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# LC3B is indispensable for selective autophagy of p62 but not basal autophagy

Yoko Maruyama [a,b], Yu-Shin Sou [a], Shun Kageyama [a], Takao Takahashi [b], Takashi Ueno [c], Keiji Tanaka [d], Masaaki Komatsu [a,e,*], Yoshinobu Ichimura [a,*]

[a] Protein Metabolism Project, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan
[b] Department of Pediatrics, School of Medicine, Keio University, Tokyo 160-8582, Japan
[c] Division of Proteomics and Biomolecular Science, Center for Biomedical Research Resources, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan
[d] Laboratory of Protein Metabolism, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan
[e] Department of Biochemistry, School of Medicine, Niigata University, Niigata 951-8510, Japan

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ABSTRACT

Autophagy is a unique intracellular protein degradation system accompanied by autophagosome formation. Besides its important role through bulk degradation in supplying nutrients, this system has an ability to degrade certain proteins, organelles, and invading bacteria selectively to maintain cellular homeostasis. In yeasts, Atg8p plays key roles in both autophagosome formation and selective autophagy based on its membrane fusion property and interaction with autophagy adaptors/specific substrates. In contrast to the single Atg8p in yeast, mammals have 6 homologs of Atg8p comprising LC3 and GABARAP families. However, it is not clear these two families have different or similar functions. The aim of this study was to determine the separate roles of LC3 and GABARAP families in basal/constitutive and/or selective autophagy. While the combined knockdown of LC3 and GABARAP families caused a defect in long-lived protein degradation through lysosomes, knockdown of each had no effect on the degradation. Meanwhile, knockdown of LC3B but not GABARAPs resulted in significant accumulation of p62/Sqstm1, one of the selective substrate for autophagy. Our results suggest that while mammalian Atg8 homologs are functionally redundant with regard to autophagosome formation, selective autophagy is regulated by specific Atg8 homologs.

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## 1. Introduction

Macroautophagy (hereafter referred to as autophagy) is an evolutionally conserved intracellular bulk degradation system. Single membrane sac called isolation membrane or phagophore elongates and sequesters cytoplasmic components including entire organelles randomly. Subsequently, the edge of isolation membrane fuses with each other to form a double membrane structure called autophagosome. Eventually, the contents in autophagosome are degradated via the fusion with lysosome [1]. This pathway is highly inducible and serves as a supplier of molecular building blocks under starved conditions and also contributes to cellular renovation during cell differentiation [2]. Besides such fundamental role, increasing evidence points to the selectivity of autophagy in sorting of vacuolar enzymes and removal of aggregate-prone proteins, unwanted organelles, and invading bacteria [3]. Such selectivity allows diverse cellular regulation, similar to the ubiquitin proteasome pathway.

Taking advantage of yeast genetics and biochemical analysis, 36 *ATG* (autophagy-related) genes have been identified so far [1]. The molecular function of each Atg protein is almost conserved in eukaryotes from yeasts to mammals. Among them, Atg8 and its mammalian homolog microtubule-associated protein 1 light chain 3/MAP1LC3 (LC3), whose structures comprise ubiquitin fold and N-terminal two α-helices, play a crucial role in autophagosome formation [4,5]. Atg4, a cysteine protease, processes the C-terminal amino acid of Atg8/LC3 to expose a glycine residue immediately

*Abbreviations:* GABARAP, gamma-aminobutyrate receptor-associated protein; GEC-1, glandularepithelial cell protein 1; GATE-16, golgi-associated ATPase enhancer of 16 kDa; ITC, isothermal titration calorimetry; LC3, microtubule-associated protein 1 light chain 3/MAP1LC3; LIR, LC3-interacting region; PE, phosphatidylethanolamine.

* Corresponding authors. Address: Protein Metabolism Project, Tokyo Metropolitan Institute of Medical Science, Tokyo 156-8506, Japan.

*E-mail addresses:* komatsu-ms@igakuken.or.jp (M. Komatsu), ichimura-ys@igakuken.or.jp (Y. Ichimura).

