Title:
Glucosamine induces autophagy via an mTOR-independent pathway

Running title:
Glucosamine induces autophagy

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Abbreviations: Atg, autophagy-related genes; LC3, microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin

Abstract

Autophagy is a cellular process that nonspecifically degrades cytosolic components and is involved in many cellular responses. We found that amino sugars with a free amino group such as glucosamine, galactosamine and mannosamine induced autophagy via an mTOR-independent pathway. Glucosamine induced autophagy at concentrations of at least 500 μM to over 40 mM. In the presence of 40 mM glucosamine, autophagy induction was initiated at 6 h and reached a plateau at 36 h. Glucosamine-induced autophagy could remove accumulated ubiquitin-conjugated proteins as well as 79-glutamine repeats. Therefore, orally administered glucosamine could contribute to the prevention of neurodegenerative diseases and promotion of antiaging effects.

Introduction

Macroautophagy (autophagy) is a cellular process that nonspecifically degrades cytosolic components. An autophagic membrane engulfs a part of the cytosol containing proteins and organelles, and fuses with the lysosome to lyse the inner components [1; 2]. Autophagy was originally described as a cellular response to starvation [3]. Later, autophagy induction was known to play critical roles in various cellular responses such as prevention of infection by bacteria and viruses [4; 5], antigen presentation [6; 7], early embryogenesis [8], survival of newborns [9], neural development [10], and lipid metabolism [11]. In contrast to induced autophagy in response to environmental signals, basal level autophagy may also have important functions in quality control and antiaging of the cells [12; 13; 14].

More than thirty genes essential for the process of autophagy have been reported [3]. Among them, microtubule-associated protein 1 light chain 3 (LC-3) has so far been the best marker protein for autophagosomes [15; 16; 17]. LC-3 is constitutively expressed as a pro-form in the cytosol and immediately processed to remove C-terminal peptides by Atg4 forming soluble LC3-I. When autophagy is induced, the newly generated carboxyl-group of the C-terminal glycine residue in LC3-I is transferred to the amide group of phosphatidylethanolamine by a sequential three-step reaction involving Atg7 and Atg3, generating autophagosomal membrane-bound LC3-II.

Induction of autophagy by drugs is one of the promising therapeutic approaches for treatment of neurodegenerative disorders such as Parkinson, Huntington and polyglutamine diseases. These diseases all involve the accumulation of insoluble degenerated proteins in the cytosol of neural cells. Autophagy inducible chemical compounds were shown to be able to clear the accumulated proteins *in vivo* [18]. The natural disaccharide trehalose was also reported to be able to induce autophagy and accelerate the clearance of mutant huntingtin and α-synuclein [19; 20].

Our interests have focused on the biofunction of chitosan/chitin and their hydrolysates as food ingredients. Glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) form the constitutional unit of chitosan and chitin, respectively, and have been reported to function in reducing arthritic pain and improving skin quality [21; 22; 23]. During the course of our studies, we found that GlcN has an autophagy induction activity. In addition, we screened various sugars and found that all tested amino sugars with a free amino group are active in autophagy induction.

Materials and methods

Materials. GlcN, chitobiose *N*,*N*'-diacetylchitobiose were purchased from Seikagaku Corporation (Japan); GalN, ManN, trehalose, rapamycin and bafilomycin A1 were from Sigma-Aldrich; lactacystin was from Cayman Chemical; the other materials were from Wako Pure Chemical Industries.

Cell culture. HeLa and COS7 cells were cultured in DMEM supplemented with 10% FCS. PC12-derived TQ15 cells stably transfected with pTet-tTAK and pTet-spliced/FLAG-tagged 79-glutamine repeats (Q79) were cultured in low glucose DMEM supplemented with 10% FCS and 5% horse serum. Sugars (0.1-80 mM) were added to 50% confluent cells and the cells were further cultured for the appropriate period. The cells were removed from the dish by Cell Scrapers, and washed with PBS twice.

