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ADDENDUM

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Lysosomal membrane protein SIDT2 mediates the direct uptake of DNA by lysosomes

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ABSTRACT

Lysosomes degrade macromolecules such as proteins and nucleic acids. We previously identified 2 novel types of autophagy, RNautophagy and DNautophagy, where lysosomes directly take up RNA and DNA, in an ATP-dependent manner, for degradation. We have also reported that SIDT2 (SID1 transmembrane family, member 2), an ortholog of the *Caenorhabditis elegans* putative RNA transporter SID-1 (systemic RNA interference defective-1), mediates RNA translocation during RNautophagy. In this addendum, we report that SIDT2 also mediates DNA translocation in the process of DNautophagy. These findings help elucidate the mechanisms underlying the direct uptake of nucleic acids by lysosomes and the physiological functions of DNautophagy.

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Lysosomes were first discovered as organelles that contain acid phosphatase, ribonuclease, deoxyribonuclease, cathepsin, and β-glucuronidase activities.[1,2] To date, lysosomes have been shown to be capable of degrading various macromolecules including proteins, RNA, DNA, and lipids.[3] Although the majority of cellular DNA is in nuclei, some types of DNA, such as mitochondrial and viral DNA, may reside in the cytoplasm during inflammation or DNA virus infection.[4,5] Therefore, cytoplasmic DNA could be a target of autophagic pathways. However, whether and how cytoplasmic DNA is transported into lysosomes has largely been unknown. We previously found, using isolated lysosomes, that RNA and DNA are directly taken up by lysosomes in an ATP-dependent manner and degraded.[6,7] We named these novel types of autophagy RNautophagy and DNautophagy. RNautophagy and DNautophagy possess some selectivity for RNA and DNA substrates in vitro.[8] Most recently, we found that SIDT2, an ortholog of the *Caenorhabditis elegans* putative RNA transporter SID-1, is a lysosomal membrane protein that mediates RNA translocation during RNautophagy.[9] Following a recent study showing that plasmid DNA can be imported into cultured silkworm cells by SID-1,[10] we hypothesized that SIDT2 also mediates the translocation of DNA during DNautophagy. In this addendum we have tested this possibility using essentially the same methods as in the previous study.[9]

To investigate whether SIDT2 mediates DNA translocation during DNautophagy, we performed gain- and loss-of-function experiments using isolated lysosomes from cultured cells, and plasmid DNA. Isolated lysosomes and plasmid DNA were incubated with ATP, lysosomes were precipitated by centrifugation, and the DNA in solution outside of the lysosomes was analyzed by agarose gel electrophoresis, as an indicator of DNA uptake activity (DNA uptake assay). We also incubated isolated lysosomes and plasmid DNA in the presence of ATP, and analyzed the total levels of DNA in samples to reveal whether DNA is degraded in lysosomes (DNA degradation assay).

To assess the effects of SIDT2 overexpression on DNA uptake and degradation, Neuro2a cells were transfected with an expression vector that produces full-length SIDT2. Lysosomes were isolated from SIDT2-overexpressing or control (empty vector transfected) cells, and overexpression of SIDT2 in the lysosomal fraction was validated (Fig. 1A). Intactness of lysosomes isolated from either SIDT2-overexpressing cells (SIDT2-overexpressing lysosomes) or control cells (control lysosomes) was confirmed, because DNA was not degraded outside of lysosomes during incubation (Fig. 1B). Then, the DNA uptake assay was performed. Lysosomes isolated from SIDT2-overexpressing cells had significantly more DNA uptake activity than those from cells transfected with empty vector (Fig. 1C). We also observed the higher uptake activity of SIDT2-overexpressing lysosomes using post-embedding immunoelectron microscopy (Fig. 1D). We have previously reported that a serine 564 to alanine mutation (S564A) inhibits RNA uptake activity of SIDT2.[9] We tested the effect of this mutation on DNA uptake by performing DNA uptake assay using lysosomes derived from mutant SIDT2-overexpressing cells. Overexpression of mutant SIDT2 did not significantly increase DNA uptake activity (Fig. 1E).