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after its synthesis [1]. Thereafter, matured Atg8/LC3 is activated by Atg7 (E1) in an ATP-dependent manner and transferred to Atg3 (E2) [1]. Subsequently, the C-terminal glycine of Atg8/LC3 conjugates to the amino group of phosphatidylethanolamine (PE) in an Atg12–Atg5 Atg16-dependent manner [1]. Atg8-PE/LC3-PE is localized in the inner and outer membranes of the isolation membrane/phagophore [1]. Atg8-PE/LC3-PE localized on the outer membrane is re-cleaved by Atg4 following completion of autophagosome formation and released Atg8/LC3 is recycled, whereas Atg8-PE/LC3-PE present on the inner membrane is degraded together with other cellular constituents by vacuolar/lysosomal proteases [1]. The Atg8-PE/LC3-PE is essential for biogenesis and closure of autophagosomal membrane [6–8].

In addition to the importance of both Atg8 and its homologs in autophagosome formation, their interaction with specific proteins plays a crucial role in selective autophagy [3]. p62/Sqstm1 (hereafter referred to as p62), which is the best characterized autophagy-substrate in mammals, localizes at the autophagosome formation site and directly interacts with Atg8 homologs through the LC3-interacting region (LIR) [9–11], and it is incorporated subsequently into the autophagosome and finally degraded. Impaired autophagy is accompanied by the accumulation of p62, followed by the formation of aggregates positive for p62 and ubiquitinated proteins because of the nature of both self-oligomerization and the ubiquitin-binding capacity of p62 [12]. This protein serves as a signaling hub for several signal transductions and as an autophagy-adaptor for ubiquitinated cargos [13].

Human has 6 genes coding for Atg8 homologs, and gene products can be grouped into two subfamilies; (1) the LC3 subfamily containing LC3A, LC3B, and LC3C, and (2) the gamma-aminobutyrate receptor-associated protein (GABARAP) subfamily containing GABARAP, GABARAPL1/GEC-1, and GABARAPL2/GATE-16. While previous studies showed that these proteins are all conjugated to PE [14], they appear to have complex non-redundant functions in membrane biogenesis of autophagosomes and in preferential binding to adaptors/targets for selective autophagy [5,8,15–17]. Herein we demonstrate that LC3 and GABARAP families are both dispensable for basal autophagy in HEK293T cells and that among of the 6 Atg8 homologs, LC3B is responsible for selective degradation of p62 through autophagy.

## 2. Materials and methods

### 2.1. Cell culture and transfection

HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 5 U/ml penicillin and 50 μg/ml streptomycin. At subconfluence, HEK293T cells were transfected with the indicated 25 nM siGENOME SMART pool siRNAs (MAP1LC3B siRNA Pool: M012846, GABARAP siRNA Pool: M012368, GABARAPL1 siRNA Pool: M014715, GABARAPL2 siRNA Pool: M006853, ULK1 siRNA Pool: M-005049, ATG3 siRNA Pool: D-015375, Non-targeting siRNA Pool: D-001206, Thermo Scientific) using DharmaFECT 1 transfection reagent according to the manufacturer's protocol (Thermo Scientific). Cells were analyzed at 48 h after transfection. The immortalized MEFs were transfected with GFP-LC3, GFP-GABARAP, GFP-GABARAPL1 or GFP-GABARAPL2 using the retrovirus vector system, and then cultured with medium in the presence of 2 μg/mL puromycin to select stable transformants.

### 2.2. RT-PCR

cDNA was synthesized from 1 μg of DNase I-treated total RNA using the SuperScript First-Strand Synthesis System (Gibco BRL) and oligo $(dT)_{12-18}$ primers. Specific primers for each gene were indicated in Supplementary Table 2.

### 2.3. Droplet digital-PCR

Using the Transcriptor First-Strand cDNA Synthesis Kit (Roche Applied Science), cDNA was synthesized from 1 μg of total RNA. Digital PCR was was performed using the QX100 Droplet Digital PCR system (BioRad) according to the manufacturer's instructions [18]. Specific primer pairs were designed by ProbeFinder software (Roche). Data from absolute quantification are shown.