Western blotting. Cells were lysed in 20 mM Tris/HCl buffer (pH 7.4) containing 1.0% Triton X-100, 150 mM NaCl, and complete protease inhibitor cocktail (Roche). After centrifugation at 12,000 rpm for 10 min, supernatants were obtained. Protein concentration was measured using the BCA Protein Assay Kit (Pierce). Each supernatant containing 10 μg of proteins was separated by 7.5% or 12% SDS-PAGE gel under reducing conditions and blotted onto a PVDF membrane. The membrane was blocked with 5.0% bovine serum albumin (Nacalai Tesque, Japan) for detection of phosphorylated proteins or with 2.0% skim milk (Wako) for other ones. The first antibodies used were rabbit anti-LC3 polyclonal (1/2000, MBL, Japan), anti-phosphorylated S6 (1/2000, Cell Signaling Technology), anti-phosphorylated 4E-BP1 (1/2000, Cell Signaling Technology), anti-ubiquitin monoclonal, clone Ubi-1 (1/1000, Zymed Laboratories), anti-FLAG monoclonal, clone M2 (1/1000, Sigma-Aldrich) and rabbit anti-actin (1/2000, Sigma-Aldrich). The second antibody was horseradish peroxidase-conjugated anti-mouse IgG (1/4000, MBL) or anti-mouse IgG (1/4000, Santa Cruz Biotechnology). Detection was carried out using West Pico Chemiluminescent Kit (Pierce) and LAS Image Analyzer (Fuji Film, Japan). The estimation of the amounts of proteins was carried out by analyzing the data of Western blotting using the Image Gauge program (Fuji Film).

Fluorescent microscopy. Cells grown on glass were fixed with

paraformaldehyde and then treated with 0.1% Triton X-100. Autophagosomes were stained with rabbit anti-LC3 antibody and Alexa Flour 594-conjugated anti-rabbit IgG (Invitrogen). Cells were analyzed using a fluorescent Olympus IX-70 microscope (Olympus, Japan).

Results

Screening of various sugars for autophagy inducible activity

We found that a mixture of acid hydrolysates from chitin showed strong activity for autophagy induction in mammalian cells. To identify the minimal structure required for the activity, we tested GlcN, GlcNβ1-4GlcN (chitobiose), GlcNAc, GlcNAcβ1-4GlcNAc (*N,N'*-diacetylchitobiose) and other various sugars unrelated to chitosan/chitin. HeLa cells were treated with 40 mM of each sugar for 24 h and then harvested. The lysates were analyzed by Western blotting using anti-LC3 antibody to detect the conversion of LC3-I to LC3-II (Fig. 1A). Among the 21 types of mono- and disaccharides tested, three amino sugars, GlcN (lane 9), mannosamine (ManN; lane 10) and galactosamine (GalN; lane 11), were highly active, and chitobiose (lane 18) and trehalose (lane 14) were moderately active. The other sugars, including GlcNAc (lane 8) and *N,N'*-diacetylchitobiose (lane 17), showed no or only trace activities. Similarly in COS7 cells, GlcN induced autophagy, which was much stronger than that induced by trehalose (Fig. 1B, lane 2 vs. lane 4). GalN and ManN also induced autophagy in COS7 cells (data not shown).

The autophagosome formation was analyzed by fluorescent microscopy using HeLa cells and the same antibody as used in Western blotting (Fig. 1C). A significant increase in the number of dots was observed in the cytosol after the treatment with GlcN compared with the control. COS7 cells were also tested for autophagosome formation and similar results were obtained (data not shown). These results clearly indicate that GlcN as well as GalN and ManN induce LC3-II conversion and concomitant autophagosome formation.

Characterization of autophagy induced by GlcN

To confirm that LC3-II-containing autophagosomes formed by treatment with GlcN flow into lysosomal degradation, we tested the effect of a lysosome inhibitor (Fig.

2A). When COS7 cells were treated with both 10 mM GlcN and 200 nM bafilomycin A1, an inhibitor for H^+-ATPase found in the ion channel on the lysosomal membrane, LC3-II was markedly accumulated compared with that in the cells treated only with GlcN (lane 4 vs. lane 2). This result confirms that GlcN does not affect the lysosomal degradation of LC3-II, but induces the conversion of LC3-I to LC3-II.

Next, we tested the dose dependency and time course of GlcN-induced autophagy in COS7 cells by Western blotting. The strength of activity (LC3-II conversion) was dose dependent up to 40 mM GlcN. Weak upregulation was observed at a minimum of 2 mM (Fig. 2B). GalN and ManN also showed detectable activity at a concentration of 2 mM, but trehalose did not (data not shown). To further analyze the minimal concentration of GlcN required for detectable activity, we treated COS7 cells with a lower concentration range of GlcN in the presence of bafilomycin A1 (Fig. 2C). In this assay, we observed significant activity at 500 μM GlcN. LC3-II conversion was first detected after 6 h of treatment of 40 mM GlcN and gradually increased almost linearly until 24 h, followed by reaching a plateau at 36 h (Fig. 2D). This indicated that this was a very slow response in comparison with autophagy induced by nitrogen starvation.