Next, we used SIDT2-overexpressing and control lysosomes to perform a DNA degradation assay. We observed that DNA

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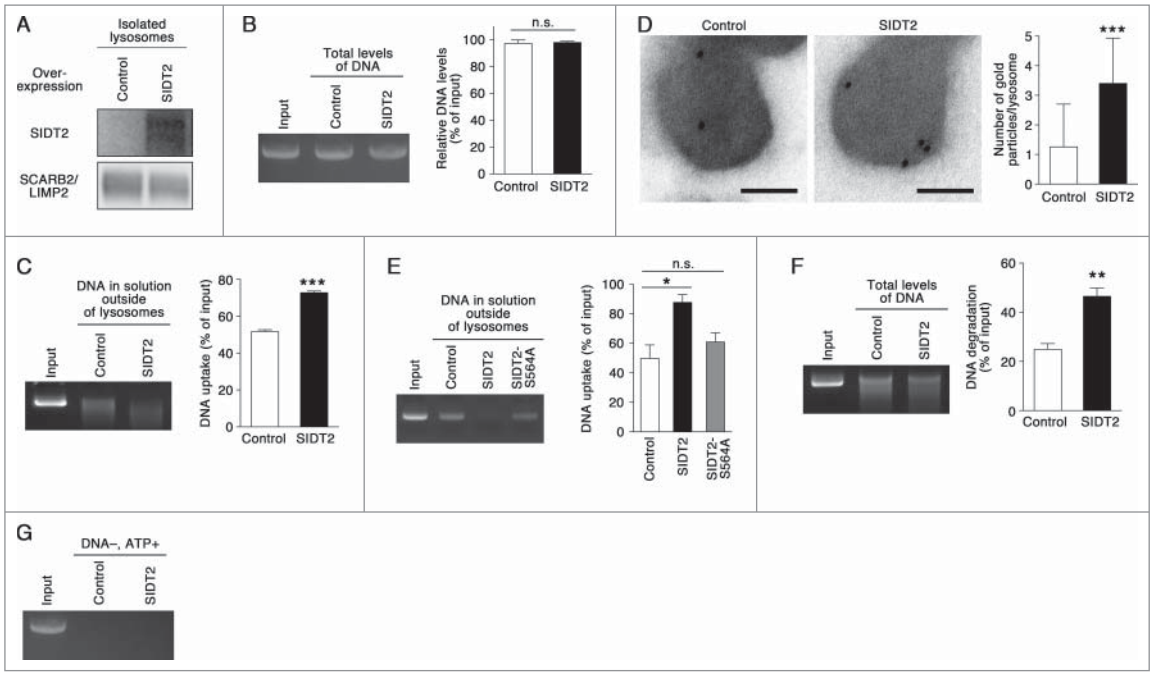

Figure 1. Effects of SIDT2 overexpression on DNA uptake and degradation by lysosomes. (A) Lysosomes were isolated from SIDT2-overexpressing cells (SIDT2-overexpressing lysosomes) or control (empty vector transfected) cells (control lysosomes) and protein levels in lysosomal samples were analyzed by immunoblotting. (B) Intactness of SIDT2-overexpressing or control lysosomes was confirmed. Isolated lysosomes (~25 μg protein) were incubated in the presence of an ATP regeneration system for 5 min at 37°C. Lysosomes were pelleted by centrifugation at 17,700 g for 1 min, and the supernatant fraction was incubated with 1 μg of plasmid DNA under the same conditions, then analyzed by agarose gel electrophoresis. DNA levels were quantified using ImageJ software. One hundred percent of input DNA was electrophoresed. Migration time was 5 min. Mean ± SEM (n = 3). n.s., not significant. (C) A DNA uptake assay was performed using SIDT2-overexpressing or control lysosomes as described in Materials and Methods. Mean ± SEM ($n = 3$). ***, $P < 0.001$. (D) SIDT2-overexpressing or control lysosomes were prepared and incubated with DNA as described in Materials and Methods. Post-embedding immunoelectron microscopy of the lysosomes was performed using an anti-DNA antibody followed by anti-mouse IgG coupled with 12-nm gold particles. Gold particles were observed in the lysosomes and the number of gold particles per lysosome counted. Mean ± SD ($n = 20$). ***, $P < 0.001$. Scale bars: 100 nm. (E) Lysosomes isolated from S564A mutant SIDT2-, wild-type SIDT2-overexpressing cells or control cells were subjected to a DNA uptake assay as described in Materials and Methods. Mean ± SEM (n = 3). *, $P < 0.05$. (F) A DNA degradation assay was performed as described in Materials and Methods using isolated lysosomes derived from Neuro2a cells transfected with SIDT2-expression or control empty vectors. Mean ± SEM ($n = 3$). **, $P < 0.01$. (G) SIDT2-overexpressing or control lysosomes were prepared and incubated without DNA as described in Materials and Methods.