### 2.4. Immunological analysis

Samples were separated using the NuPAGE system (Invitrogen) on 12% Bis-Tris gels in MOPS-NuPAGE buffer, and then transferred to polyvinylidene difluoride (PVDF) membranes. The antibodies for LC3B (#2775, Cell Signaling Technology), GABARAP (PM037, MBL), GABARAPL1 (ab86497, Abcam), GABARAPL2 (PM038, MBL), Atg3 (PM034, MBL), ULK1 (H-240, Santa Cruz), p62 (GP62-C, Progen), and actin (MAB1501, Millipore) were purchased from the indicated suppliers. Band intensities were quantified by ImageJ 1.42q software (NIH, Bethesda, MD). Statistical significance was calculated by two-tailed Student's $t$ test. A $p$ value less than 0.05 denoted the presence of significant difference. For LC3- or GABARAP- and p62-staining, cells were fixed and stained with anti-LC3B (4E12, MBL) or anti-GABARAP (PM037, MBL) and anti-p62 (GP62-C, Progen) antibodies, respectively, as described previously [10]. Images were acquired with a laser scanning confocal microscope (FV1000-D, Olympus). After image acquisition, image contrast and brightness were adjusted using Photoshop CS4 (Adobe).

### 2.5. Long-lived protein degradation assay

The assay was performed essentially as described previously [19].

### 2.6. Computer-based structure modeling

Structural models of GABARAP-LIR and GABARAPL2-LIR complex were created based on those of the rat GABARAP [20] and bovine GABARAPL2/GATE-16 [21], respectively, using the MOE program (version 2005.06; Chemical Computing Group, Montreal, Canada). The models shown in Fig. 2A were generated by PyMOL.

### 2.7. Isothermal titration calorimetry

GST-tagged LC3 and GABARAPL2 were purified as described previously [10]. LIR peptide (*N-acetyl*-GGDDDWTHLS-amide) was purchased from Toray Research Center. ITC experiments were performed in PBS at 25 °C on a MicroCal-iTC$_{200}$ system (GE Healthcare). In each run, 40 μl of 1.0 mM LIR was injected 39 times at 2 min intervals from a stirring syringe (1000 rpm) into the sample cell containing 200 μl of 0.1 mM LC3 or GABARAPL2/GATE-16. Binding data were analyzed using Origin 5.0 (MicroCal Inc.) and standard deviations were derived from three separate runs.

### 2.8. Pull down analysis

HEK293T co-expressing One-Strep-FLAG-p62 and GFP-LC3, GFP-GABARAP, or GFP-GABARAPL2 cells were lysed in TNE buffer and centrifuged at 15,000 rpm for 5 min at 4 °C. The resulting supernatants were incubated with Strep-Tactin Sepharose (IBA) at 4 °C for 1 h. The protein complexes were washed three times with lysis buffer, and eluted with elution buffer (50 mM Tris–HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, and 2.5 mM D-desthiobiotin [Sigma–Aldrich]).

