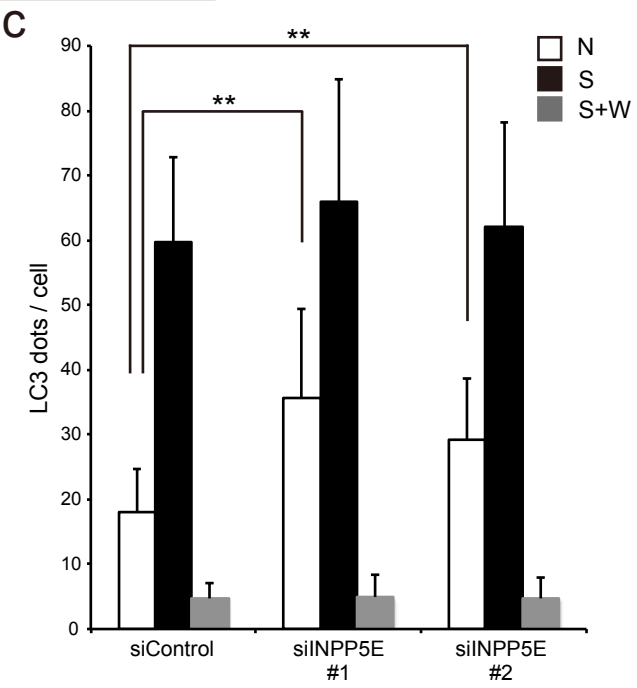

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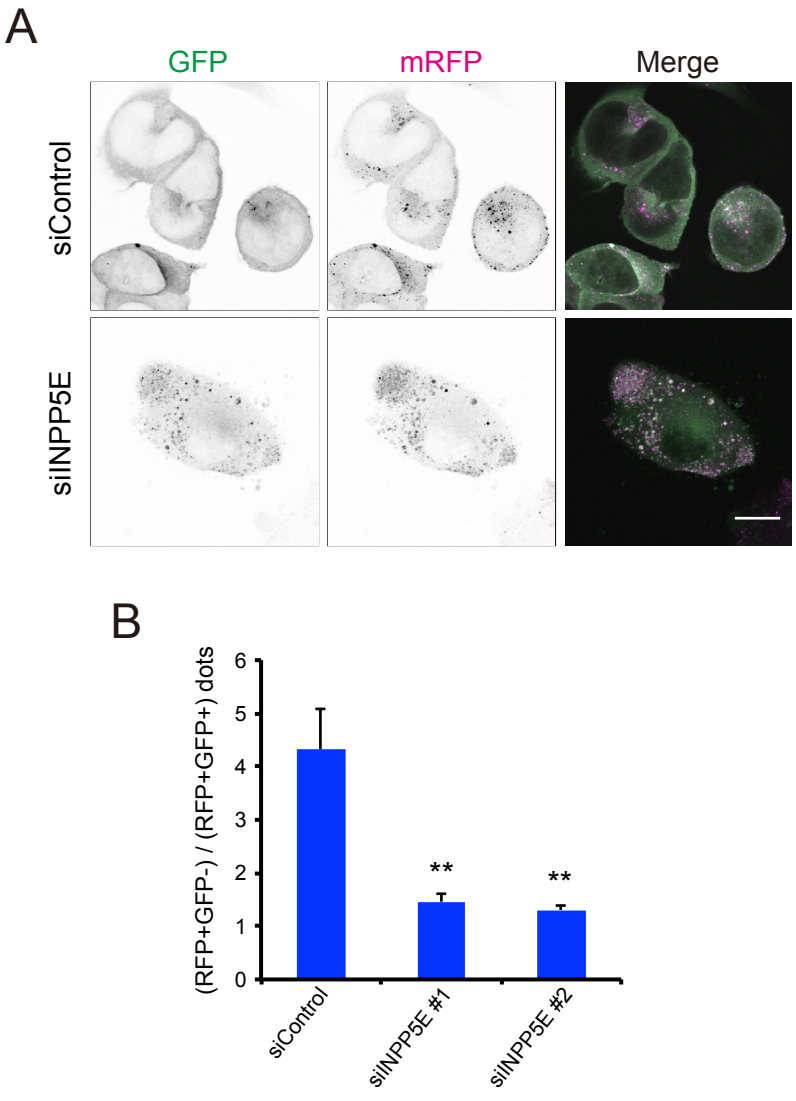

