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Supplemental Material to:

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Discovery of a novel type of autophagy targeting RNA

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Supplementary Information

Materials and Methods

Calculation of the identity levels of amino acid sequences

The identity levels of the cytosolic sequences of fly and nematode LAMPs to those of human LAMP2s were calculated using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Antibodies and peptides

The following primary antibodies were used: rat monoclonal anti-HA (3F10, Roche, 11867423001), mouse monoclonal anti-HNRNPA1 (F-8, Santa Cruz, sc-365486), rabbit polyclonal anti-RPL8 (Lifespan, LS-C31782), monoclonal rabbit anti-RPS6 (Cell Signaling, 2217), rabbit polyclonal anti-SNCA/α-synuclein (Chemicon, AB5038), mouse monoclonal anti-GAPDH (6C5, Chemicon, MAB374), and mouse monoclonal anti-ACTB/β-actin (AC15, Sigma, A5441). The doublet observed on the immunoblot with anti-HNRNPA1 antibody corresponds to HNRNPA1.[1]

The rabbit polyclonal anti-LAMP2C antibody was raised in rabbit against synthetic peptides (CGRRKSRTGYQSV and CSYMIGRRKSRTGYQSV) containing an amino acid sequence corresponding to the cytosolic region of LAMP2C. The specificity of the anti-LAMP2C antibody was confirmed as shown in **Fig. S2**.

Biotin-conjugated peptides were synthesized by Invitrogen as a custom service. The sequences of the peptides containing the cytosolic sequence of *C. elegans* LMP-1 or Drosophila LAMP-1 are as follows. *C. elegans*: [Biotin]-GSRARAKRQGYASV; Drosophila:

[Biotin]-GSRRRSTSRGYMSF.

Construction of plasmids

pCI-neo-LAMP2A plasmid was prepared as described previously.[2] Human *RPL8* cDNA was purchased from Kazusa DNA Research Institute (FHC08670), and human *HNRNPA*1 and *LAMP2B* cDNAs were purchased from Open Biosystems. *Lamp2c* cDNA was generated from total RNA from mouse brain. For construction of pCI-neo-*LAMP2B* and pCI-neo-*Lamp2c* vectors, each cDNA was amplified by PCR, and the PCR products were subcloned into pCI-neo mammalian expression vector (Promega, E1841) using XhoI and NotI sites. pCI-neo-*HNRNPA1*-HA for expressing HNRNPA1 with an HA tag at the C-terminus was also constructed in PCI-neo. The primers used were as follows. For *LAMP2B*: 5'-AAA ACT CGA GCC GCC ACC ATG GTG TGC TTC CGC CTC TTC C-3' and 5'-AAA AGC GGC CGC TTA CAG AGT CTG ATA TCC AGC ATA AT-3'. For *Lamp2c*: 5'-AAA ACT CGA GCC GCC ACC ATG TGC CTC TCT CCG GTT AAA GGC-3' and 5'-AAA AGC GGC CGC TTA GAC AGA CTG ATA ACC AGT ACG AC-3'. For *HNRNPA1*: 5'-AAA ACT CGA GCC GCC ACC ATG TCT AAG TCA GAG TCT CCT AAA G-3' and 5'-AAA AGC GGC CGC TTA AGC GTA ATC TGG AAC ATC GTA TGG GTA AAA TCT TCT GCC ACT GCC ATA GC-3'. For construction of a pGEX-*RPL8* plasmid, cDNA was amplified by PCR, and the PCR products were subcloned into pGEX6P-1 (GE Healthcare, 28-9546-48) using EcoRI and XhoI sites. The following primers were used in this experiment. 5'-AAA AGA ATT CAT GGG CCG TGT GAT C-3' and 5'-AAA ACT CGA GCT AGT TCT CTT TCT C-3'.

For amino acid substitutions, mutations were introduced using the Quik-Change Mutagenesis Kit according to the manufacturer's instructions (Stratagene, 200518). All resulting

constructs were confirmed by sequencing.

Cell culture and transfection

HeLa, Neuro-2a and J774A.1 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, 12701-017) supplemented with 10% fetal bovine serum (Cell Culture Bioscience, 171012). Transient transfection with each vector was performed using Lipofectamine LTX with Plus Reagent (Invitrogen, 15338-100), according to the manufacturer's instructions.

Preparation of recombinant RPL8 protein

Recombinant RPL8 protein was prepared as previously described.[3] *Escherichia coli* BL21 was transformed with GEX6p-1-*RPL8*. Production of the protein was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. The cells were then incubated in 37°C. After 2 h of induction, the cells were harvested and lysed by sonication in PBS containing 1% Triton X-100 and protease inhibitors. GST-tagged RPL8 was purified using glutathione Sepharose 4B (GE Healthcare, 17-0756-01), and RPL8 was released from GST by digestion using PreScission Protease (GE Healthcare, 27-0843-01).

LAMP2-deficient mice

LAMP2-deficient mice, which lack expression of all isoforms of LAMP2,[4] were backcrossed to C57BL/6J mice for more than 11 generations. These mice were propagated at the National Institute of Neuroscience, National Center of Neurology and Psychiatry. All animal experiments were performed in strict accordance with the guidelines of the National Institute of

Neuroscience, National Center of Neurology and Psychiatry, and were approved by the Animal Investigation Committee of the Institute.

