Autophagy sequesters damaged lysosomes to control lysosomal biogenesis and kidney injury

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Diverse causes, including pathogenic invasion or the uptake of mineral crystals such as silica and monosodium urate (MSU), threaten cells with lysosomal rupture, which can lead to oxidative stress, inflammation, and apoptosis or necrosis. Here, we demonstrate that lysosomes are selectively sequestered by autophagy, when damaged by MSU, silica, or the lysosomotropic reagent L-Leucyl-L-leucine methyl ester (LLOMe). Autophagic machinery is recruited only on damaged lysosomes, which are then engulfed by autophagosomes. In an autophagy-dependent manner, low pH and degradation capacity of damaged lysosomes are recovered. Under conditions of lysosomal damage, loss of autophagy causes inhibition of lysosomal biogenesis *in vitro* and deterioration of acute kidney injury *in vivo*. Thus, we propose that sequestration of damaged lysosomes by autophagy is indispensable for cellular and tissue homeostasis.

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Introduction

The lysosome, a single-membrane acidic organelle, is present in all eukaryotic cells. Lysosomes act as a cellular ‘digestive apparatus’ that degrades materials delivered either from outside via the endocytic pathway or from inside via the

autophagic pathway. These compartments provide cells with nutrients, including amino acids and lipids, by degrading proteins and other macromolecules, and they also function in plasma membrane repair, defense against pathogens, antigen presentation, and cell death (Saftig and Klumperman, 2009). Lysosomal rupture results in leakage of contents, including Cathepsins, from the lysosomal lumen into the cytosol; in the worst cases, this can cause apoptotic or necrotic cell death (Boya and Kroemer, 2008). Even when the damage is not lethal, lysosomal rupture provokes oxidative stress due to the release of H⁺ from the lysosomal lumen into the cytosol, DNA damage, and reduction in the catabolic capacity of the lysosome, which might in turn affect the cellular functions in which lysosomes are involved (Boya and Kroemer, 2008; Hornung *et al*, 2008; Johansson *et al*, 2010). Emerging evidence indicates that lysosomal rupture activates the NLRP3 inflammasome, which induces the secretion of proinflammatory cytokines including IL-1 β , promoting inflammation and enhancing pathogenesis (Hornung *et al*, 2008; Salminen *et al*, 2012). Therefore, lysosomal rupture is a potentially harmful and stressful event for cells. Diverse substances, including mineral crystals such as silica and monosodium urate (MSU), bacterial or viral toxins, lipids, β -Amyloid, lysosomotropic compounds, and cell death effectors can impair lysosomal membranes *in vivo*. These substances can cause pathologies, including neurodegenerative disorders such as Parkinson’s disease, inflammation, and the development of hyperuricemic nephropathy (Emmerson *et al*, 1990; Kroemer and Jäättelä, 2005; Boya and Kroemer, 2008; Dehay *et al*, 2010; Salminen *et al*, 2012).

Autophagy is an intracellular bulk degradation system that is drastically induced under several cellular stress conditions such as nutrient starvation, and plays diverse physiological and pathological roles as a prosurvival mechanism of cells through maintaining cellular and tissue homeostasis (Mizushima and Levine, 2010). Autophagy is initiated by *de novo* generation of the isolation membrane in the cytoplasm. This membrane elongates to engulf cytoplasmic macromolecules and organelles, and encloses these cargos to form the autophagosome. The autophagosome itself does not have the ability to degrade its contents. Fusion with the lysosome provides an acidic environment and hydrolases, enabling degradation of autophagosomal contents. LC3, a mammalian Atg8 homologue, is a ubiquitin-like protein that is essential for autophagy. The cysteine proteinase Atg4 processes the C-terminal 22 residues of newly synthesized precursor LC3, producing a soluble form of LC3 (LC3-I) that exposes the C-terminal glycine residue (Kabeya *et al*, 2000). The exposed C-terminus of LC3 is conjugated to the head group amine of phosphatidylethanolamine (PE) by a ubiquitin-like system composed of Atg7 (E1 enzyme), Atg3 (E2 enzyme), and the Atg12–Atg5–Atg16L complex (E3 enzyme) (Ichimura *et al*, 2000; Hanada *et al*, 2007; Fujita *et al*, 2008b). Lipidated LC3 (LC3-II), a widely used autophagic

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marker, is anchored to the forming autophagosome membrane. Upon membrane closure, LC3-II is cleaved by Atg4, and LC3 dissociates from PE (Kirisako *et al*, 2000; Kim *et al*, 2001). Thus, Atg4 is involved in both lipidation and delipidation of LC3. Furthermore, overexpression of Atg4B^{C74A}, an inactive mutant of Atg4B (a mammalian Atg4 homologue), strongly inhibits lipidation of LC3 by sequestering LC3 orthologues including LC3A, LC3B, GABARAP, GATE16, and Atg8L prior to lipidation (Fujita *et al*, 2008a).

Although autophagy is considered to be a non-selective degradation system, growing evidence has revealed autophagic pathways that selectively degrade aggregation-prone proteins, invading pathogens, and damaged or superfluous organelles such as mitochondria, peroxisomes, and endoplasmic reticulum (ER) in order to maintain cellular homeostasis (Komatsu and Ichimura, 2010; Tanida, 2011). In mammalian cells, ubiquitination plays a crucial role in determining the target of selective autophagy, working in conjunction with adaptor proteins such as p62/SQSTM1, which enable interaction between ubiquitin and LC3 paralogues. Following interaction with adaptors, ubiquitinated substrates are engulfed by autophagosomes via binding between adaptors and LC3.