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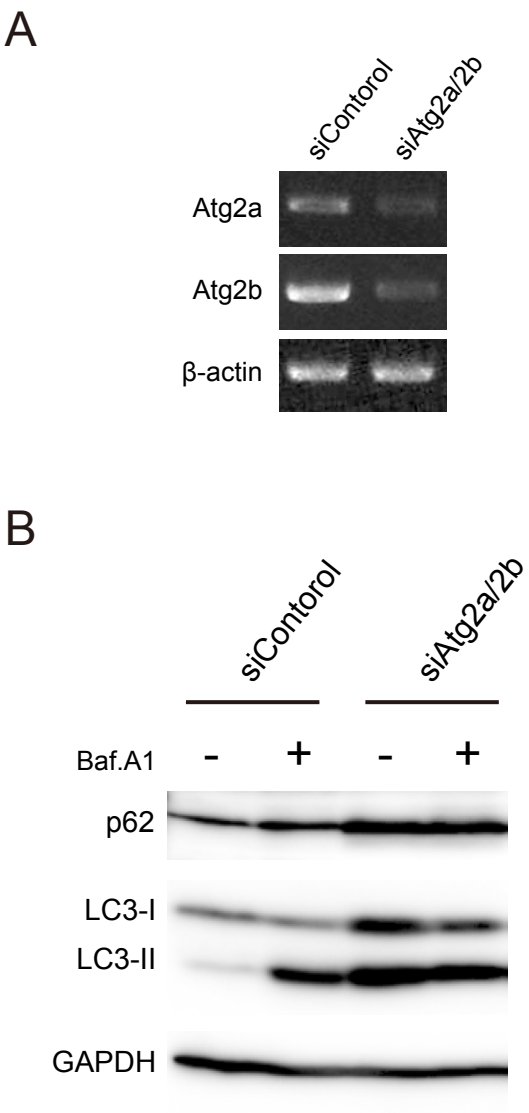

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