degradation levels were significantly higher in samples containing lysosomes derived from SIDT2-overexpressing Neuro2a cells, compared with those from control cells (Fig. 1F). We confirmed that the DNA remaining in the samples was derived from the plasmid DNA that was added to the isolated lysosomes, because DNA was not detected in isolated lysosomes when lysosomes were incubated in the absence of DNA (Fig. 1G). Collectively, these results indicate that overexpression of SIDT2 enhances DNA uptake and degradation by lysosomes.

To investigate the effects of *SIDT2* knockdown on DNA uptake and degradation, HeLa cells were transfected with siRNA targeting *SIDT2* (*SIDT2*-A) or control siRNA, then lysosomes were isolated and decreased levels of SIDT2 in the lysosomal fraction were verified (Fig. 2A). Intactness of lysosomes isolated from either *SIDT2*-knockdown or control cells was confirmed, because DNA was not degraded outside of lysosomes during incubation (Fig. 2B). Then, DNA uptake and degradation assays were performed. Lysosomes isolated from *SIDT2* knockdown cells had significantly less DNA uptake activity than lysosomes isolated from control siRNA-transfected cells (Fig. 2C). Additionally, we observed significantly reduced levels of DNA degradation in samples containing lysosomes from *SIDT2* knockdown cells (Fig. 2D). Again, we confirmed that the DNA remaining in the samples was derived from the plasmid DNA that was added to the isolated lysosomes, because DNA was not detected in isolated lysosomes when lysosomes were incubated in the absence of DNA (Fig. 2E). Similar results were obtained when we used another siRNA (*SIDT2*-B) against *SIDT2* (Fig. 2F-J).

Taken together, these results demonstrate that SIDT2 has a critical role in the process of DNautophagy.

Next, we assessed the efficacy of SIDT2 to mediate translocation of several types of nucleic acids. We performed DNA uptake assays using SIDT2-overexpressing or control lysosomes and circular or linear DNA as substrate. Interestingly, uptake levels of linear DNA were significantly higher than those of circular DNA, when the uptake assay was performed with either SIDT2-overexpressing or control lysosomes (Fig. 3A). We also conducted uptake assays using SIDT2-overexpressing or control lysosomes and mouse total RNA or linear DNA. Uptake levels of RNA were similar to those of DNA when either of the SIDT2-overexpressing or control lysosomes were used (Fig. 3B). Thus, under our in vitro experimental conditions, SIDT2 is more efficacious in mediating the uptake of linear DNA than the uptake of circular DNA, but exhibits similar uptake efficacy toward RNA and DNA.

In this addendum, we have shown that SIDT2 mediates translocation of DNA across the lysosomal membrane in the process of DNautophagy, in addition to translocation of RNA during RNautophagy. We previously reported that a single-pass lysosomal transmembrane protein, LAMP2C (lysosomal-associated