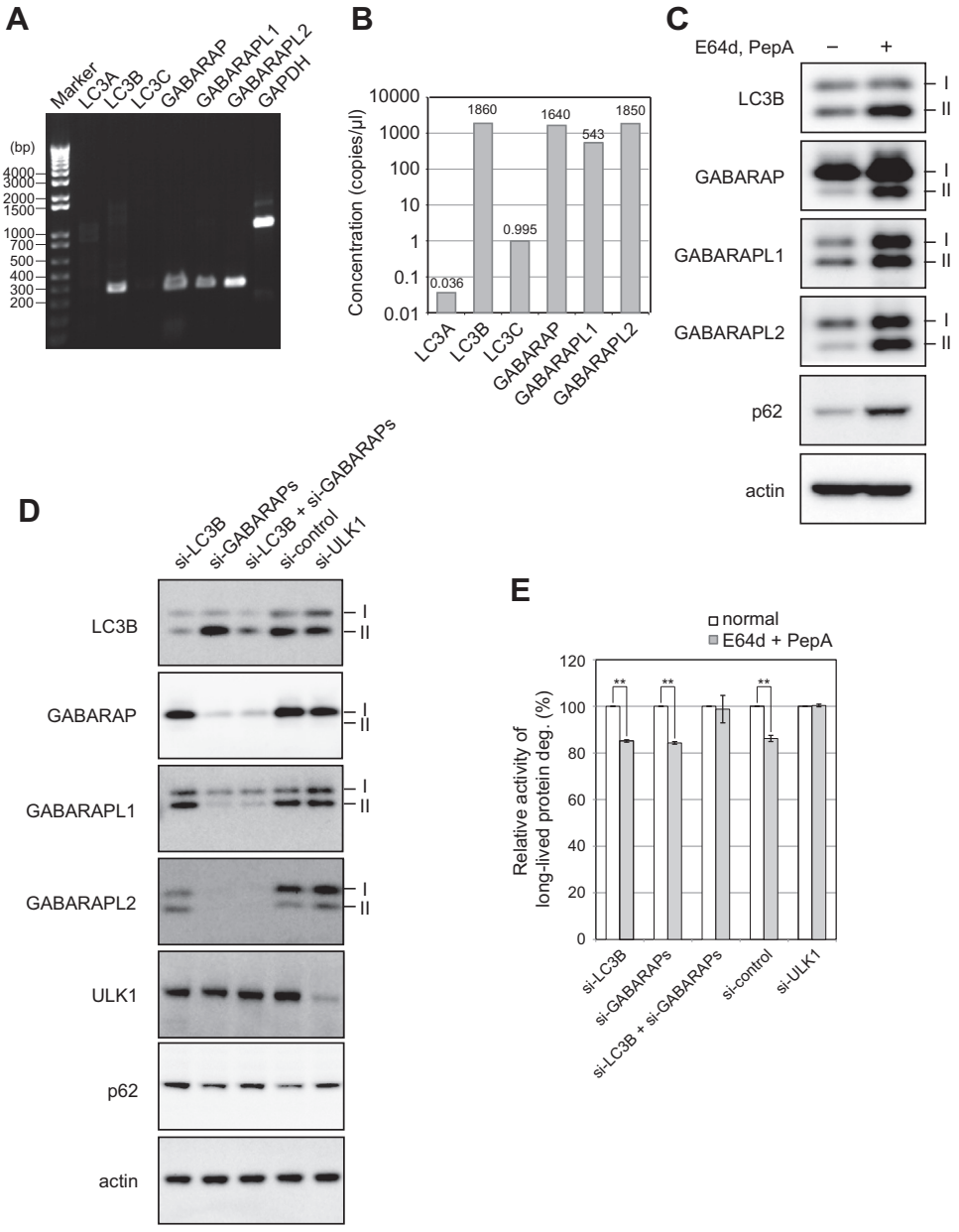


**Fig. 1.** Neither LC3 nor GABARAP is needed for basal autophagy. (A) RT-PCR analysis of *Atg8* homologues in HEK293T cells. (B) Droplet digital PCR analysis of *Atg8* homologues in HEK293T cells. (C) Immunoblot analysis. HEK293T cells were cultured in DMEM containing 10% FCS with or without E64d and pepstatin A (PepA) for 18 h, and then lysates were conducted to immunoblot analysis with the indicated antibodies. I: mature form, II: lipidated form. (D) Knockdown analysis. At 48 h after introduction of the indicated siRNA, the cell lysates were analyzed by immunoblotting with the indicated antibodies. (E) Long-lived protein degradation assay. HEK293T cells shown in (D) were labeled with [$^{14}$C] leucine for 24 h, and degradation of long-lived protein in basal condition was measured. E64d and pepstatin A (E64d + PepA) was added as indicated. Degradation rate of each knockdown cells under normal conditions is indicated as 100%. Data are means ± SE of triplicate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Samples were subjected to NuPAGE and detected by CBB staining or immunoblotting with anti-GFP (A6455, Invitrogen) and anti-p62 (GP62-C, Progen) antibodies.

## 3. Results

### 3.1. Expression of Atg8 homologs in HEK293T cells

All mammalian Atg8 homologs seem to function at different steps of membrane biogenesis of autophagosomes under starvation conditions though they are all conjugated to PE. For instance, LC3 family is involved in elongation of the isolation/phagophore membrane whereas the GABARAP family is essential for a later stage of autophagosome maturation [8]. Is there any functional redundancy among mammalian Atg8 homologs in basal autophagy? To answer this question, we first examined their gene expression in HEK293T cells by RT-PCR analysis. Gene expression of *LC3B* and all *GABARAP* family but not *LC3A* and *LC3C* were recognized in HEK293T cells under normal culture conditions (Fig. 1A). Further, absolute quantification using droplet digital PCR showed that there is quite less expression of *LC3A* and *LC3C* (Fig. 1B). We next sought to determine the protein level of Atg8 homologs in HEK293T cells. To do this, we tested specificity of antibodies against each Atg8 homologs with the recombinant proteins and verified their specificities, though anti-GABARAP and anti-GABARAPL1 antibodies exhibited only modest cross-reactivity with GABARAPL1 and GABARAP, respectively (Fig. S1). Immunoblot analysis with the antibodies showed that LC3B and all GABARAP family proteins