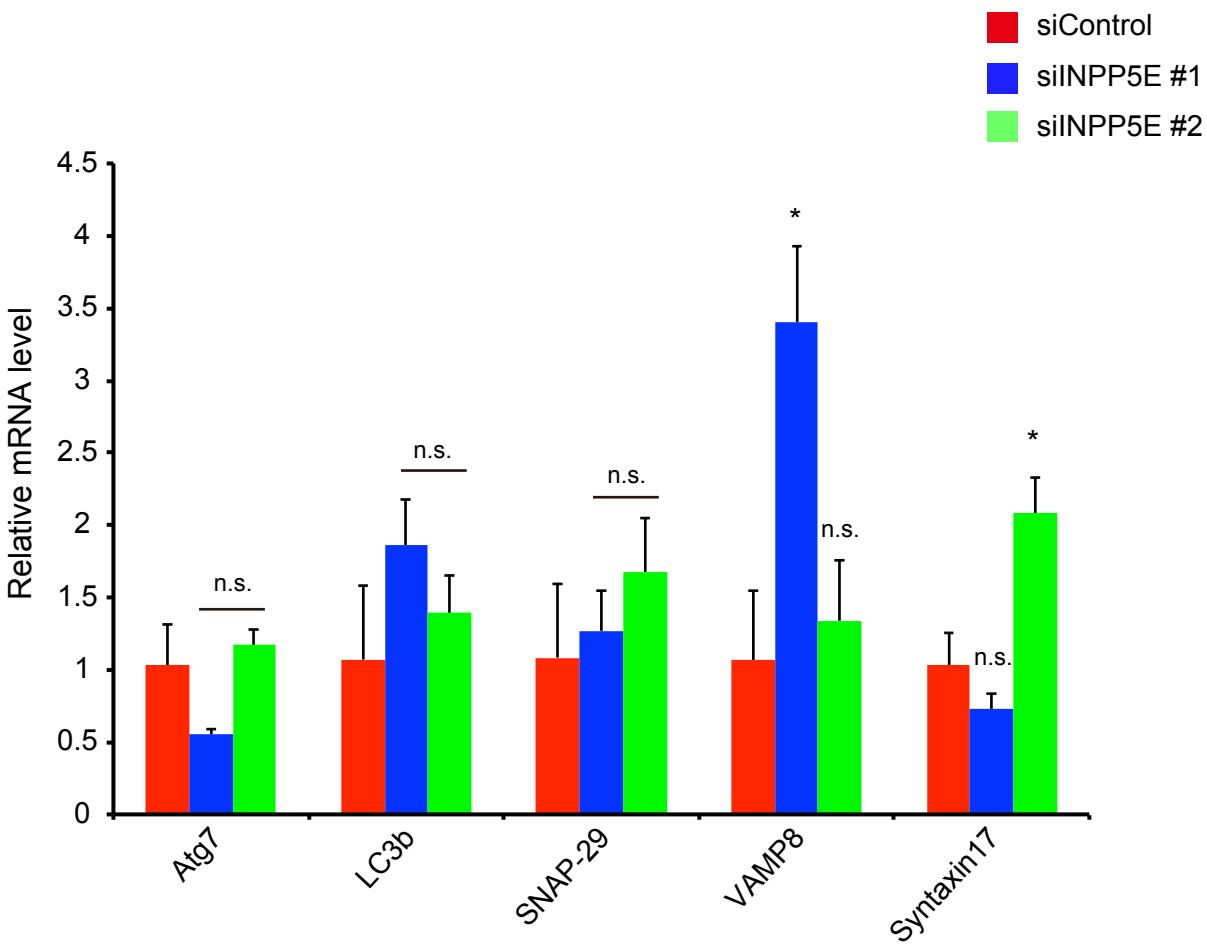

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