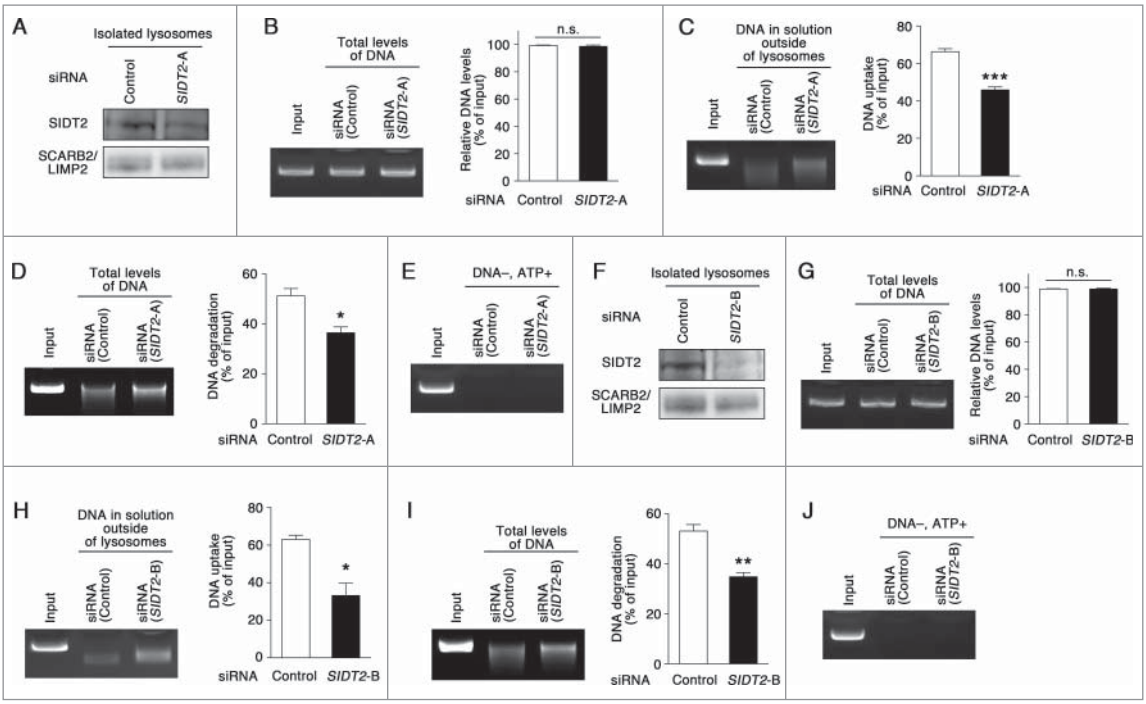

Figure 2. Effects of *SIDT2* knockdown on DNA uptake and degradation by lysosomes. (A and F) Lysosomes derived from *SIDT2*-knockdown HeLa cells were analyzed by immunoblotting as described in Fig. 1A. (B and G) Intactness of lysosomes isolated from HeLa cells was confirmed as described in Fig. 1B. (C and H) DNA uptake assays were performed using isolated lysosomes derived from *SIDT2*-knockdown HeLa cells (transfected with *SIDT2* siRNA) or control HeLa cells (transfected with control siRNA) as described in Materials and Methods. Mean ± SEM ($n = 3$). ***, $P < 0.001$. (D and I) DNA degradation assays were performed using isolated lysosomes derived from *SIDT2*-knockdown HeLa cells or control HeLa cells as described in the Materials and Methods. Mean ± SEM ($n = 3$). *, $P < 0.05$. (E and J) Isolated lysosomes derived from *SIDT2* knockdown HeLa cells or control HeLa cells were prepared and incubated without DNA as described in Materials and Methods.

membrane protein 2C), can function as a receptor for DNA and RNA in the process of DNautophagy and RNautophagy.[6,7,11] LAMP2C is unlikely to be a transporter for DNA and RNA, because channels are generally formed by multipass transmembrane proteins.[12] In contrast, the multipass transmembrane protein SIDT2 is a strong candidate for a nucleic acids transporter, particularly considering that SID-1 has been reported to be an RNA transporter.[13]

In this study, we demonstrate that SIDT2 can mediate uptake of linear DNA by lysosomes (Fig. 3A-B). The size of the linear DNA we used in this study is ~5.47 kbp and is estimated to be 1.86-μm long. Considering that the diameter of lysosomes is approximately 200 nm (Fig. 1D), it is more likely for this linear DNA to be taken up by lysosomes through a transporter than through a microautophagy-like process. Hence, our results support the hypothesis that SIDT2 functions as a nucleic acids transporter.

We show that uptake levels of circular DNA were significantly lower than those of linear DNA, when DNA was incubated with either SIDT2-overexpressing or control lysosomes (Fig. 3A). Linear DNA structures may be easier to be translocated by SIDT2 than circular structures. We also conducted comparative uptake assays using RNA (mainly single-stranded) and DNA (double-stranded), and found that there was no significant difference in uptake efficiencies of RNA and DNA (Fig. 3B). These results suggest that SIDT2 may have similar translocation activity for single-stranded and double-stranded nucleic acids.