GlcN induces autophagy via an mTOR-independent pathway

To date, the mTOR-dependent pathway is the only known intracellular signaling pathway of autophagy induction. However, several autophagy inducers are known to employ an mTOR-independent pathway. Thus, we investigated whether the autophagy induced by GlcN is mediated by an mTOR-dependent or independent pathway. To test mTOR dependency, we detected the activation of the downstream molecules of mTOR (Fig. 3). By treating COS7 cells with rapamycin, an inhibitor for mTOR, the phosphorylation levels of S6 and 4E-BP1 proteins decreased, which are known signaling molecules downstream of mTOR (lane 2). By contrast, after treatment with 40 mM GlcN, the phosphorylation levels of S6 and 4E-BP1 proteins were not markedly changed compared with the control and Glc treatments (lane 3 vs. lanes 1 and 4). This result suggests that GlcN-induced autophagy is mediated by an mTOR-independent pathway.

Autophagy induced by GlcN enhanced degradation of accumulated proteins in the

cytosol

First, we tested whether GlcN-induced autophagy enhanced degradation of ubiquitinated proteins (Fig. 4A). After treatment of the cells for 48 h with 200 μM lactacystin, a proteasome inhibitor, ubiquitinated proteins accumulated in the cytosol were detected as smear bands by Western blotting using anti-ubiquitin antibody (lane 4). The amount of accumulated ubiquitinated proteins was estimated as 4.9 times that of the control (lane 1). When both 40 mM GlcN and 200 μM lactacystin were added to the cells, the smear bands of ubiquitinated proteins clearly decreased (lane 3). The amount of ubiquitinated proteins was 3.1 times that of the control: i.e., 40% of ubiquitinated proteins were removed by addition of GlcN.

Next, we tested the degradation of polyglutamines, a causative protein of many types of polyglutamine diseases. PC12 cells expressing FLAG-tagged 79-glutamine repeats (Q79) under the control of the Tet-off system (TQ15 cells) [24] were used. We induced autophagy in the cells before expression of Q79 because polyglutamine aggregates once formed in the cytosol and then cannot be degraded by autophagy [25]. After 12 h of treatment with GlcN, cells were transferred to a new medium containing no tetracycline to induce Q79 and further incubated for 24 h. The cell lysate was analyzed by Western blotting using anti-LC3 and anti-FLAG antibodies. Under these conditions, LC3 conversion occurred, indicating that GlcN induced autophagy in PC12 cells (Fig. 4B). Concomitantly, polyglutamine aggregates in the stacking gel as well as a 30 kDa Q79 band corresponding to a monomer in the separating gel [26] indicated that an enhanced degradation had taken place (Fig. 4C). The amounts of insoluble and soluble polyglutamines were estimated as 25% and 30% of the control, respectively. Taken together, these results suggest that autophagy induced by GlcN could enhance the removal of neurodegenerative proteins accumulated in the cytosol.

Discussion

Chitin is one of the most abundant natural biopolymers on earth and is produced by arthropods, fungi and a portion of cephalopods. GlcN is produced commercially by the hydrolysis of crustacean exoskeletons mainly composed of chitin, and is commonly used as a complementary or alternative medicine for the treatment of

osteoarthritis. Since GlcN can be a precursor of UDP-GlcNAc, orally administered GlcN is considered to enhance the biosynthesis of glycosaminoglycans and proteoglycans, both crucial components of cartilage.

In this study, we have identified a novel function of GlcN as an autophagy inducer. We confirmed that GlcN induced the conversion of LC3 and formation of LC3-positive autophagic vesicles, and concomitantly enhanced the degradation of ubiquitinated proteins and polyglutamine in the cytosol. GlcN-induced autophagy was mediated via an mTOR-independent pathway because the phosphorylation levels of S6 and 4E-BP1 proteins were not changed in the presence of GlcN. However, a more detailed mechanism by which GlcN induces autophagy remains unclear. As already known, GlcN is incorporated into the cells via glucose transporters, and then is metabolized into UDP-GlcNAc through GlcN-6-phosphate. GlcN was reported to cause increasing metabolite flux through the hexosamine biosynthetic pathway with concomitant reduction of intracellular ATP levels [27]. It is possible that a reduced intracellular ATP level causes autophagy. In fact, 2-deoxy-Glc, a known inhibitor for ATP biosynthesis, has been shown to induce autophagy [28; 29]. However, 2-deoxy-Glc-induced autophagy was reported to be via an mTOR-dependent pathway [28], which is quite different from GlcN- and trehalose-induced autophagy [19].