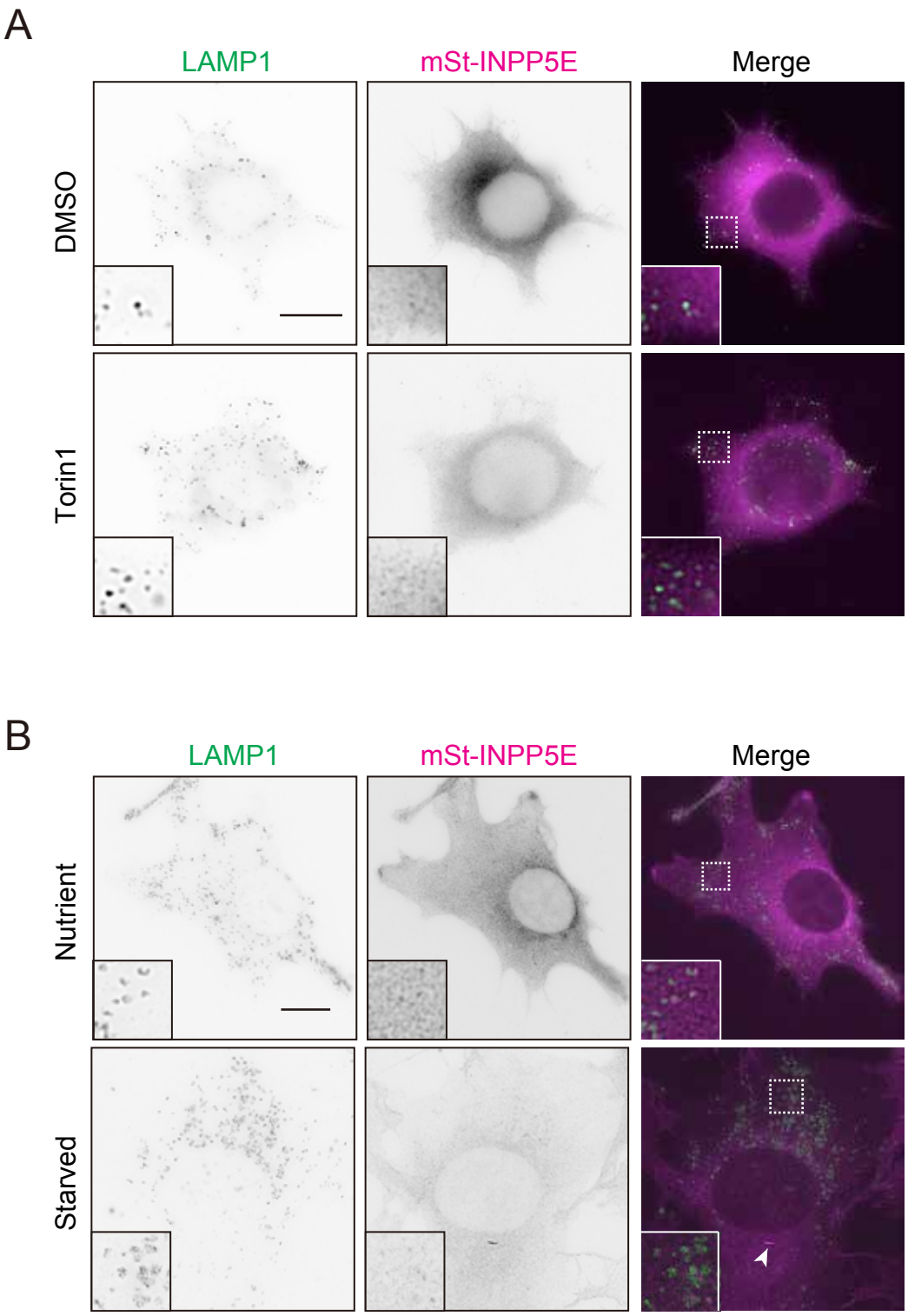

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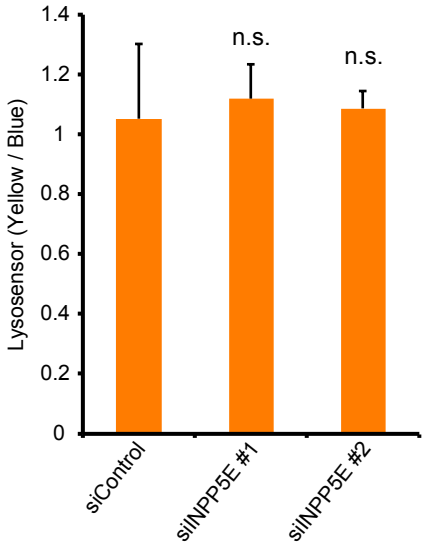