Cell and brain lysis

HeLa cells were harvested and lysed on ice in 1% Triton X-100 lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitors (Complete, EDTA free; Roche Applied Science, 1873580). For the experiments shown in **Fig. 4E**, 40 to 48-week-old mouse brains were used. Each mouse brain was homogenized in 10 ml of the lysis buffer. Lysates were centrifuged at 17,700 × *g* for 10 min at 4°C, and the supernatants were pooled.

Pull-down assay

Pull-down assays were performed as previously described.[2] For assays using lysates of HeLa cells or brains, cell lysate or 10- to 12-week-old mouse brain lysate containing 3 mg of proteins were prepared, and incubated with 8 nmol of biotin fusion peptides and 40 μl of Streptavidin–Sepharose (GE Healthcare, 17-5113-01). After 2 h of incubation at 4°C, beads were washed three times with lysis buffer. Pulled-down proteins were eluted with SDS-PAGE sample buffer (10 mM Tris, pH 7.8, 3% SDS, 5% glycerol, 0.02% bromophenol blue and 2% 2-mercaptoethanol), and analyzed by SDS-PAGE followed by silver staining, Coomassie brilliant blue (CBB) staining or immunoblotting. RNA pulled down with the beads was extracted using phenol-chloroform, and analyzed by electrophoresis in agarose gels containing ethidium bromide.

For assays using purified RNA, Streptavidin–Sepharose beads were blocked with 3%

bovine serum albumin for 15 h. Ten micrograms of purified total RNA was incubated with 8 nmol of biotin fusion peptides and 40 μl of the beads in PBS containing 0.05% Triton X-100. After 2 h of incubation at 4°C, beads were washed three times with PBS containing 0.05% Triton X-100. RNA pulled down with the beads was extracted using phenol-chloroform or TRIzol (Life Technologies, 15596-018), and analyzed by electrophoresis in agarose gels containing ethidium bromide.

In some experiments, lysates were preincubated with RNase A (200 μg/ml in final concentration) for 1 h before the peptides, beads and lysates were mixed.

Immunoblotting

SDS-PAGE was performed under reducing conditions. Immunoblotting was performed according to standard procedures. After overnight incubation with primary antibodies at 4°C, each blot was probed with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive signals were visualized with SuperSignal West Dura extended duration substrate (Pierce, 34076) or SuperSignal West Femto extended duration substrate (Pierce, 34095) and detected using a chemiluminescence imaging system (FluorChem; Alpha Innotech). The signal intensity was quantified by densitometry using FluorChem software (Alpha Innotech).

Primary culture of neurons and glial cells

Primary culture of neurons and glial cells was performed according to standard procedures. For neurons, cerebral cortices were dissected from pups of a C57BL/6J mouse at embryonic day 14. Cortices were mechanically dissociated by trituration and trypsinized at 37°C

in 0.25 % trypsin- 1 mM EDTA. One microliter of trypsin inhibitor (3.5 BAEE units/μl, GIBCO, 17075029) and 1 ml of 1 mg/ml DNase I were added after 5 min, and centrifuged for 3 min at 4°C (190 × *g*). The supernatant was removed, and the cells were plated on culture dishes coated with 10 μg/ml poly-D-lysine. Cells were incubated at 37°C with 5% CO_2 in Neurobasal Medium (GIBCO, 21103-049500) supplemented with 0.5 mM L-glutamin, 2% B27 supplement (GIBCO, 0050129SA), and 100 units/ml penicillin-streptomycin.

For glial cells, cerebral cortices were dissected from the pups of a C57BL/6J mouse at postnatal day 1 to 2. Cortices were mechanically dissociated by trituration and trypsinized at 37 °C in 0.25% trypsin-1 mM EDTA. Afer 5 min, 10% ES Cell Qualified Fetal Bovine Serum (FBS; GIBCO, 16141) and 1 ml of 1 mg/ml DNase I were added; then, cells were filtered using a 70-μm cell strainer (Falcon, 352350) and centrifuged for 3 min at 4°C (427 × *g*). The supernatant was removed, and the cells were plated on 10-cm dishes at a density of 5×10^6 cells/dish with Dulbecco's modified Eagle's medium containing 10% FBS. Cells were incubated at 37°C with 5% CO_2 for 7 days and then detached in 0.25% trypsin-1 mM EDTA and reseeded in 150-cm^2 bottles (Techno Plastic Products, 90151) at a density of 1×10^7 cells/bottle. After a further 7 days, these mixed glial cultures were shaken at 80 rpm for 5 min at 37°C to eliminate non-astrocytic cells (referred to as "mixed non-astrocytic cultures"). The 150-cm^2 bottles were shaken again at 250 rpm for 16 h at 37°C to eliminate any remaining oligodendrocytes. Astrocytes adherent to the bottles were trypsinized and reseeded in culture dishes with DMEM containing 10% FBS. After 48 h, the medium was changed to DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin-streptomycin, and 1% N2 supplement (GIBCO, 17502-048). The mixed non-astrocytic cultures eliminated from the 150-cm^2 bottles were plated on culture dishes with medium for 30

min at 37°C to attach microglia to the dishes. The medium was retrieved and oligodendrocytes were plated on culture dishes coated with 10 μg/ml poly-D-lysine.

Purification of total RNA from mice brains

Total RNA was purified from 10 to 12-week-old mouse brains using an RNeasy Lipid Tissue Mini Kit (QIAGEN, 74804) according to the manufacturer's instructions.