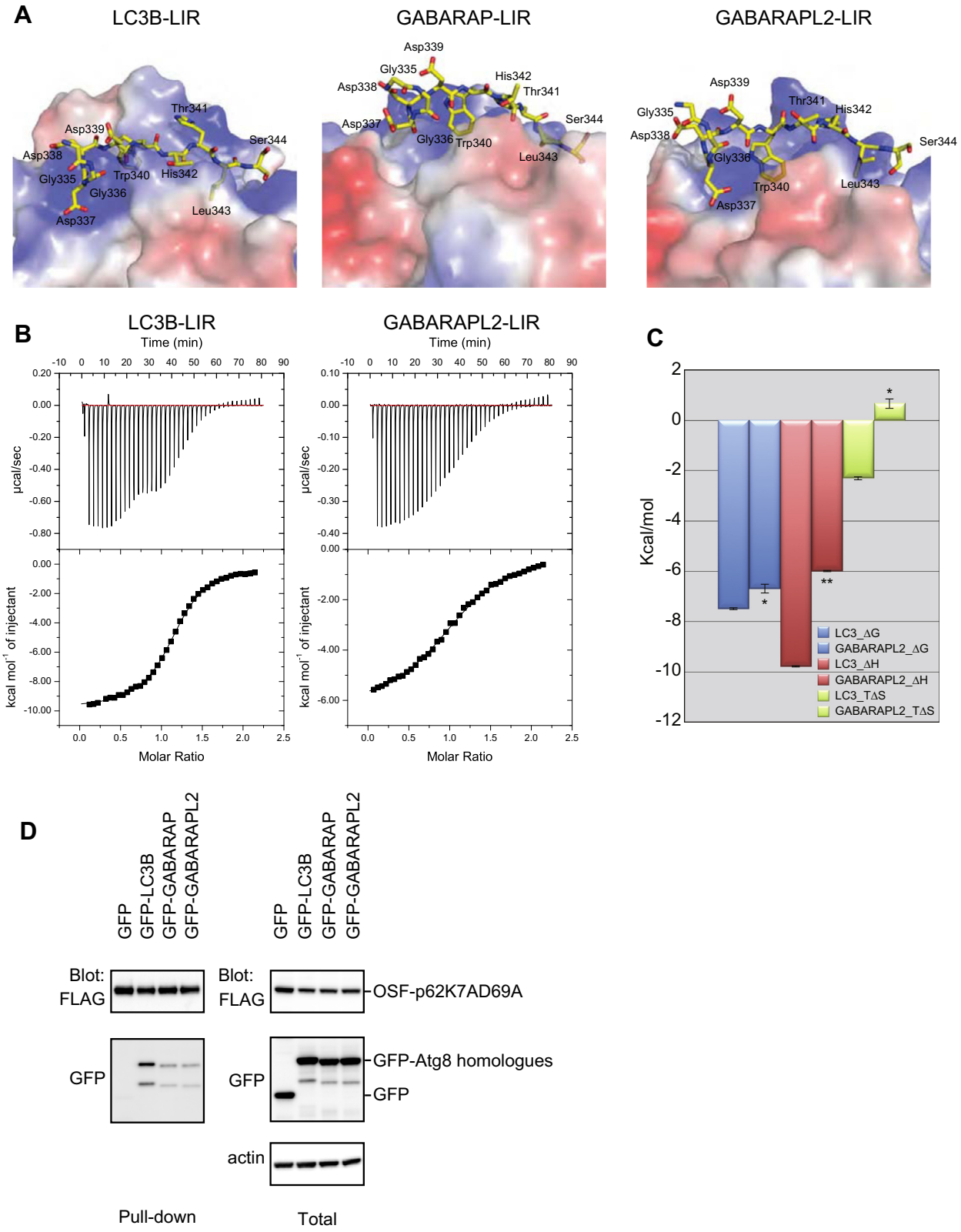


**Fig. 2.** LC3B binds to LIR of p62 at higher affinity than GABARAPs *in vitro*. (A) X-ray crystal structure of LC3B-LIR (2zjd) and models of the GABARAP-LIR and GABARAPL2/GATE-16-LIR complex from published structures (1eo6 and 1kgt). The molecular surface of each LIR interacting region is displayed with a transparent electrostatic potential (red: negative electrostatic potentials, blue: positive electrostatic potentials). Yellow stick model: LIR peptide consisting of 8 amino acids (337-DDDWTHLS-344) in mouse p62. (B) Representative isothermal calorimetric profiles of LC3B and GABARAPL2 titrated by LIR peptide. Top: raw ITC thermograms, Bottom: fitted binding isotherms. (C) Kinetic data obtained from ITC shown in (B). Each bar represents the means ± SE of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. Note the higher affinity of LC3B for LIR compared with GABARAPL2. (D) Precipitation assay. HEK293T cells were transfected with GFP-tagged LC3B, GABARAP or GABARAPL2 together with One-Strep-FLAG tagged p62 K7A/D69A mutant lacking ability of self-oligomerization [23]. At 48 h after transfection, the cell lysates (Total) were subjected to pull down analysis with Strep-Tactin Sepharose, followed by immunoblotting analysis. (Pull-down).

exist in HEK293T (Fig. 1C). Treatment of HEK293T cells with lysosomal inhibitors, E64d and pepstatin A, resulted in the accumulation of their PE-conjugated forms as well as p62 (Fig. 1C), suggesting their association with the autophagosomes followed by lysosomal degradation.

### 3.2. LC3B and GABARAPs are dispensable for basal autophagy

To investigate the effect of loss of each Atg8 homolog on basal autophagic activity, we carried out small interfering RNA (siRNA)-mediated knockdown of *LC3B*, *GABARAP*, *GABARAPL1*, *GABARAPL2* or *ULK1* in HEK293T. The reduction in each Atg8 homolog and ULK1 protein was confirmed by immunoblot analysis (Fig. 1D). ULK1 and 2 are serine/threonine protein kinases essential for autophagy, and the knockdown of ULK1 in case of HEK293 is sufficient for suppression of autophagy [22]. Long-lived protein degradation assays revealed that while the degradation in HEK293T cells harboring each control-, LC3B- or GABARAPs-siRNA, was significantly suppressed by exposure to E64d and pepstatin A, such inhibitory effect was cancelled in HEK293T cells knocked-down for both *LC3B* and *GABARAPs* or *ULK1* (Fig. 1E), implying that either LC3B or GABARAP family is dispensable for basal autophagy.