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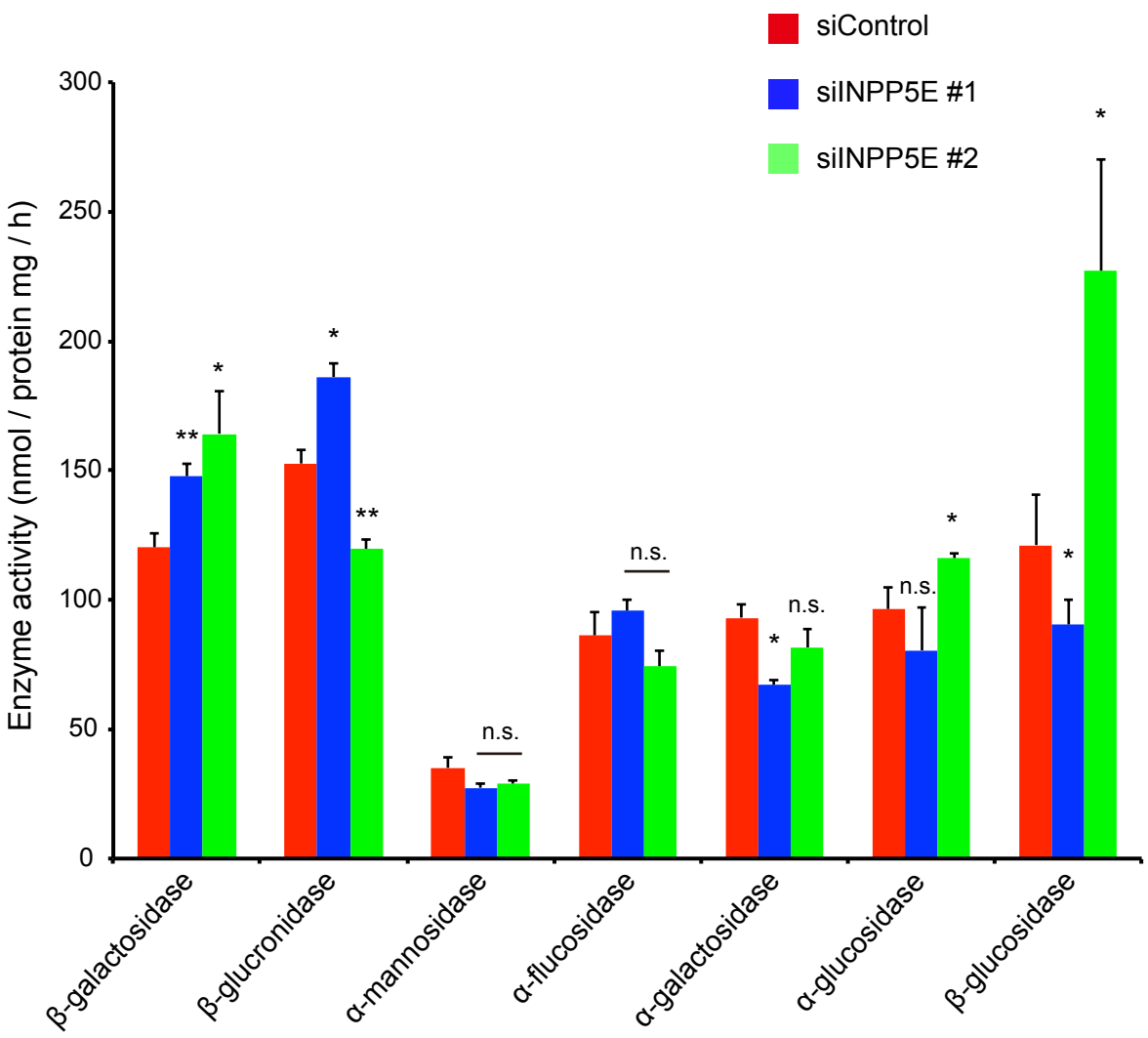

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