Here, we examined the possibility that cells induce autophagy in order to avoid the associated risks that is caused by lysosomal rupture. We found that lysosomes damaged by lysosomotropic reagents are selectively isolated by autophagy. Furthermore, we suggest a possibility that this selective isolation plays critical roles in lysosomal biogenesis in cells and in suppression of acute kidney injury *in vivo*.

Results

Lysosomal rupture induces autophagy

To disrupt the lysosomal membrane, we used either the lysosomotropic compound L-Leucyl-L-leucine methyl ester (LLOMe) or crystalline silica. LLOMe accumulates in lysosomes and is converted into its membranolytic form (Leu-Leu)_n-OMe ($n > 3$) by a lysosomal thiol protease, dipeptidyl peptidase I (DPP1) (Thiele and Lipsky, 1990; Uchimoto *et al*, 1999). Crystalline silica, also known as silicon dioxide (SiO₂), exists in nature as sand or quartz. Although silica itself is usually harmless, crystalline silica has been shown to disrupt the lysosomal membrane in alveolar macrophages and activate lung inflammation *in vivo* (Mossman and Churg, 1998; Hornung *et al*, 2008).

To test whether autophagy is induced by lysosomal rupture, we measured lipidation of endogenous LC3 by immunoblotting in the murine macrophage cell line J774 cells and mouse embryonic fibroblasts (MEFs). LLOMe treatment stimulated cytosolic release of Cathepsin D in a dose-dependent manner (Supplementary Figure S1A), and the level of lipidated LC3 dose dependently increased upon either LLOMe or silica treatment (Supplementary Figure S1B and C). These results suggest that lysosomal rupture induces autophagy. Then, we found the colocalization of LC3 and Lamp1 in LLOMe- or silica-treated J774 cells (Supplementary Figure S1D) using confocal microscopy. In particular, silica-treated cells exhibited obvious recruitment of LC3 to silica particles surrounded by Lamp1. These results raise the possibility that autophagy selectively targets damaged lysosomes.

Autophagic machinery is selectively recruited to damaged lysosomes

To determine whether LC3 is specifically recruited only to damaged lysosomes, we used Galectin-3 (Gal3), a recently established marker of damaged endomembranes (Paz *et al*, 2010). Galectin-3/Mac-2 is a member of the Galectins, a lectin protein family defined by conserved sequence and affinity for β -galactosides. Gal3 is distributed throughout the cytoplasm and nucleus, whereas β -galactose-containing glycoconjugates are present only on the cell surface and in the lumens of endocytic compartments, the Golgi apparatus, and post-Golgi secretory compartments (Houzelstein *et al*, 2004). Therefore, these proteins normally do not interact with each other; however, endosomal membrane rupture allows Gal3 to access the luminal glycoproteins of these compartments (Paz *et al*, 2010).

We generated Atg7-deficient MEFs, in which Atg5–Atg12 conjugation is impaired, stably expressing GFP-Gal3 to investigate the recruitment of LC3 to membrane-damaged lysosomes. Under untreated conditions, GFP-Gal3 and LC3 were diffusely distributed throughout the cytosol and did not colocalize with Lamp1 (Figure 1A). However, upon LLOMe or silica treatment, several GFP-Gal3 puncta appeared in the cytosol. The number of these punctate structures increased in an LLOMe dose-dependent manner (Supplementary Figure S2A) and Gal3 puncta extensively colocalized with Lamp1 in LLOMe- or silica-treated Atg7^{+/+} and Atg7^{-/-} MEFs; conversely, we could not observe Lamp1-negative Gal3 puncta. GFP-Gal3-positive Lamp1 puncta were not stained with LysoTracker (Supplementary Figure S2B). Video microscopy observations showed that GFP-Gal3 puncta were never stained with LysoTracker throughout its recruitment in the presence of LLOMe (Supplementary Figure S3A; Supplementary Movie1), indicating that Gal3 is recruited to lysosomes that have lost their acidic interior environment following membrane damage. Gal3 was also recruited to Lamp1 and colocalized with LC3 under oxidative stress conditions, which is also known to cause lysosomal membrane damage (Supplementary Figure S2C) (Boya and Kroemer, 2008), suggesting that Gal3 can be used as a general marker of damaged lysosomes. LC3 was specifically recruited to GFP-Gal3-positive Lamp1 puncta upon LLOMe or silica treatment in Atg7^{+/+} MEFs, but not in Atg7^{-/-} MEFs (Figure 1A). Since LC3 was recruited to Gal3 puncta, even when only a few lysosomes were damaged at the low concentration of LLOMe (Supplementary Figure S2D), autophagy is highly sensitive to lysosomal damage.