### 3.3. Preferential interaction of p62 with LC3 family in vitro

In next series of experiments, we investigated the difference between LC3- and GABARAP-family in their interaction with p62. Previous structural analyses of LC3B-LIR of p62 complex revealed hydrophobic interactions of Trp-340 and Leu-343 of the LIR with two different hydrophobic pockets on the LC3B surface, and electrostatic interaction of Asp-337 and Asp-338 in LIR with Arg-10 and Arg-11 in LC3 N-terminal region (Fig. 2A) [10,11]. Based on the crystal structure of LC3B-LIR, models of GABARAPs-LIR complexes were generated by using the MOE program (Chemical Computing Group) (Fig. 2A). Like LC3B, GABARAPs form the major interaction sites of Trp-340 and Leu-343, however; the basic surface on α1 helix of LC3B that interacts with the acidic cluster of Asp-337–Asp-339 was different from that of the corresponding GABARAPs region (Fig. 2A). These models suggest that LC3B have a greater affinity for the LIR than GABARAPs. To test this hypothesis, we assessed the binding energy by isothermal titration calorimetry (ITC) using recombinant LC3B, GABARAPL2 and LIR peptide. Expectedly, the affinity of LIR for LC3B was markedly higher compared with that of the GABARAPL2 (Fig. 2B, C and Supplementary Table 1), and the binding stoichiometry among each complex was 1:1 (Fig. 2B). These results suggest preferential interaction of p62 with LC3 family *in vitro*. We further examined the interaction of Atg8 homologs to p62 in cells. HEK293T cells were transfected with GFP-tagged LC3B, GABARAP or GABARAPL2 together with One-Strep-FLAG tagged p62 K7A/D69A mutant lacking ability of self-oligomerization [23], followed by pull-down with Strep-Tactin Sepharose and immunoblotting analysis (Fig. 2D). While there was little interaction between p62 K7A/D69A and GABARAP or GABARAPL2, LC3B was found to bind to the p62 with high affinity (Fig. 2D).