Quantitative real-time PCR analysis

Real-time PCR was carried out as previously described.[5] Total RNA was prepared from 10 to 12-week-old mouse tissues or cultured cells, and cDNA was synthesized using a Quantitect Reverse Transcription Kit (QIAGEN, 205313). Relative levels of mRNA expression were measured by real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, 4344463) and Mx3000P (Stratagene). Primers specific for mouse *Lamp2c* (forward: 5'-AAA GAG CAG GTG CTT TCT GTG-3'; reverse; 5'-ACA CCC ACT GCA ACA GGA AT-3') were used. *Actb/β-actin* was amplified with each sample for normalization. Primers used in a quantification of *Actb* levels were as follows. Forward: 5'-CGT GCG TGA CAT CAA AGA GAA-3'; reverse: 5'-CAA TAG TGA TGA CCT GGC CGT-3'.

Mass spectrometry analysis

Protein bands in the molecular weight range 30 to 50 kDa, which interacted with LAMP2C peptide in a pull-down assay using HeLa cells, were analyzed by LC-MS/MS at Shimadzu Techno-Research, Inc. as a custom service. The identified proteins are shown in **Table**

S1.

All protein samples detected in a pull-down assay using brain lysate were analyzed by LC-MS/MS at Oncomics Co., Ltd as a custom service. The identified proteins in control samples were subtracted from the identified proteins in the sample pulled down with LAMP2C peptide. Identified proteins with a Mascot score over 150 are shown in **Table S1**.

Uptake and degradation of RNA and proteins by isolated lysosomes

Isolation of lysosomes and monitoring of CMA were performed as described.[6] Briefly, lysosomes were isolated from 10 to 12-week-old mouse brains using a Lysosome Enrichment Kit (Pierce, 89839). Lysosomes were preincubated on ice in 0.3 M sucrose with or without 10 mM chymostatin for 10 min, and then incubated with 5 μg of purified SNCA/α-synuclein (ATGen, SNA2001), ovalbumin (Albumin from chicken egg white, Sigma, A7641) or RPL8 for 20 min at 37°C in 30 μl of 0.3 M sucrose containing 10 mM MOPS buffer (pH 8.0), energy regenerating system (10 mM ATP, 10 mM $MgCl_2$, 2 mM phosphocreatine, and 50 μg/ml creatine phosphokinase) and 1 μg of active HSPA8 (Stress Marq, SPR-106). After the incubation, lysosomes were precipitated, and proteins remaining with lysosomes were detected by immunoblotting. The uptakes of the proteins were detected as difference of protein levels between chymostatin-treated and untreated samples.[7, 8]

For monitoring RNA uptake, isolated lysosomes (25 to 50 μg of protein) were incubated with 5 or 10 μg of purified total RNA at 37°C for 5 or 20 min in 30 μl of 0.3 M sucrose containing 10 mM MOPS buffer (pH 8.0) with or without energy regenerating system (ATP- or ATP+). In some experiments, 1 μg of active HSPA8 was added in the solution. After the

incubation, lysosomes were precipitated by centrifugation and RNA remaining in the supernatant was analyzed by electrophoresis in agarose gels containing ethidium bromide. RNA in agarose gels was detected using FluorChem, and the levels of RNA were quantified by densitometry using FluorChem software. To assess the presence of uptaken RNA inside lysosomes, RNA outside of lysosomes was degraded by 200 μg of RNase A after the incubation. RNase A treatment was carried out for 20 min on ice; then, RNA was extracted using TRIzol. Levels of RNase A-resistant RNA were analyzed in agarose gels. In a time course analysis, isolated lysosomes were incubated with 10 μg of total RNA for 0 to 20 min as indicated in **Fig. 3C**.

Degradation of RNA by isolated lysosomes was investigated as follows. Isolated lysosomes and 5 μg of total RNA were incubated with or without energy regenerating system for 5 min, and the total levels of RNA in the samples were analyzed.

For assays using HeLa cells, lysosomes were isolated from ~1.4×10^8 cells transfected with pCI-neo-*Lamp2c* or empty vector, and subjected to analysis as described above.

Measurement of RNA turnover in HeLa cells

RNA turnover in HeLa cells was measured as described,[9] with slight modifications. Briefly, 7.0×10^4 cells/well of HeLa cells were incubated in 24-well dishes (BD Falcon, 353047) 24 h before transfection, and 0.2 μg/well of plasmids was transfected into these cells. To prelabel RNA, 0.3 uCi/ml of [^{3}H]uridine was added to the cells 24 h after the transfection. After 24 h of labelling, cells were washed with 500 μl of medium containing 5 mM non-labelled uridine, and then incubated in another 500 μl of the same medium (time zero). After 6 h of incubation, media were collected and floating cells were removed by centrifugation (900 × *g*). Then, acid-soluble

radioactivity in medium was measured using Tri-Carb 3100TR (Packard).

At time zero, the cells were trypsinized and acid-insoluble radioactivity was measured to access levels of labelled RNA in cells. We confirmed that, at time zero, overexpression of each LAMP2 had no significant effect on levels of labelled RNA (**Fig. S8**).

Quantitation of total RNA in mice brains

Total RNA was extracted from 40 to 48-week-old mouse brains using TRIzol Reagent according to the standard procedure. The levels of RNA were then quantified by measuring OD_{260} values, normalized by brain weight, and these were compared between WT and KO littermates.