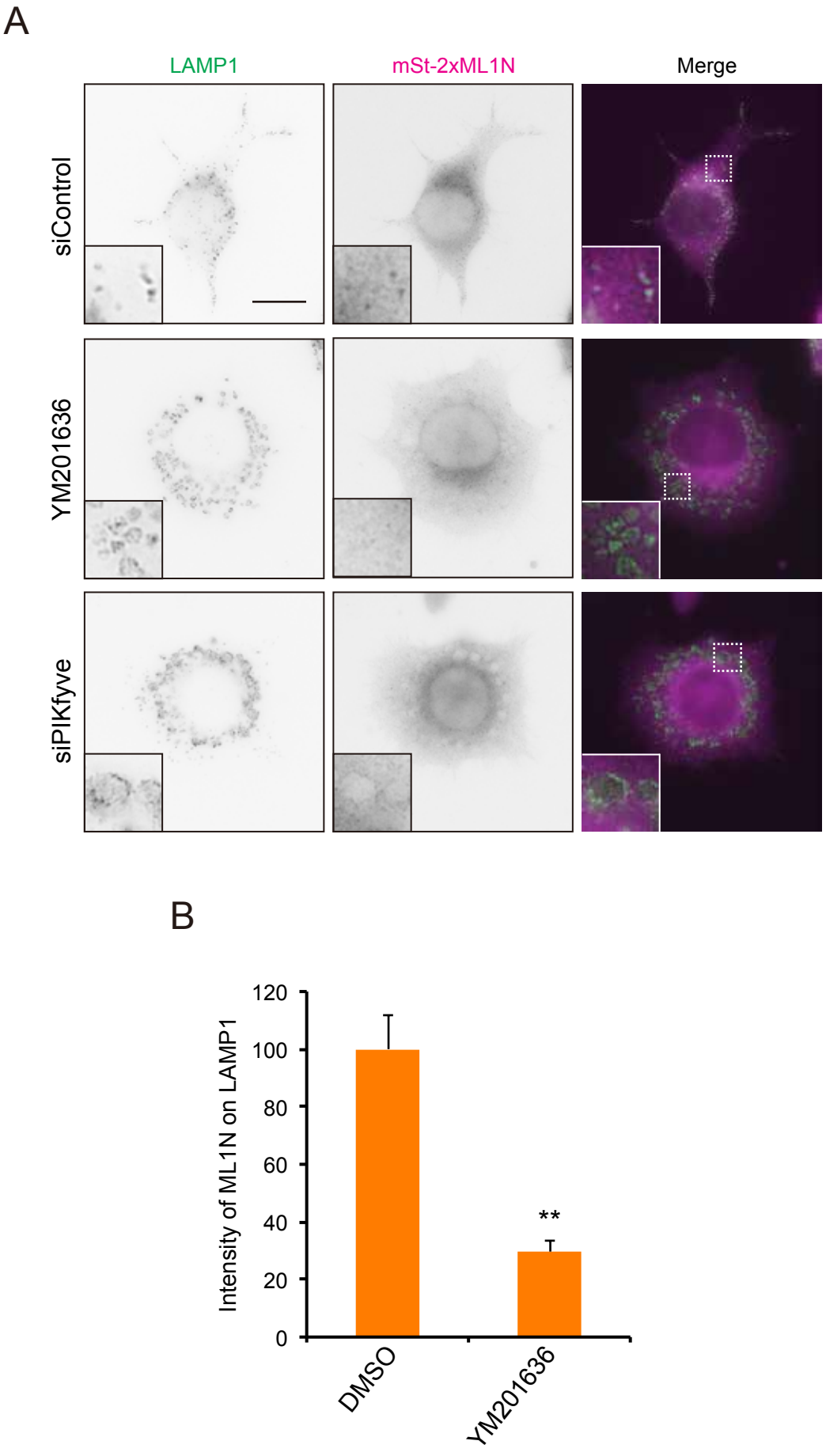

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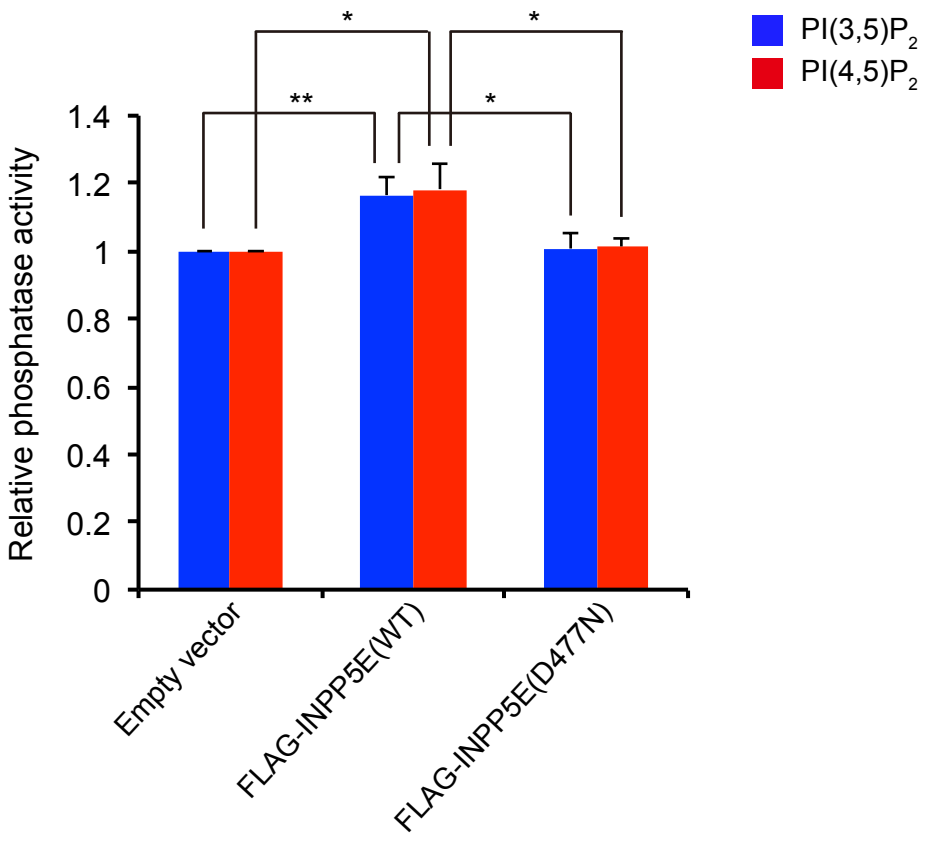