Gal3-positive damaged membranes were ubiquitinated and colocalized with p62 in both MEFs (Figure 1B and C). These results suggest that ubiquitination and the recruitment of p62 occurs in a manner similar to that observed for selective autophagy against invading bacteria. We also confirmed the colocalization of other GFP-tagged upstream Atg proteins (ULK1, Atg9L1, Atg14L, WIPI1, and Atg5) and Gal3-positive lysosomes upon either LLOMe or silica treatment (Supplementary Figure S3C). Furthermore, to clarify the cause–effect relationship between lysosomal rupture and autophagy induction, we observed puncta formation of GFP-Atg5, a marker of the isolation membrane, and mStrawberry-Gal3 using video microscopy (Supplementary Figure S3B; Supplementary Movie 2). Upon LLOMe treatment, the recruitment of GFP-Atg5 always followed the formation of mStrawberry-Gal3 puncta, suggesting that autophagy is induced after lysosomal rupture. Taken together,

Figure 1 The recruitment of ubiquitin and LC3 to GFP-Gal3-positive damaged lysosomes. (A–C) *Atg7*^{+/+} and *Atg7*^{-/-} MEFs stably expressing GFP-Gal3 (A, C) or GFP-Ub (B) were treated with 1000 μ M LLOMe or 250 μ g/ml silica for 3 h. Cells were subjected to immunocytochemistry using the following antibodies: anti-LC3 and anti-Lamp1 (A), anti-p62 and anti-Lamp1 (B), or anti-FK2 and anti-p62 (C). Bars: 10 μ m.

these results indicate that the core autophagy machinery is selectively recruited to damaged lysosomes.

GFP-Gal3-positive lysosomes decrease in an autophagy-dependent manner

What happens in damaged lysosomes after specific recruitment of Atg proteins? We exposed cells to 1000 μ M LLOMe for 1 h, and after washing out the reagent cultured the cells for an additional 24 h in the absence of LLOMe. Then, we evaluated the percentage of GFP-Gal3-positive Lamp1 puncta at the indicated time points (Figure 2A and B; Supplementary Figure S4A and B). Three hours after LLOMe washout, almost 30% of Lamp1 puncta colocalized with GFP-Gal3 (Figure 2B). These Gal3-positive Lamp1 puncta dramatically decreased

within 10 h, and completely disappeared until 24 h after LLOMe washout. In contrast to control cells, in cells stably expressing an inactive *Atg4B* mutant, *Atg4B*^{C74A}, that sequesters LC3 paralogues prior to lipidation and strongly blocks autophagy (Fujita *et al*, 2008a), GFP-Gal3-positive Lamp1 puncta slightly decreased but were then maintained at a high level even 24 h after LLOMe washout (Figure 2B). These results suggest that disappearance of GFP-Gal3 puncta is due to autophagy. The total number of Lamp1 puncta remained stable in both control and *Atg4B*-mutant cells (Supplementary Figure S4A). Similar results were obtained in *Atg7*-deficient MEFs (Supplementary Figure S5A–C). LysoTracker staining showed that there remained LysoTracker-positive Lamp1 puncta representing acidic intact

Figure 2 Decrease in the number of GFP-Gal3 puncta is dependent on time and autophagy. (A, B) NIH3T3 cells stably expressing GFP-Gal3 and either empty vector (control) or mStrawberry-Atg4B^{C74A} (Atg4B mutant) were treated with 1000 μ M LLOMe for 1 h. After LLOMe washout, cells were fixed at the indicated time points and subjected to immunocytochemistry for Lamp1 and DAPI (blue) (A). The number of GFP-Gal3 or Lamp1 puncta per cell was quantified using G-Count (see also Supplementary Figure S4A and B). Then, the percent of GFP-Gal3-positive Lamp1 puncta was determined (B). The data represent means $\pm$ s.d. At least 70 cells were counted ($n = 3$). Bars: 20 μ m. Source data for this figure is available on the online supplementary information page.

lysosomes at 3 h after LLOMe washout (Supplementary Figure S5D).

We also tested continuous treatment of cells with LLOMe for longer periods of time. NIH3T3 cells were treated with 1000 μ M LLOMe for the indicated time, and analysed the percentage of GFP-Gal3-positive Lamp1 puncta (Supplementary Figure S6A–C). Interestingly, even in the continuous presence of LLOMe, GFP-Gal3-positive Lamp1 puncta significantly decreased, although there remained some at 24 h. Presumably, autophagic sequestration of damaged lysosomes overcomes continuous damage of them.

Acidity of damaged lysosomes recovers in an autophagy-dependent manner

At least two phenomena could explain the disappearance of GFP-Gal3 puncta after LLOMe washout: release of GFP-Gal3 from the damaged membrane, and quenching of the GFP

signal in an acidic environment such as that generated during autophagy. To distinguish between these two possibilities, we constructed mRFP- and GFP-tandem-tagged Gal3 (tandem fluorescent-tagged Galectin-3, tfGal3) (Figure 3A). GFP and mRFP are differentially sensitive to acidic environments (Kneen *et al*, 1998; Campbell *et al*, 2002). GFP fluorescence is rapidly quenched, and GFP is degraded by lysosomal hydrolases, whereas mRFP fluorescence remains relatively stable. Thus, as shown in Figure 3B, tfGal3 makes it possible to monitor the pH change in damaged lysosomes. The change in the surrounding environment from neutral to acidic pH causes attenuation of GFP puncta signals, while mRFP puncta stably persist. Therefore, if GFP signal is quenched after LLOMe washout, the number of mRFP⁺GFP⁺ puncta should decrease, whereas the number of mRFP⁺ puncta (i.e., regardless of the presence or absence of GFP signal) should not be changed. In contrast, if Gal3 is released from damaged