It is also well known that upregulated hexosamine biosynthesis causes an increase of *O*-GlcNAc modified proteins in the nucleocytoplasm. In our studies, GlcN was active whereas GlcNAc was not, suggesting that GlcN-induced autophagy may not be related to the upregulated flux of hexosamine biosynthesis and *O*-GlcNAc modification of proteins.

GlcN was also reported to induce ER stress by unknown mechanisms [30; 31]. A reduction in intracellular ATP levels may be one of the reasons of ER-stress upregulation, because the ER chaperon BiP requires ATP. Unfolded proteins in the ER lumen are transported to the cytosol through the ERAD (ER-associated degradation) system and accumulation of unfolded proteins in the cytosol may result in the induction of autophagy. In yeast, ER-stress was confirmed to induce autophagy [32; 33].

Since all amino sugars with a free amino group tested here were active in autophagy induction, we speculate that the amino group in the sugars is involved in their activity. Recently, spermidine, a type of polyamine, was reported to induce autophagy in *Drosophila* and promote life-span longevity [34]. It is highly possible that

amino sugars and polyamines share a common mechanism of autophagy induction via the amino group in their molecules. On the other hand, amino acids are well known to be strong negative regulators for autophagy. Thus it needs to be clarified which amino compounds are active and whether amino groups are involved in induction of autophagy.

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Figure legends

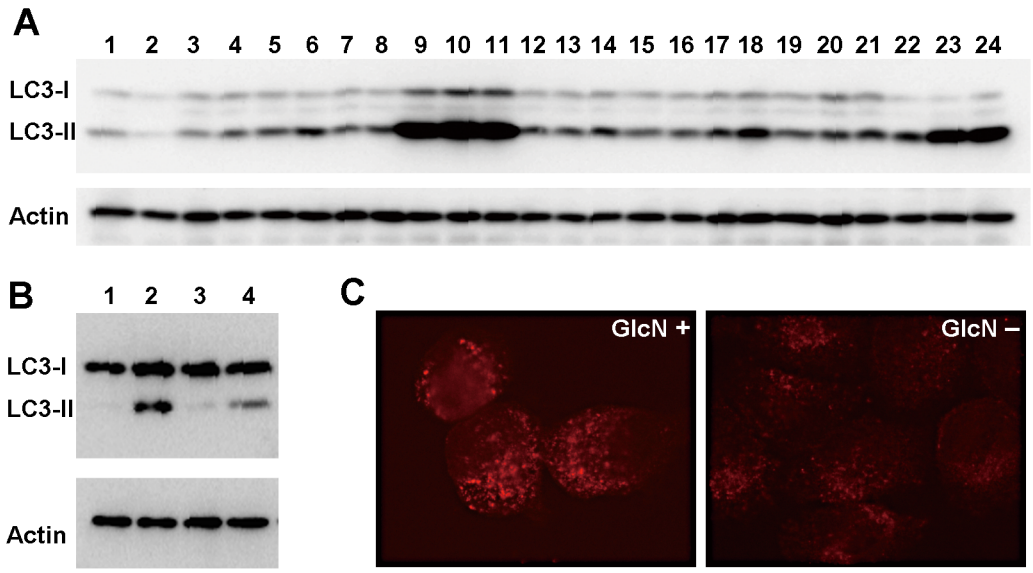
Fig. 1. Amino sugars induce autophagy. (A) Various sugars were screened for autophagy inducible activity. HeLa cells were incubated with indicated sugars (40 mM) for 24 h, and conversion of LC3-I to LC3-II was analyzed by Western blotting. Actin was stained as the loading control. Lane 1, none; 2, glucose; lane 3, mannose; lane 4, galactose; lane 5, fructose; lane 6, rhamnose; lane 7, fucose; lane 8, GlcNAc; lane 9, GlcN; lane 10, ManN; lane 11, GalN; lane 12, arabinose; lane 13, xylose; lane 14, trehalose; lane 15, maltose; lane 16, cellobiose; lane 17, *N*,*N*'-diacetylchitobiose; lane 18, chitobiose; lane 19, sucrose; lane 20, melibiose; lane 21, lactose; lane 22, lactulose; lane 23, 0.2 μM rapamycin; lane 24, 0.4 μM rapamycin. (B) COS7 cells were incubated with GlcN, GlcNAc and trehalose, and analyzed for LC3 conversion by Western blotting. Lane 1, none; lane 2, 40 mM GlcN; lane 3, 40 mM GlcNAc; lane 4, 80 mM trehalose. (C) Autophagosome formation was analyzed by fluorescent microscopy. HeLa cells were cultured for 30 h in the presence (left panel) or absence (right panel) of GlcN, and then stained with anti-LC3 and Alexa594-conjigated anti-rabbit IgG.