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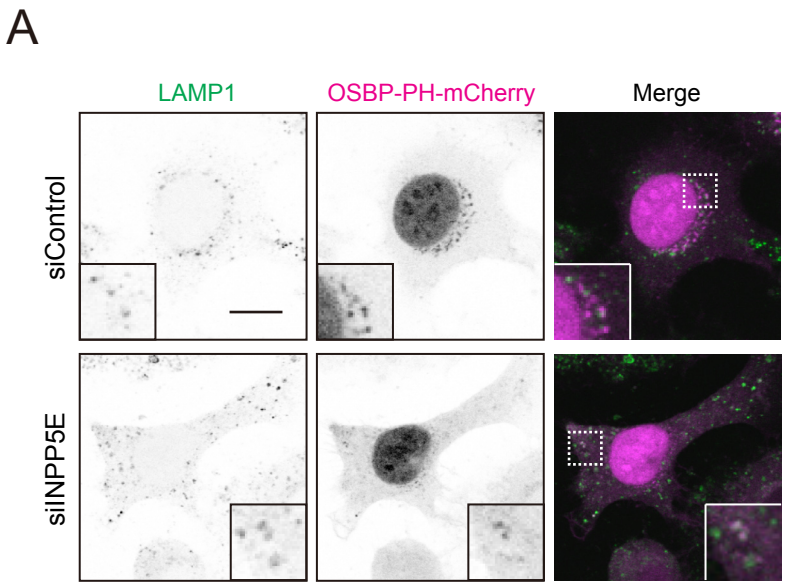

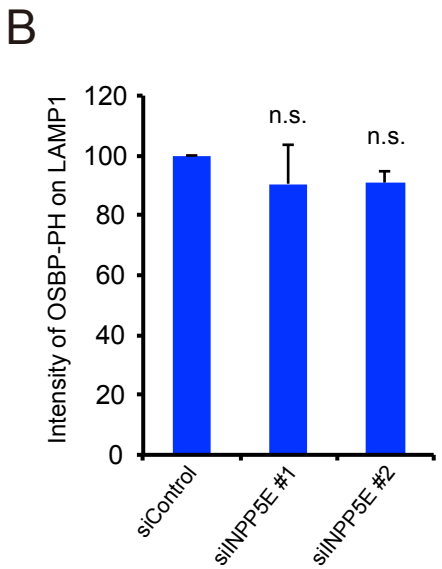