Electron microscopy

Isolated lysosomes were fixed in 0.1% glutaraldehyde and 4% paraformaldehyde in PBS for 16 h at 4°C, and post-fixed with 1% osmium for 1 h at room temperature. Samples were then dehydrated in a series of water/ethanol mixtures to 100% ethanol, and embedded in Epon (TAAB, 3402). The embedded samples were sectioned at 60 nm, collected on 400-mesh copper grids, and viewed on a Tecnai Spirit transmission electron microscope (FEI, Hillsboro, OR) at 120 kV.

For immunogold electron microscopy, isolated lysosomes were incubated with or without purified total RNA in the presence of ATP (energy regenerating system) for 5 min, precipitated by centrifugation, and fixed in 0.1% glutaraldehyde and 4% paraformaldehyde in PBS for 16 h at 4°C. Samples were then dehydrated in a series of water/ethanol mixtures to 100% ethanol, and embedded in LR White (Nisshin EM Co., Ltd., 3962). The embedded samples were

sectioned at 100 nm, and collected on 400-mesh nickel grids. Immunogold labelling was performed using an anti-rRNA antibody (1:50, Y10b, Abcam, ab37144) followed by anti-mouse IgG coupled with 10 nm of gold particles, and viewed using a Tecnai Spirit transmission electron microscope (FEI) at 80 kV. The Y10b antibody (anti-rRNA antibody) specifically recognizes several different rRNAs from diverse species, including 28S, 18S and 5.8S rRNAs.[10]

Statistical analyses

For comparisons between two groups, the statistical significance of differences was determined by Student's t test. For comparisons of more than two groups, Dunnett's multiple comparison test was used. * and ** indicate $P < 0.05$ and $P < 0.01$, respectively.

Supplementary References

1. Raffalli-Mathieu F, Glisovic T, Ben-David Y, Lang MA. Heterogeneous nuclear ribonucleoprotein A1 and regulation of the xenobiotic-inducible gene Cyp2a5. Mol Pharmacol 2002; 61:795-9.

2. Kabuta T, Furuta A, Aoki S, Furuta K, Wada K. Aberrant interaction between Parkinson disease-associated mutant UCH-L1 and the lysosomal receptor for chaperone-mediated autophagy. J Biol Chem 2008; 283:23731-8.

3. Kabuta T, Setsuie R, Mitsui T, Kinugawa A, Sakurai M, Aoki S, et al. Aberrant molecular properties shared by familial Parkinson's disease-associated mutant UCH-L1 and carbonyl-modified UCH-L1. Hum Mol Genet 2008; 17:1482-96.

4. Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R, et al.

Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. Nature 2000; 406:902-6.

5. Kodomari I, Wada E, Nakamura S, Wada K. Maternal supply of BDNF to mouse fetal brain through the placenta. Neurochem Int 2009; 54:95-8.

6. Ali AB, Nin DS, Tam J, Khan M. Role of chaperone mediated autophagy (CMA) in the degradation of misfolded N-CoR protein in non-small cell lung cancer (NSCLC) cells. PLoS One 2011; 6:e25268.

7. Aniento F, Roche E, Cuervo AM, Knecht E. Uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase by rat liver lysosomes. J Biol Chem 1993; 268:10463-70.

8. Kaushik S, Cuervo AM. Methods to monitor chaperone-mediated autophagy. Methods Enzymol 2009; 452:297-324.

9. Sameshima M, Liebhaber SA, Schlessinger D. Dual pathways for ribonucleic acid turnover in WI-38 but not in I-cell human diploid fibroblasts. Mol Cell Biol 1981; 1:75-81.

10. Garden GA, Hartlage-Rubsamen M, Rubel EW, Bothwell MA. Protein masking of a ribosomal RNA epitope is an early event in afferent deprivation-induced neuronal death. Mol Cell Neurosci 1995; 6:293-310.