Fig. 2. Characterization of autophagy induced by GlcN. (A) Flux of autophagy induced by GlcN was examined. COS7 cells were treated with 10 mM GlcN and/or 200 μM bafilomycin A1 for 48 h, and then conversion of LC3-I to LC3-II was analyzed by Western blotting. Lane 1, none; lane 2, GlcN; lane 3, bafilomycin A1; lane 4, both GlcN and bafilomycin A1. (B) Dose dependency of GlcN on autophagy induction was analyzed by LC3 Western blotting. COS7 cells were incubated with various concentrations of GlcN for 36 h. Lanes 1-8, 0, 2, 5, 8, 10, 20, 40, and 80 mM GlcN, respectively. (C) Dose dependency of GlcN on autophagy induction in the presence of 200 μM bafilomycin A1. Lane 1, none; lanes 2-7, 200 μM bafilomycin A1 plus 0, 0.1, 0.2, 0.5, 1.0, and 2.0 mM GlcN, respectively. (D) Time course of autophagy induction by GlcN was analyzed by LC3 Western blotting. COS7 cells were incubated with 40 mM GlcN for various periods. Lanes 1-8, 0, 3, 6, 12, 24, 36, and 48 h, respectively.

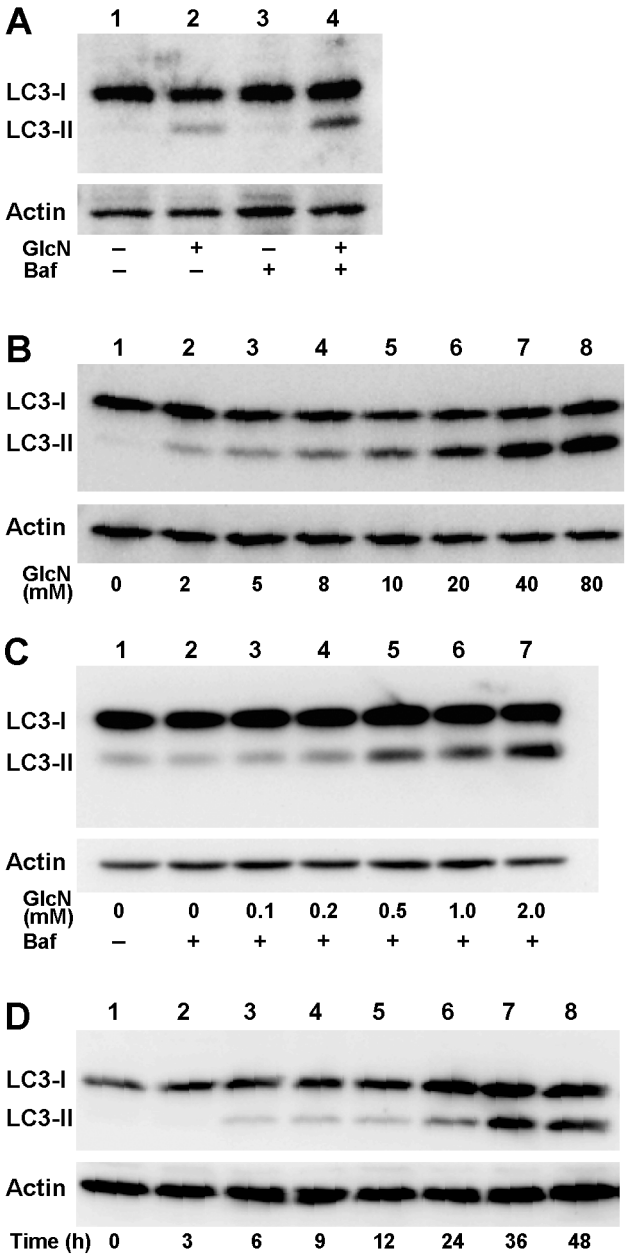
Fig. 3. Autophagy is induced by GlcN via an mTOR independent pathway. COS7 cells were treated with either 40 mM GlcN, 40 mM glucose or 0.4 μM rapamycin for 24 h. Phosphorylation of S6 and 4E-BP1 proteins, which are the downstream signaling