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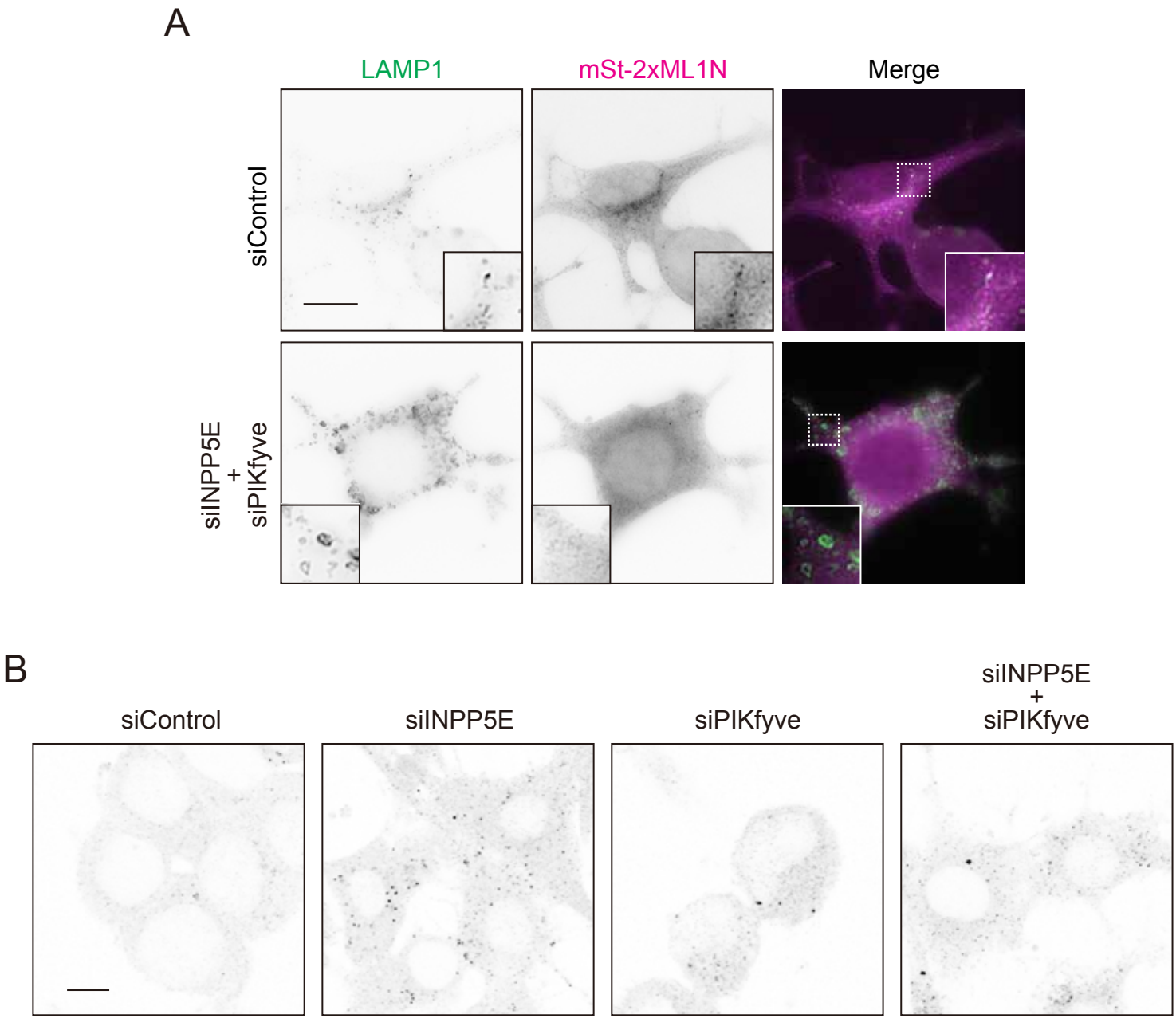

Appendix Figure S14