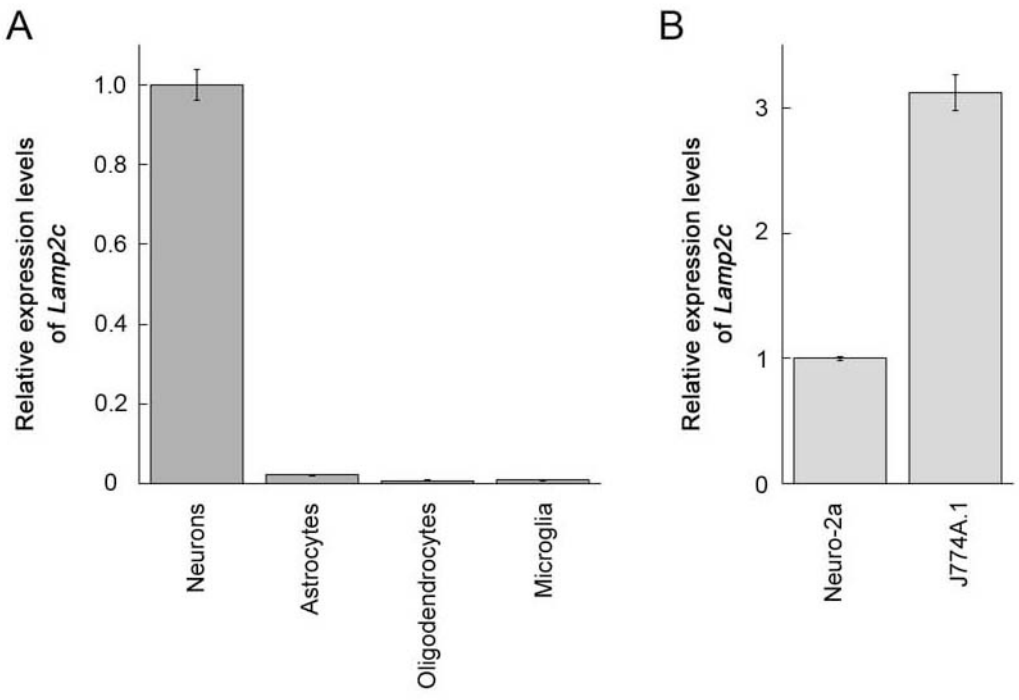

Figure S1. Expression levels of *Lamp2c* mRNA in various types of cells.

(A and B) Total RNA was prepared from primary cultured neurons, astrocytes, oligodendrocytes and microglia (A), or from Neuro-2a cells (neuronal cell line) and J774A.1 cells (macrophage cell line) (B). Relative levels of *Lamp2c* mRNA were measured by quantitative real-time PCR (n=2-3).

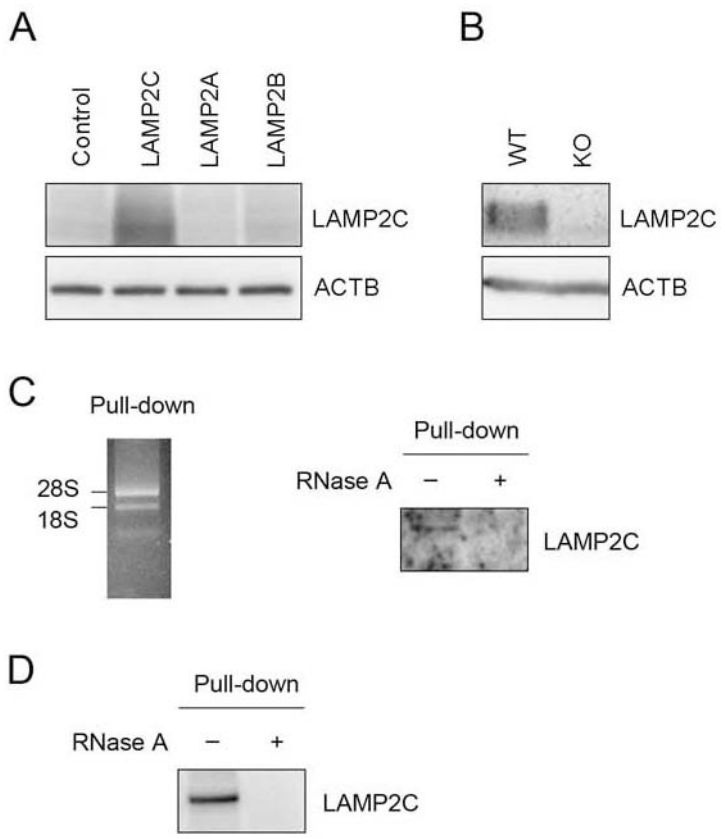

Figure S2. Interaction between endogenous LAMP2C and RNA

(A and B) Specificity of the anti-LAMP2C antibody. HeLa cells were transfected with the indicated constructs (control: empty vector). Cell lysates were prepared and immunoblotted using anti-LAMP2C and ACTB/β-actin antibodies (A). Brain lysates were prepared from WT and LAMP2 KO mice (6 weeks of age), and immunoblotted (B). (C) Brain lysate containing 4 mg/ml protein was prepared from a 12-week-old mouse. After 1 ml of lysate was incubated with or without RNase A (final concentration: 0.2 mg/ml), total RNA was pulled down using biotin-labelled random DNA nonamers (5 nmol/sample). RNA could be pulled-down by random DNA nonamers (left panel). Endogenous LAPM2C in the pulled-down samples was detected by

immunoblotting using an anti-LAMP2C antibody. (D) Brain lysate containing 4 mg/ml protein was prepared from a 12-week-old mouse. After 1 ml of lysate was incubated with or without RNase A (final concentration: 0.2 mg/ml), total RNA was pulled down using the same method as described in the legend for Fig. 2C with biotin-labelled peptide containing the cytosolic sequence of LAMP2C (8 nmol/sample), which is indicated in Fig. 1B. Endogenous LAMP2C in the pulled-down samples was detected by immunoblotting using an anti-LAMP2C antibody. In both (C) and (D), LAMP2C was detected in the pulled-down samples only in the absence of RNase A, indicating that endogenous LAMP2C interacts with RNA.

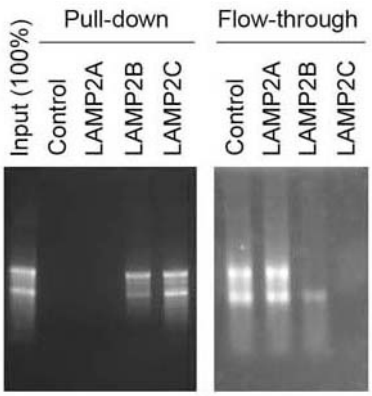

Figure S3. Interactions of purified total RNA with cytosolic sequence of LAMP2A, LAMP2B and LAMP2C.