Figure 3 tfGal3 GFP signal in puncta attenuates in an autophagy-dependent manner. (A) Diagram of the primary structure of tandem fluorescence-tagged Galectin-3 (tfGal3). (B) Schematic diagram of the fate of tfGal3 recruited to damaged lysosomes. (C–E) HeLa cells transfected with tfGal3 and either One-STREP-FLAG-tagged Atg4B^{C74A} (Atg4B mutant) or empty vector (control) were observed by confocal microscopy after treatment as shown in Figure 2A and B. The number of GFP[±]RFP⁺ or GFP⁺RFP⁺ puncta per cell was quantified using G-Count (D). Then, the percent of GFP⁺RFP⁺ tfGal3 puncta was calculated (E). The data represent means $\pm$ s.d. At least 30 cells were counted ($n = 3$). Bar: 10 μ m. (F–H) NIH3T3 cells stably expressing empty vector (control) or mStrawberry-Atg4B^{C74A} (Atg4B mutant) were treated with 1000 μ M LLOMe for 1 h. After LLOMe washout, cells were cultured in the presence or absence of both 10 μ g/ml E64d and Pepstatin A, fixed at the indicated time points, and subjected to immunocytochemistry for Gal3 (green) and DAPI (blue) (F). The number of endogenous Gal3 puncta per control (G) or Atg4B-mutant (H) cells was quantified by G-Count. The data represent means $\pm$ s.d. At least 50 cells were counted ($n = 3$). Bars: 20 μ m. Source data for this figure is available on the online supplementary information page.

lysosomes, then the number of both mRFP⁺GFP⁺ and mRFP⁺ puncta should decrease.

We transfected HeLa cells with a plasmid encoding tfGal3, with or without a plasmid encoding Atg4B^{C74A}, and then subjected the cells to the experimental procedure as in Figure 2. As we expected, upon LLOMe treatment, tfGal3 formed several mRFP⁺GFP⁺ puncta in both control and Atg4B-mutant cells (Figure 3C). In control cells, the GFP⁺ puncta were almost completely abolished 24 h after LLOMe washout, whereas the mRFP⁺ puncta could be observed at the same level as at time 0 (Figure 3D and E). The attenuation of GFP puncta signals was cancelled by Bafilomycin A1, a specific inhibitor of the vacuolar-type ATPase. By contrast, tfGal3 puncta did not lose GFP signal in Atg4B-mutant cells. The total number of mRFP⁺ puncta did not decrease even 24 h after LLOMe depletion in either cell types (Figure 3D). From these data, we conclude that GFP-Gal3 is not released from damaged membranes, and that the acidity of damaged lysosomes recovers in an autophagy-dependent manner.

We also tried to corroborate our conclusion by other approaches than those using tagged Gal3. In Supplementary Figure S5D, Lysotracker-positive intact lysosomes seemed to increase while Lysotracker-negative lysosomes were colocalized with decreased GFP-Gal3. Therefore, we next measured % of Lysotracker-negative lysosomes in total lysosomes representing damaged lysosomes leaking protons (Supplementary Figure S5E). Lysotracker-negative lysosomes increased after 1.5 h from LLOMe washout but decreased after 6 h from LLOMe washout in control cells, suggesting recovery of low pH in damaged lysosomes. On the other hand, in autophagy-deficient Atg7^{-/-} MEFs, the increase in Lysotracker-negative lysosomes was higher after 1.5 h from LLOMe washout but their decrease after 6 h from LLOMe washout was small compared to control. The result also supports that acidity is recovered by autophagy.

Endogenous Gal3 on damaged lysosomes is degraded by autophagy

Endogenous Gal3 puncta that appeared upon LLOMe treatment exhibited similar decrease as exogenous-tagged Gal3 puncta in control and Atg4B-mutant cells (Figure 3F–H), and in Atg7^{+/+} and Atg7^{-/-} MEFs (Supplementary Figure S5F and G). The number of Gal3 puncta was not affected in Atg4B-mutant cells by protease inhibitors, whereas Gal3 puncta accumulated in control cells in the presence of protease inhibitors (Figure 3G and H). Most likely, these results indicate that endogenous Gal3 on damaged lysosomes is degraded in an autophagy-dependent manner. We also measured the amount of endogenous Lamp1 in the presence of cycloheximide to inhibit synthesis of new proteins up to 10 h after LLOMe washout (Supplementary Figure S5H). There was no significant change observed. Presumably, Lamp1 on damaged lysosomes turns over very slowly due to its resistance to lysosomal proteases. Indeed, the half-life of Lamp1 was reported to be 1.6 days (Meikle *et al*, 1999).

In addition, the increase in LC3 lipidation upon LLOMe treatment decreased after LLOMe washout, returning to the basal level within 10 h after LLOMe washout (Supplementary Figure S4C and D), consistent with the kinetics of change in the number of Gal3-positive lysosomes. This is not due to impaired autophagy flux, since we could observe autophagy flux by using mRFP-GFP-LC3, which is an established probe

for autophagy flux (Kimura *et al*, 2007). In this assay, mRFP⁺GFP⁺ LC3 puncta represent forming autophagosomes or autophagosomes, and mRFP⁺-only puncta indicate autolysosomes. After LLOMe washout, the percentage of mRFP⁺GFP⁺ LC3 puncta decreased with increased mRFP⁺-only puncta in a time-dependent manner (Supplementary Figure S4E and F), indicating that autophagy flux (formation of autolysosome) is not significantly hindered in the experimental condition.