We have previously reported that S564A mutant SIDT2 lacks the ability to mediate translocation of RNA.[9] SIDT2 has

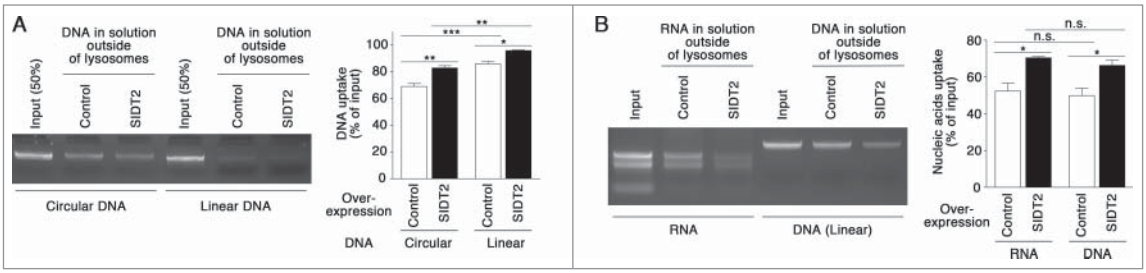

Figure 3. Uptake efficacy of SIDT2 toward different types of nucleic acids. (A) A DNA uptake assay was performed using SIDT2-overexpressing and control lysosomes as described in Materials and Methods. One microgram of circular (plasmid) DNA or linear DNA was used as a substrate. Mean ± SEM (n = 3). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. (B) RNA and DNA uptake assays were performed. SIDT2-overexpressing or control lysosomes were incubated with 5 μg of mouse total RNA or linear DNA in the presence of an ATP regeneration system for 5 min at 37°C. Lysosomes were pelleted by centrifugation at 17,700 g for 1 min, and nucleic acids in the supernatant fraction were analyzed by agarose gel electrophoresis. Migration time was 15 min. Mean ± SEM (n = 3). *, $P < 0.05$.

been suggested to possess hydrolase activity and S564 seems to be important for this function.[14] In this study, we showed that S564A mutant SIDT2 also lacks the ability to mediate DNA uptake by lysosomes (Fig. 1E). Further studies on the putative hydrolase activity of SIDT2 should contribute to the understanding of mechanisms underlying direct uptake of nucleic acids by lysosomes.

We have reported that constitutive degradation of cellular RNA is one of the physiological functions of RNautophagy.[9] Currently, the physiological roles of DNautophagy are unclear and warrant further examination. As we have previously proposed,[7] one of the physiological or pathophysiological roles of DNautophagy may be the degradation of cytosolic mitochondrial DNA. We have shown that mitochondrial DNA can be a substrate of DNautophagy in vitro.[7] Mitochondrial DNA has been reported to be released into the cytosol during inflammasome activation.[4] Another physiological or pathophysiological role of DNautophagy could be the degradation of exogenous DNA, such as viral DNA, which is found in the cytosol during infection with various DNA viruses.[5] Considering that a lysosomal deoxyribonuclease, DNASE2 (deoxyribonuclease II) has been reported to function as a lysosomal barrier to transfection,[15] it is also possible that DNautophagy is involved in the efficiency of the transfection, although this possible function is less physiological. The findings of the current study contribute to the elucidation of the physiological and pathophysiological roles of DNautophagy.

Materials and methods

Plasmid generation

Plasmids were prepared as described previously.[9]

Cell culture and transfection

The human adenocarcinoma cell line HeLa and the mouse neuroblastoma cell line Neuro2a were maintained in Dulbecco's modified Eagle's medium (Gibco-Life Technologies, C11995500) supplemented with 10% fetal bovine serum (Sigma-Aldrich, 172012) at 37°C, 5% CO_2. Transient transfection of Neuro2a cells with each vector was performed using Lipofectamine LTX with PLUS reagent (Life Technologies, 15338100). For siRNA-mediated knockdown of *SIDT2*, HeLa cells were transfected with *SIDT2*-targeting, or control siRNAs using Lipofectamine RNAiMAX (Life Technologies, 13778075) for 72 h. The target sequences are 5′-GAG GAU GAC UAC GAC ACA U-3′ (human *SIDT2* siRNA-A), 5′-CUA UGG UUG CAU UUC CGU U-3′ (human *SIDT2* siRNA-B), and 5′-CAG CAC GAC UUC UUC AAG U-3′ (*EGFP* siRNA). *EGFP* siRNA was used as a control.