A pull-down assay was performed using purified total RNA as described in Materials and Methods.

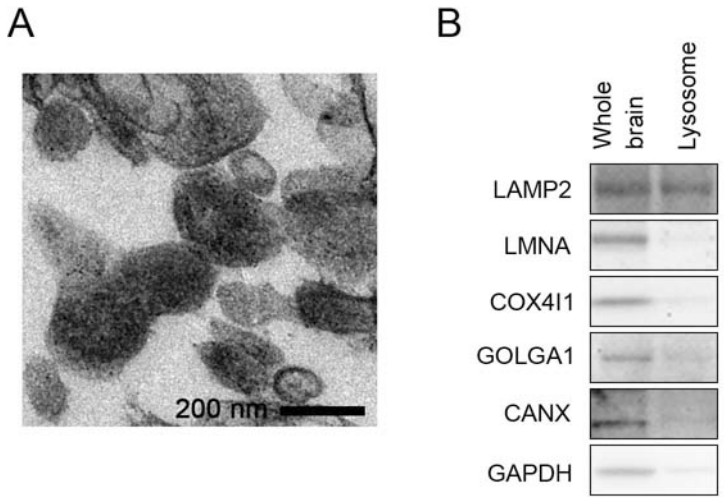

Figure S4. Ultrastructural and biochemical characterization of isolated lysosomes

(A) Ultrastructural analysis of isolated lysosomes. (B) Immunoblot of lysates prepared from whole brain or isolated lysosomes using different organelle markers: LAMP2 (lysosomal marker), LMNA/lamin A (nuclei), COX4I1/COX IV (mitochondrial marker), GOLGA1/Golgin-97 (Golgi apparatus marker), CANX/calnexin (endoplasmic reticulum marker), and GAPDH (cytosolic marker).

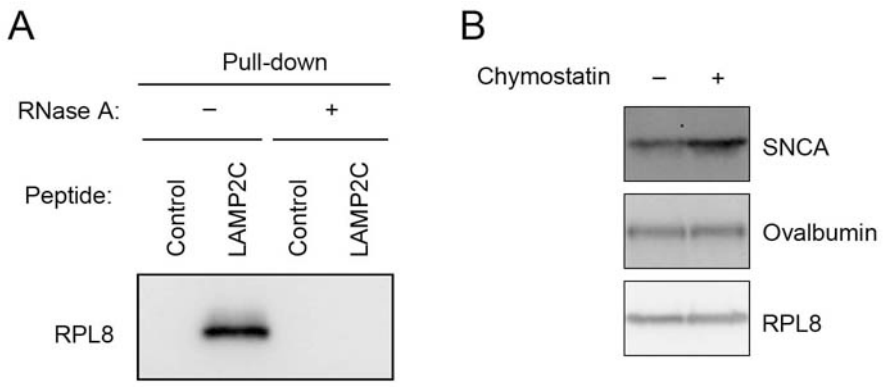

Figure S5. Uptake of proteins by isolated lysosomes.

(A) Purified RPL8 was mixed with a biotin-labelled peptide matching the cytosolic tail of LAMP2C, and a pull-down assay was performed in the presence or absence of RNase A. RPL8 interacted with the peptide in the absence of RNase A, but not in the presence of RNase A, indicating that purified RPL8 contained RNA and interacted with the LAMP2C peptide through RNA. (B) Freshly isolated lysosomes were prepared from mouse brain, and a lysosomal uptake assay was performed using purified SNCA/α-synuclein, ovalbumin and RPL8. The lysosomal uptake of SNCA, but not of ovalbumin and RPL8, was detected, even though purified RPL8 interacts with LAMP2C peptide.

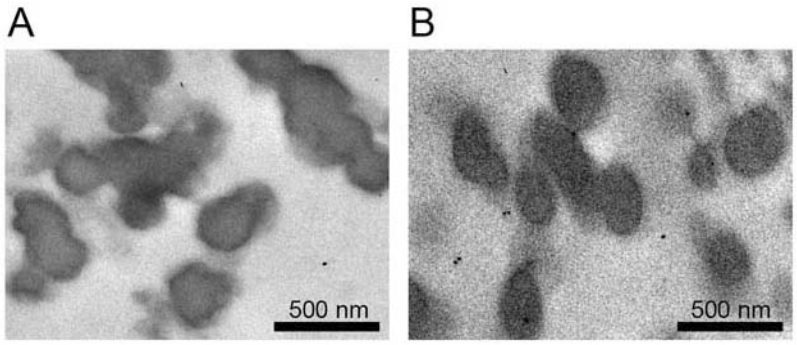

Figure S6. Control assays for immunogold electron microscopy.

(A) Isolated lysosomes were incubated with ATP in the presence of RNA, and then immunogold labelling was performed without primary antibody. No gold particles were observed in the lysosomes. (B) Isolated lysosomes were incubated with ATP in the absence of RNA, and then immunostained with an anti-rRNA primary antibody, followed by immunogold labelling. Virtually no gold particles were observed in the lysosomes.

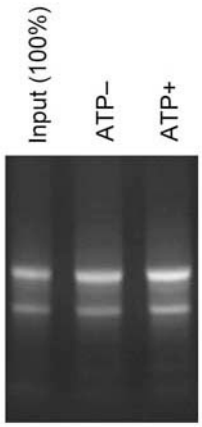

Figure S7. RNA was not degraded in the solution outside lysosomes incubated either with or without ATP.