Autophagosomes engulf damaged lysosomes

To reveal how autophagy restores the acidic and proteolytic environment in damaged lysosomes, we observed the ultra-structure of GFP-LC3- and mStrawberry-Gal3-positive damaged lysosomes by correlation of light and electron microscopy (CLEM). HeLa cells expressing GFP-LC3 and mStrawberry-Gal3 were treated with LLOMe for 1 h, and immediately fixed (Figure 4A–F). CLEM revealed that LC3- and Gal3-positive puncta are double membranes, a typical autophagosome structure, tightly sequester swollen lysosomes (Figure 4C–F). The single-membrane vesicles were partially or completely sequestered in double-membrane structures (Figure 4G and H). We could not find such structures in cells that were not treated with LLOMe (Supplementary Figure S7A–D). In contrast to control cells, all of the obtained images in Atg4B-mutant-expressing cells treated with LLOMe showed that lysosomes were partially attached with flattened membranous sacs but were never enclosed in double-membrane structures (Figure 4I). These results suggest that damaged lysosomes are selectively engulfed by autophagosomes. Presumably, autophagosomes containing damaged lysosomes fuse with remaining intact lysosomes, resulting in the recovery of acidity and proteolysis activity.

Autophagy suppresses development of acute hyperuricemic nephropathy in mice

Finally, we examined the pathophysiological importance of autophagic isolation of damaged lysosomes *in vivo*. Acute hyperuricemic nephropathy is a type of acute kidney injury observed in patients with tumour lysis syndrome, caused by chemotherapeutic treatment of haematopoietic malignancies (Ejaz *et al*, 2006). In this disease, oversaturation of uric acid (UA) in urine causes precipitation of UA and MSU in the renal tubule (Nickeleit and Mihatsch, 1997; Ejaz *et al*, 2006). A previous study showed that urate crystals cause the cytosolic release of lysosomal enzymes in MDCK cells, and that this lysosomal damage is involved in the development of hyperuricemic nephropathy (Emmerson *et al*, 1990). Therefore, we examined whether autophagy protects against acute hyperuricemic nephropathy *in vivo*.

First, we tested the induction of autophagy in renal tubular cells in acute hyperuricemic nephropathy, using transgenic mice expressing GFP-LC3 (Kuma *et al*, 2004). Administration of UA and an inhibitor of urate oxidase, oxonic acid (OA), induced accumulation of GFP-LC3 puncta in kidney of GFP-LC3 transgenic mice, whereas GFP-LC3 puncta were rarely observed in vehicle-treated mice (Supplementary Figure S8A). Immunostaining with megalin, a marker of the proximal tubular brush border, revealed that GFP-LC3 puncta increased in proximal tubules. These results suggest that

Figure 4 CLEM analysis of mSt-Gal3- and GFP-LC3-associated membranes. (A–F) HeLa cells stably expressing GFP-LC3 were transfected with mStrawberry-Gal3, and treated with 1000 μ M LLME for 1 h. Then, cells were fixed and observed by confocal microscopy (A). The same specimens were further examined by transmission electron microscopy (B–F). Green: LC3; magenta: Gal3; blue: DAPI. (G–I) NIH3T3 cells stably expressing CFP-Gal3 and YFP-LC3, and either empty vector (control) (G, H) or mStrawberry-Atg4B^{C74A} (I) were treated with 1000 μ M LLME for 2 h, fixed, and observed by confocal microscopy. The electron micrographs were taken in the same sample field as the transmission electron microscope. Green: LC3; blue: Gal3 and DAPI; black arrow: single membrane; white arrow: autophagosome; white arrowhead: ER membrane.

autophagy is induced in proximal tubules in acute hyperuricemic nephropathy.

Next, we analysed the role of autophagy in the development of acute hyperuricemic nephropathy, using proximal tubule-specific Atg5-deficient mice (Atg5^{F/F};KAP) (Kimura *et al*, 2011). In these mice, Cre recombinase is expressed under the control of the kidney androgen-regulated protein (KAP) promoter. Plasma uric acid concentrations were comparable between Atg5^{F/F};KAP and control Atg5^{F/F} mice after UA and OA administration (Supplementary Figure S8B).

The kidney function of UA- and OA-treated Atg5^{F/F};KAP mice was significantly deteriorated compared with that of UA- and OA-treated Atg5^{F/F} mice, as assessed by plasma urea nitrogen levels (47 ± 4 mg/dl versus 34 ± 5 mg/dl, respectively; $P < 0.05$) and plasma creatinine levels (0.69 ± 0.07 mg/dl versus 0.48 ± 0.06 mg/dl, respectively; $P < 0.05$) (Figure 5A). Periodic acid-Schiff (PAS) staining demonstrated that Atg5^{F/F};KAP mice exhibited severer loss of the brush border, vacuolization, tubular dilation, and cast formation in proximal tubules, compared with Atg5^{F/F} mice, following UA and OA administration