Immunoblotting

Isolated lysosomes were lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (Sigma-Aldrich, T8787) and protease inhibitors (Complete, EDTA-free; Roche Diagnostics, 11873580001) at 4°C and samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Following transfer to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc., 162-0177), the blots were incubated with primary antibodies overnight at 4°C, then probed with secondary antibodies for 1 h at room temperature. Immunoreactive signals were visualized using the SuperSignal West Dura extended duration substrate (Thermo Scientific, 34075) and detected with a FluorChem 8000 imaging system (AlphaInnotech, CA, USA). The primary and secondary antibodies used are: polyclonal goat anti-SIDT2 (N-20; Santa Cruz Biotechnology, sc-19991) to detect murine SIDT2, monoclonal rabbit anti-SCARB2/LIMP2 (EPR12080; Abcam, ab176317), horseradish peroxidase-conjugated anti-goat IgG (Jackson ImmunoResearch, 305-036-003) and horseradish peroxidase-conjugated anti-rabbit IgG (Thermo Scientific, 31460). The rabbit polyclonal anti-SIDT2 antibody used to detect human SIDT2 was prepared as described previously.[9] The Can Get Signal Immunoreaction Enhancer Solution (Toyobo, NKB101) was used for blots against murine SIDT2.

DNA uptake assay

Intact isolated lysosomes (~25 μg protein) were incubated with 1 μg of substrate DNA in the presence of an ATP regeneration system for 5 min at 37°C. DNA in solution outside of lysosomes was recovered by centrifugation at 17,700 g for 1 min, and analyzed by agarose gel electrophoresis with ethidium bromide staining and UV illumination. The migration time was 5 min. DNA levels were quantified and DNA uptake levels were calculated by subtracting DNA levels remaining in solution outside of lysosomes from input DNA levels. Unless otherwise specified, 100% of input DNA was electrophoresed. DNA levels were quantified using ImageJ software. Plasmid DNA (pCI-neo mammalian expression vector; Promega, E1841) or linear DNA was used as substrate. Linear DNA was generated by cutting circular DNA with EcoRI restriction enzyme.

DNA degradation assay

Intact isolated lysosomes were incubated with plasmid DNA in the presence of an ATP regeneration system for 5 min at 37°C. Following incubation, DNA was extracted as follows: A proteinase K solution containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS, and 0.1 mg/ml proteinase K (New England Biolabs Inc., P8102S) in saline sodium citrate buffer was added and incubated for 2 h at 37°C, followed by phenol-chloroform extraction and ethanol precipitation. Total levels of DNA were analyzed by agarose gel electrophoresis with ethidium bromide staining and UV illumination. The migration time was 5 min. DNA degradation levels were calculated by subtracting the DNA levels remaining in samples from the levels of input DNA. 100% of input DNA was electrophoresed. DNA levels were quantified using ImageJ software.

Electron microscopy

Immunogold electron microscopy was conducted as previously described.[6] In brief, SIDT2-overexpressing or control

lysosomes were incubated with plasmid DNA in the presence of an ATP regeneration system for 5 min at 37°C, then lysosomes were pelleted by centrifugation and fixed with 0.1% glutaraldehyde and 4% paraformaldehyde in PBS overnight at 4°C. Next, samples were dehydrated and then embedded in LR White (Nisshin EM Co., Ltd., 3962). Sectioning at 100 nm and collection on 400-mesh nickel grids coated with collodion (Nisshin EM Co., Ltd., custom-made) followed by immunogold labeling was performed. We used a mouse monoclonal anti-DNA primary antibody (Abcam, ab27156) and an anti-mouse IgG coupled with 12-nm gold particles secondary antibody (Jackson ImmunoResearch, 115-205-166). Visualization was performed with a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, The Netherlands) at 80 kV.

Abbreviations

DNASE2	deoxyribonuclease II, lysosomal
LAMP2	lysosomal-associated membrane protein 2
SID-1	systemic RNA interference defective-1
SIDT2	SID1 transmembrane family, member 2

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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