Isolated lysosomes were incubated with or without ATP for 5 min at 37°C. The lysosomes were removed by centrifugation, and then the solution outside the lysosomes was incubated with 10 μg of RNA for 5 min at 37°C. RNA was analyzed by electrophoresis in agarose gels containing ethidium bromide.

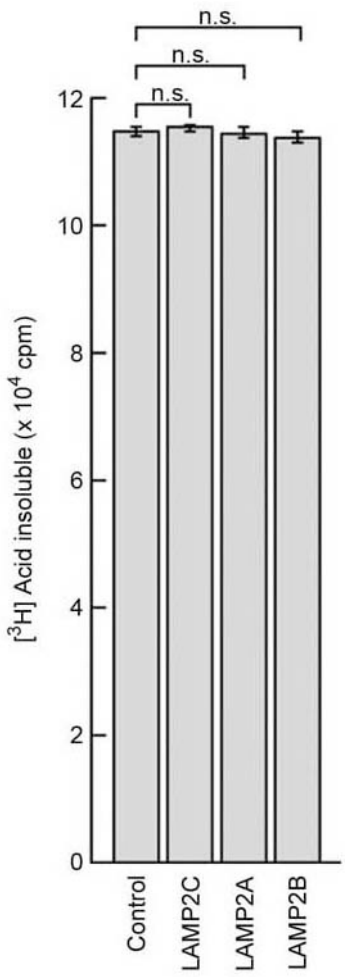

Figure S8. Acid-insoluble radioactivity in HeLa cells.

HeLa cells transfected with each LAMP2 isoform or empty vector. Cells were labelled with [^{3}H]uridine for 24 h, and then acid-insoluble radioactivity in cells was measured (n=3).

Table S1. LC-MS/MS analysis of proteins that interacted with the cytosolic sequence of LAMP2C.

GenInfo Identifier (GI) and protein names are shown.

HeLa cells

Accession number	Protein name
gi|4506661	RPL7A, ribosomal protein L7a
gi|4506667	RPLP0, 60S acidic ribosomal protein P0
gi|14591909	RPL5, ribosomal protein L5
gi|12653649	RPL14, ribosomal protein L14
gi|36102	HNRNPA1, heterogenous nuclear ribonucleoprotein A1
gi|356168	HIST1H1B, histone H1b
gi|337930	RPS4X, ribosomal protein S4e
gi|35903	RPL7, ribosomal protein L7
gi|4506743	RPS8, ribosomal protein S8
gi|825671	NPM1, B23 nucleophosmin (280 AA)
gi|4885381	HIST1H1B, histone H1.5
gi|337580	RPL3, ribosomal protein L3
gi|337457	LARP1, ribonucleoprotein La
gi|306553	RPS3A, ribosmal protein S3A

gi|16579885	RPL4, ribosomal protein L4
gi|4506663	RPL8, ribosomal protein L8
gi|337514	RPS6, ribosomal protein S6
gi|3088335	RPS2, ribosomal protein S2
gi|306875	HNRNPC, heterogenous nuclear ribonucleoprotein C
gi|531171	RPL10A, ribosomal protein L10a

Mouse brain

Accession number	Protein name	Group
gi|56205529	CLTC, clathrin, heavy polypeptide (Hc)	Other
gi|74177681	NCL, unnamed protein product (nucleolin)	RNA-binding
gi|695638	RPL6, M-TAXREB107	Ribosomal
gi|6679573	PURA, transcriptional activator protein Pur-alpha	DNA-binding
gi|6671569	RPLP0, 60S acidic ribosomal protein P0	Ribosomal
gi|200770	RPL7, ribosomal protein	Ribosomal
gi|74219852	HNRNPU, unnamed protein product (heterogeneous nuclear ribonucleoprotein U)	RNA-binding
gi|30794450	RPL4, 60S ribosomal protein L4	Ribosomal
gi|6755252	PURB, transcriptional activator protein Pur-beta	DNA-binding
gi|7305443	RPL7A, 60S ribosomal protein L7a	Ribosomal
gi|74185347	RPL3, unnamed protein product (ribosomal protein L3)	Ribosomal
gi|149265827	RPS3A, PREDICTED: 40S ribosomal protein S3a-like	Ribosomal
gi|12846287	RPL15, unnamed protein product (ribosomal protein	Ribosomal

	L15)	
gi|18087805	RPS2, 40S ribosomal protein S2	Ribosomal
gi|53754	PABPC1, poly(A) binding protein	RNA-binding
gi|4506725	RPS4X, 40S ribosomal protein S4, X isoform X isoform	Ribosomal
gi|4506743	RPS8, 40S ribosomal protein S8	Ribosomal
gi|6671561	AP2A1, AP-2 complex subunit alpha-1 isoform a	Other
gi|148696938	RPL18A, mCG14783, isoform CRA_a	Ribosomal
gi|31559916	HNRNPA3, heterogeneous nuclear ribonucleoprotein A3 isoform a	RNA-binding
gi|13592053	RPL10, 60S ribosomal protein L10	Ribosomal
gi|6755372	RPS3, 40S ribosomal protein S3	Ribosomal
gi|74222288	RPS3, unnamed protein product (ribosomal protein S3)	Ribosomal
gi|309263268	GM5045, PREDICTED: 60S ribosomal protein L7 isoform 4	Ribosomal
gi|13592009	RPL10A, 60S ribosomal protein L10a	Ribosomal
gi|14141193	RPS9, 40S ribosomal protein S9	Ribosomal
gi|6677809	RPS6, 40S ribosomal protein S6	Ribosomal
gi|21313640	AP2B1, AP-2 complex subunit beta isoform b	Other
gi|74226881	RPL8, unnamed protein product (ribosomal protein L8)	Ribosomal
gi|74139958	G3BP2, unnamed protein product (GTPase activating protein (SH3 domain) binding protein 2)	RNA-binding
gi|31560517	RPL27A, 60S ribosomal protein L27a	Ribosomal
gi|60334810	RPS16, Rps16 protein	Ribosomal
gi|16741485	RPL23A, Rpl23a protein	Ribosomal