Figure 5 Autophagy-deficient renal tubules exhibit severe injury and dysfunction under hyperuricaemia. (A) Plasma creatinine and urea nitrogen in $Atg5^{F/F}$ and $Atg5^{F/F};KAP$ mice treated with vehicle or UA + OA. The values displayed represent means $\pm$ s.e. Statistically significant differences (* $P < 0.05$) are indicated. F/F: $Atg5^{F/F}$ mice; F/F;KAP: $Atg5^{F/F};KAP$ mice. (B, C) PAS-stained renal cortical region of $Atg5^{F/F}$ and $Atg5^{F/F};KAP$ mice treated with vehicle or UA + OA ($n = 4-7$). Bars: 40 μ m (B). Kidney injury score of $Atg5^{F/F}$ and $Atg5^{F/F};KAP$ mice treated with vehicle or UA + OA (C). (D) Kidney cortices from $Atg5^{F/F}$ and $Atg5^{F/F};KAP$ mice treated with vehicle or UA + OA were subjected to immunohistochemical analysis of Lamp1 and LC3. Green: LC3; magenta: Lamp1; blue: DAPI. F/F: $Atg5^{F/F}$ mice; F/F;KAP: $Atg5^{F/F};KAP$ mice. Bar: 5 μ m. (E) Electron micrographs of kidney proximal tubules in $Atg5^{F/F}$ and $Atg5^{F/F};KAP$ mice treated with UA + OA. Yellow arrowhead: autophagosome; asterisk: lysosome; Mt: mitochondrion. Bar: 1 μ m. (F) Model of autophagic clearance of damaged lysosomes. Source data for this figure is available on the online supplementary information page.

(Figure 5B). Semi-quantitative analysis of the severity of the tubular injury revealed that $Atg5^{F/F};KAP$ mice exhibited significantly higher injury than $Atg5^{F/F}$ mice (2.4 ± 0.4 versus 0.9 ± 0.2 , respectively; $P < 0.05$) (Figure 5C). UA- and OA-treated $Atg5$ -conditional knockout mice showed abnormality of lysosomes (Supplementary Figure S8C). Usually, Lamp2-

positive lysosomes localize beneath the brush border in proximal tubules, but Lamp2 diffused in UA- and OA-treated $Atg5^{F/F};KAP$ mice. Furthermore, the number of enormous ubiquitin aggregates in proximal tubules increased >10-fold in $Atg5$ -conditional knockout mice compared with control mice upon administration of UA and OA (Supplementary

Figure S8D and E), indicating that a deficiency in autophagy strongly enhances the development of acute hyperuricemic nephropathy in mice.

To confirm that MSU indeed damages lysosomes in the renal tubule and that autophagosomes sequester the damaged compartment, we cultured proximal tubular cells isolated from Atg5^{F/F};KAP mice (Atg5 negative) and its Atg5-revertant cells (Atg5 positive) (Takahashi *et al*, 2012) in the presence of MSU crystals. MSU crystals treatment increased the level of lipidated LC3 in Atg5-positive proximal tubular cells in a dose- and time-dependent manner (Supplementary Figure S9A). A subpopulation of the Lamp1 signal surrounding needle-shaped MSU crystals colocalized with GFP-Gal3 in either Atg5-positive or -negative cells (Supplementary Figure S9B). In both cell types, Gal3-positive Lamp1 signals surrounding MSU crystals colocalized with ubiquitin and p62 (Supplementary Figure S9C), although LC3 was recruited to damaged lysosomes only in Atg5-revertant cells (Supplementary Figure S9B). These data indicate that lysosomal membranes are disrupted by MSU crystals, and targeted by autophagy *in vitro*.

In agreement with the data obtained in isolated proximal tubular cells, we found that a subpopulation of Lamp1 puncta beneath the brush border colocalized with ubiquitin *in vivo* under UA and OA treatment, which was more prominent in Atg5-deficient mice than in control mice (Supplementary Figure S8F), indicating that lysosomal rupture occurs under hyperuricaemia. We also found that LC3 was recruited to Lamp1-positive lysosomes in proximal tubules of UA- and OA-administrated control mice but not in Atg5-deficient mice (Figure 5D), suggesting autophagic engulfment of lysosomes *in vivo*. Finally, electron microscopic observations showed that the abnormal lysosome-like structures were indeed engulfed by the double-membranous isolation membrane in UA- and OA-treated control mice but not in Atg5-deficient mice (Figure 5E). These data exhibit that membrane-damaged lysosomes are sequestered by autophagosomes *in vivo*. All of these data are consistent with the idea that autophagy plays a protective role in acute hyperuricemic nephropathy by isolating damaged lysosomes.

Discussion

Lysosomes play an important role in autophagy, but we show here that even lysosomes themselves can be targeted for autophagy when they are damaged. Under conditions of lysosomal damage, autophagy induction and sequestration of damaged lysosomes occurs after the leakage of lysosomal contents such as Cathepsin D. Thus, these results indicate that the role of autophagy is to isolate damaged lysosomes rather than to prevent cytosolic release of the contents. We observed that the total number of lysosomes did not change in Atg4B-mutant-expressing cells after treatment with LLOMe, indicating that cells maintain the overall number of lysosomes in the cytoplasm even if some of them are broken. As a result, if the damaged population is not isolated, the total capacity of lysosomal degradation will be reduced, leading to reduced cellular activities. In addition, a previous report suggested that membrane remnants of endosomes broken by *Shigella* could induce inflammatory immune responses (Dupont *et al*, 2009). Therefore, autophagy is highly sensitive to emergence of damaged lysosomes.