molecules of mTOR, were analyzed by anti-phosphorylated S6 and anti-phosphorylated 4E-BP1. Rapamycin is an inhibitor for mTOR. Lane 1, none; lane 2, rapamycin; lane 3, GlcN; lane 4, glucose.

Fig. 4. GlcN enhances degradation of ubiquitinated proteins and polyglutamines. (A) COS7 cells were treated with 40 mM GlcN and/or 10 μM lactacystin, a proteasome inhibitor, for 48 h. Accumulated ubiquitinated proteins (Ub-Ps) were detected by Western blotting using an anti-ubiquitin antibody. Lane 1, none; lane 2, GlcN only; lane 3, lactacystin and GlcN; lane 4, lactacystin only. (B) PC12 cells expressing FLAG-tagged 79-glutamine repeats (Q79) under the control of the Tet-off system (TQ15 cells) were treated with 40 mM GlcN for 36 h, and then polyglutamine expression was started by removing tetracycline from the GlcN-containing medium followed by culturing for a further 24 h. LC3 conversion was examined by Western blotting. Lane 1, none; lane 2, GlcN. (C) In the same lysates of TQ15 cells, Q79 was detected by Western blotting using an anti-FLAG antibody. The same amount of proteins as in the previous panel was loaded onto each lane. Insoluble Q79 was detected in the stacking gel as smear bands, and a 30 kDa Q79 band was identified in the separating gel. Lane 1, none; lane 2, GlcN.



Shintani et al. Fig. 1



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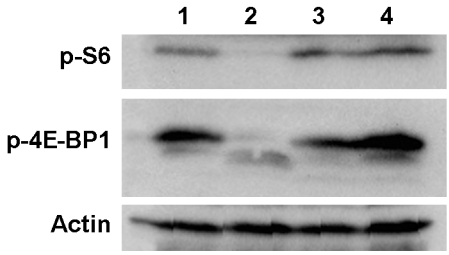

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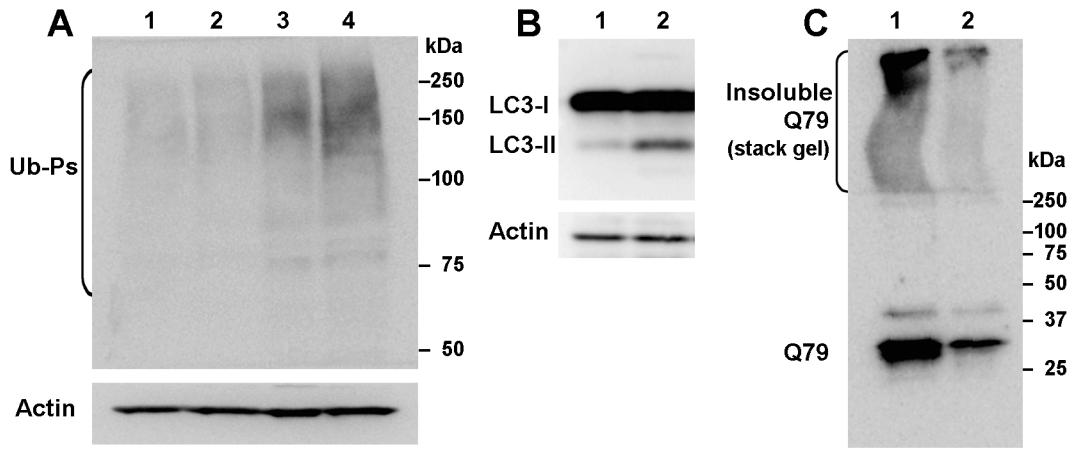

Shintani et al. Fig. 4