gi|4506621	RPL26, 60S ribosomal protein L26	Ribosomal
gi|31981945	RPL13A, 60S ribosomal protein L13a	Ribosomal
gi|19111156	DHX30, putative ATP-dependent RNA helicase DHX30	RNA-binding
gi|309266869	RPL30, PREDICTED: 60S ribosomal protein L30-like	Ribosomal
gi|55562721	DDX5, Ddx5 protein	RNA-binding
gi|74182280	FXR2, unnamed protein product (fragile X mental retardation, autosomal homolog 2)	RNA-binding
gi|12836885	UPF1, nonsense mRNA reducing factor 1 NORF1	RNA-binding
gi|12846159	RPL14, unnamed protein product (ribosomal protein L14)	Ribosomal
gi|56205563	RPL26, ribosomal protein L26	Ribosomal
gi|12840700	RPL18, unnamed protein product (ribosomal protein L18)	Ribosomal
gi|244790087	MYEF2, myelin expression factor 2 isoform 1	RNA-binding
gi|22001904	RPL17, RecName: Full=60S ribosomal protein L17	Ribosomal
gi|21313308	HNRNPM, heterogeneous nuclear ribonucleoprotein M isoform a	RNA-binding
gi|74219241	SDHA, unnamed protein product (succinate dehydrogenase complex, subunit A, flavoprotein)	Other
gi|4506741	RPS7, 40S ribosomal protein S7	Ribosomal
gi|27502351	FAM164A, hypothetical protein LOC67306	DNA-binding
gi|6678143	SSB, lupus La protein homolog	RNA-binding
gi|244790095	MYEF2, myelin expression factor 2 isoform 3	RNA-binding
gi|148697602	RPS6, mCG22088	Ribosomal

gi|12836501	HP1BP3, unnamed protein product (heterochromatin protein 1, binding protein 3)	DNA-binding
gi|13435603	HNRNPR, Hnrpr protein	RNA-binding
gi|148671985	HNRNPA1, mCG15678, isoform CRA_c	RNA-binding
gi|14149647	RPL9, 60S ribosomal protein L9	Ribosomal
gi|4506681	RPS11, 40S ribosomal protein S11	Ribosomal
gi|149233872	RPL23A, PREDICTED: 60S ribosomal protein L23a-like	Ribosomal
gi|4504301	HIST2H4, histone H4	DNA-binding
gi|149263352	RPL21, PREDICTED: 60S ribosomal protein L21	Ribosomal
gi|4506701	RPS23, 40S ribosomal protein S23	Ribosomal
gi|899445	RPL13, 60S ribosomal protein	Ribosomal
gi|148688875	RPL21, mCG115628	Ribosomal
gi|66912162	HIST2H2BB, histone H2B type 2-F isoform a	DNA-binding
gi|83699420	RBMXL1, heterogeneous nuclear ribonucleoprotein G	RNA-binding
gi|198643	RPL19, ribosomal protein L19	Ribosomal
gi|4506703	RPS24, 40S ribosomal protein S24 isoform c	Ribosomal
gi|387207	HSPA2, heat shock protein	Other
gi|82918395	RPS13, PREDICTED: 40S ribosomal protein S13-like	Ribosomal
gi|6981488	RPS26, 40S ribosomal protein S26	Ribosomal
gi|198578	RPS18, ribosomal protein	Ribosomal
gi|817939	HIST2H2AA2, histone H2A	DNA-binding
gi|148694930	GM13509, mCG9691	Other
gi|4506619	RPL24, 60S ribosomal protein L24	Ribosomal
gi|148669266	FMR1, fragile X mental retardation syndrome 1	RNA-binding

	homolog, isoform CRA_a	
gi|6576815	SYNCRIP, synaptotagmin binding, cytoplasmic RNA interacting protein	RNA-binding
gi|13385044	RPL35, 60S ribosomal protein L35	Ribosomal
gi|148693239	ILF3, interleukin enhancer binding factor 3, isoform CRA_c	RNA-binding
gi|152061323	ETL4, RecName: Full=Sickle tail protein; AltName: Full=Enhancer trap locus 4	Other
gi|226443091	HNRNPA0, heterogeneous nuclear ribonucleoprotein A0	RNA-binding
gi|12859322	RPL31, unnamed protein product (ribosomal protein L31)	Ribosomal
gi|10946928	HNRNPH1, heterogeneous nuclear ribonucleoprotein H	RNA-binding
gi|9845253	HNRNPH2, heterogeneous nuclear ribonucleoprotein H2	RNA-binding
gi|6679108	NPM1, nucleophosmin	RNA-binding