Since autophagosomes can fuse with lysosomes, they might restore damaged lysosomes by direct fusion events that seal holes. However, this seems not to be the case. We frequently observed double-bilayer isolation membranes surrounding lysosomes, suggesting that canonical autophagic processes eliminate damaged lysosomes. Once lysosomal rupture occurs, autophagosomes selectively sequester the damaged lysosomes. Since we also observed ubiquitin recruitment to damaged lysosomes, ubiquitin may be involved in the recognition of damaged lysosomes like other selective autophagy. When a subpopulation of lysosomes remains undamaged (under the condition we used, about 70% of lysosomes were intact), autophagosomes that contain damaged lysosomes can fuse with the remaining intact lysosomes (Figure 5F). This results in the recovery of low pH and degradation capacity. While Gal3 on damaged lysosomes was degraded rapidly (Figure 3F–H), Lamp1 on them was stable after 10 h from the damage (Supplementary Figure S5H). Lamp1 is likely to be degraded slowly, as hyperglycosylation protects itself from the attack by proteases (Eskelinen, 2006). It is conceivable that the importance of autophagy against damaged lysosomes is sequestration rather than degradation. Isolation of the harmful material is critical since, as mentioned above, existence of damaged lysosomes in the cytoplasm inhibits lysosomal biogenesis and could induce inflammatory immune responses.

How do cells recognize damaged lysosomal membranes? We previously showed that *Salmonella* are engulfed by autophagosomes while they are still contained within endosomes. *Salmonella* injures endosomal membranes by injecting a type-III secretion system. Thurston *et al* (2012) recently reported that Galectin-8 (distinct from Galectin-3, which we used here as a marker of the damaged membrane) is involved in autophagy against *Salmonella*. They suggested that Galectin-8 serves as a versatile receptor for vesicle-damaged pathogens, since it can recruit NDP52, a p62-like adaptor protein that binds to LC3. Galectin-8 may be involved generally in detection of endosomes and lysosomes that have been damaged, by pathogens or other materials, and targeting of these damaged organelles for autophagic clearance. Future works should address this issue as well as other questions, for example, the mechanisms underlying ubiquitination of the damaged membranes.

Many lines of evidence have shown that various pathogens that invade cells are targets of autophagy. To escape to the cytoplasm and/or to manipulate host functions, these pathogens often injure the endosomal membranes through which they invade by expression of pore-forming toxins, secretory systems, lipases, or other mechanisms. In many cases, cells may target damaged endosomes rather than the pathogens themselves; it is possible that cells do not distinguish between pathogens and the endosomes that contain them, but rather engulf the pathogens along with the surrounding compartments. The identification of Galectin-8 as a danger receptor also supports this idea. This may explain why diverse bacteria that do not share any common surface features can be targeted by autophagy; however, we do not exclude the possibility that pathogens are directly recognized in some cases.

Our cells and tissues constantly face danger from lysosomal damage originating from various causes, including

crystals such as silica and MSU as well as infection by pathogens, all of which injure the host membranes. Thus, the isolation of damaged lysosomes by autophagy is crucial for suppressing the onset of diseases and for maintaining health through avoidance of accumulation of damaged lysosomes. In fact, we showed here that lysosomes in mouse renal proximal tubules were damaged under hyperuricaemia, and that damaged lysosomes were sequestered by autophagosomes. However, autophagy-deficient proximal tubules could not isolate such damaged lysosomes, causing severe nephropathy.

It is noteworthy that irritative particles such as human islet amyloid polypeptide (IAPP), cholesterol crystals, and MSU that lead lysosomal damage are major causes of lifestyle-related diseases, including type 2 diabetes, arteriosclerosis, and gout, which are serious social problems. Hence, the findings in this paper suggest that novel therapeutic approaches are not only for nephropathy but also for such diseases.

Materials and methods

Antibodies and reagents

The following antibodies were used: anti-LC3 (MBL), anti-p62 (MBL), anti- α -tubulin (clone B5-1-2; Sigma), anti-Transferrin receptor (Zymed), anti-GAPDH (Millipore), anti-PDI (a gift from Dr A Yamamoto), anti-Cathepsin D (Calbiochem), anti-poly ubiquitin (clone FK2; BIOMOL, clone P4D1; Cell Signaling), anti-Galectin-3 (Santa Cruz Biotechnology), anti-Lamp1 (Santa Cruz Biotechnology), anti-Lamp2 (eBioscience), anti-EGFR (Fitzgerald), and anti-megalin (a gift from Dr T Michigami). Leu-Leu methyl ester hydrobromide, silicon dioxide, hydrogen peroxide, UA sodium salt, UA, OA, and Bafilomycin A1 were purchased from Sigma-Aldrich. Pepstatin A and E64d were from Peptide Institute. Lysotracker was from Molecular Probes. Cycloheximide was obtained from Nakarai. Uric acid C-test Wako and BUN-Test-Wako were from Wako. CRE-EN Kainos was from Kainos.

Cell culture, plasmid construction, transfections, and generation of stable cell lines

J774, MEF, NIH3T3, HeLa, and Plat-E cells were cultured in DMEM (Sigma-Aldrich) containing 10% fetal bovine serum and appropriate antibiotics. Transient transfections were carried out using LipofectAMINE 2000 (Invitrogen). Stable transformants were established by retrovirus infection and selection in growth media containing 1–4 μ g/ml puromycin or 1 μ g/ml blasticidin (Invitrogen).

Fluorescence and immunofluorescence microscopy

Cells were cultured on coverslips and fixed with 4% paraformaldehyde for 10 min, quenched with 50 mM NH_4Cl in PBS, permeabilized with 50 μ g/ml digitonin in PBS, blocked with 0.2% gelatin in PBS, and then incubated with indicated primary antibodies. After secondary antibody treatment, samples were observed using an FV1000 confocal microscope (Olympus). The number of Gal3, Lamp1, or Lysotracker puncta was analysed by G-Count (G-Angstrom).

Western blotting

Cells were washed with ice-cold PBS, collected by centrifugation at 4°C and lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 1 $\times$ protease inhibitor cocktail; Roche). Cell lysates were centrifuged at 15000 g for 15 min at 4°C, and supernatants were collected. Samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membranes were blocked with 1% skim milk in TBST and incubated with primary antibodies. Immunoreactive bands were detected using horseradish peroxidase-conjugated antibodies and ECL Plus Western Blotting Detection Reagent (GE Healthcare).

Correlative light microscopy-electron microscopy

NIH3T3 cells stably expressing CFP-Gal3 and YFP-LC3, and either empty vector or mStrawberry-Atg4B^{C74A} and HeLa cells stably expressing GFP-LC3 were cultured on glass-bottom dishes with a grid pattern (P35G-2-14-C-GRID; MatTek). HeLa cells stably expressing GFP-Gal3 were transfected with mStrawberry-Gal3 according to the manufacturer's protocol. After incubation with or without LLOMe for 1 or 2 h, cells were fixed with 4% formaldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 100 mM NaCl, 2 mM CaCl_2 , and 1 μ g/ml Hoechst 33342 for 30 min at room temperature; washed in 0.1 M cacodylate buffer (pH 7.4) containing 7% sucrose on ice; and observed using a confocal laser scanning microscope (FV1000; Olympus). The same specimens were incubated with 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 100 mM NaCl, 2 mM CaCl_2 at 4°C overnight. After three washes in 0.1 M cacodylate buffer (pH 7.4) containing 7% sucrose, the samples were post-fixed for 1 h with 1% Osmium tetroxide containing 0.8% potassium ferrocyanide, washed three times in distilled water, dehydrated in ethanol, and embedded in Epon812 (TAAB Laboratories Equipment). Ultrathin sections (70 nm thick) were stained with saturated uranyl acetate and Reynold's lead citrate solution. The electron micrographs were taken with a JEOL JEM-1011 transmission electron microscope (JEOL).

Hyperuricemic animal model

GFP-LC3 transgenic mice and renal tubule-specific Atg5-deficient mice (Atg5^{F/F};KAP) have been described previously (Kuma *et al*, 2004; Kimura *et al*, 2011). All experiments were performed using 8-week-old male mice. Mice were allowed access to standard chow *ad libitum*. All animal experiments were approved by the institutional committee of the Animal Research Committee of Osaka University and the Japanese Animal Protection and Management Law (No. 25). Hyperuricaemia was induced by injection with UA (100 mg/kg) and OA (50 mg/kg) three times with an interval of 12 h. Water intake was restricted for 24 h after the first UA injection in order to facilitate hyperuricaemia. As a control, vehicle (water)-injected mice were followed concurrently. These mice were sacrificed 24 h after the last injection of UA + OA or vehicle.

Biochemical measurements

The serum urea nitrogen concentration was measured using the BUN-test-Wako (Wako Pure Chemical Indust.), and the creatinine concentration was determined using the CRE-EN Kainos Kit (Kainos Laboratories, Inc.).

Histological analysis

Mice were transcardially perfused with 4% paraformaldehyde in phosphate buffer (pH 7.4). Tissues were post-fixed and embedded in paraffin or frozen in OCT compound (Miles Laboratories). Paraffin or frozen sections were immunolabelled, and the immunofluorescence images were collected using Olympus FV1000-D (Olympus). The number of ubiquitin-positive puncta was counted in at least 10 high power fields for each sample.

Evaluation of kidney tubular damage

From PAS-stained samples, kidney tubular damage was graded as previously described with some modification (Takahashi *et al*, 2012). In brief, PAS-stained sections were scored according to the percent of damaged tubules: 0, no damage; 1, <5% damage; 2, 5–10% damage; 3, 10–25% damage; 4, 25–50% damage; and 5, >50% damage. Tubular damage was defined as loss of brush border, vacuolization, tubular dilation, and cast formation. At least 10 fields ($\times 200$) were reviewed for each tissue sample.

Electron microscopy

Kidneys from Atg5^{F/F} and Atg5^{F/F};KAP mice treated with UA + OA were fixed with 2.5% glutaraldehyde and subjected to conventional electron microscopic analysis as previously described (Kimura *et al*, 2011).

Statistical analyses

All values for *in vitro* experiments except for Supplementary Figure S5E were presented as means $\pm$ s.d. Results in Supplementary Figure S5E were presented as means $\pm$ s.e. All results from *in vivo* experiments were presented as means $\pm$ s.e. Statistical analyses

were conducted using the JMP software (SAS institute). Multiple group comparisons were performed using analysis of variance (ANOVA) with post-testing according to Dunnett's test. A *P*-value of <0.05 was considered as statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: IM, TN, and TY designed the experiments. IM performed most of the experiments. AT performed the mice experiments. TK, YT, and YI advised the mice experiments. HO performed correlative light and electron microscopy. AT, MH, and AY performed conventional electron microscopy. TS and TN gave conceptual advice. IM, AT, YI, and TY wrote the manuscript. TY supervised the project.

Conflict of interest

The authors declare that they have no conflict of interest.

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