### 3.4. LC3B but not GABARAP family is indispensable for selective turnover of p62

To examine whether Atg8 homologs co-localize with p62, GFP-LC3B, GFP-GABARAP, GFP-GABARAPL1 and GFP-GABARAPL2 were introduced into mouse embryonic fibroblasts (MEFs). Endogenous p62 co-localized with the puncta positive for GFP-LC3B (Fig. 3A), and approximately 50% of the GFP-LC3B puncta were positive for p62 (Fig. 3B). We also observed co-localization of GABARAP family with p62, but the ratio was quite low compared with case of GFP-LC3B (Fig. 3A and B). To next test the co-localization of endogenous p62 and Atg8 homologs in HEK293T cells, we conducted double immunofluorescence analysis with antibodies against p62 and LC3B or GABARAP. Several LC3B-positive dots corresponding to autophagosomes were detected even under nutrient-rich conditions, and almost p62-positive structures were extensively co-localized with the LC3B-positive structures (Fig. 3C). In contrast, co-localization of p62 was hardly noted on structures positive for GABARAPs though the two were occasionally localized in the immediate vicinity of each other (Fig. 3C), suggesting the association of endogenous p62 with endogenous LC3B but not GABARAP. These results suggested that the majority of p62 is entrapped with LC3B followed by its lysosomal degradation. Actually, siRNA-mediated knockdown of LC3B but not GABARAPs in HEK293T cells caused significant accumulation of p62 (Fig. 4A and B), and the level was comparable with that in *ATG3*-knocked down HEK293T cells (Fig. 4A and B). Taken together, these results indicated that p62 is degraded through autophagy in an LC3B-dependent manner.

## 4. Discussion

In contrast to a previous report that LC3 and GABARAP families are both required for autophagosome formation under starvation conditions [8], our results suggest a functional overlap between LC3B and GABARAP family in at least basal autophagy. A possible explanation for this discrepancy with respect to LC3 is the different experiment setting (i.e., non-starvation versus starvation conditions). The number and size of autophagosome markedly increase in response to nutrient-deprivation [6,8,24,25]. Because LC3 family seems to contribute to elongation of isolation membrane/phagophore [8], LC3B might be prerequisite for membrane biogenesis only in starvation-induced autophagy. Meanwhile, because GABARAPs are thought to be involved in completion of autophagosomes [8], they would be necessary for both basal and starvation-induced autophagy. However, we did not observe any defects in degradation of long-lived proteins through the lysosomes in GABARAPs-knocked down HEK293T cells. Although we cannot exclude the presence of a small amount of GABARAPs in the knocked down cells that allows for the maturation of autophagosomes, our data suggest that LC3B compensates the function of GABARAP family.

It has become clear that a great variety of proteins including autophagy-related proteins, autophagy-adaptor proteins and RAB GAPs interact with Atg8 homologs through their LIR motifs [3]. Based on comparison of LIR domains in more than 20 proteins, its core motif conforms to the formula Θ-X-X-Γ—where Θ is an aromatic amino acid (W/F/Y), Γ is hydrophobic (L/I/V), and X can be any amino acid [3]. Although not an absolute requirement, usually there is at least one acidic residue upstream of the W/F/Y-site. The W/F/Y- and the L/I/V-sites form hydrophobic interactions with two different hydrophobic pockets that are configured on the surface of every Atg8 homologs, implying that the LIR-containing proteins interacts with both LC3 and GABARAP family members. But, in this study, we showed that LC3B selectively recognizes p62 for its autophagic degradation. In support of our results, Elazar group has also reported that LC3 but not GABARAPs is responsible for the final stages of p62 incorporation into autophagosomes [8]. Furthermore, certain proteins possessing the LIR motif show preferential binding to LC3- or GABARAP family [15,26]. What are the mechanisms that regulate such specificity? Our structural models indicate that the acidic cluster (Asp-337–Asp-339) adjacent to core LIR of p62 interacts with the basic surface on α1 helix of LC3B, but not with that of the corresponding GABARAPs region, suggesting that amino acid residues close to core LIR of p62 enable the

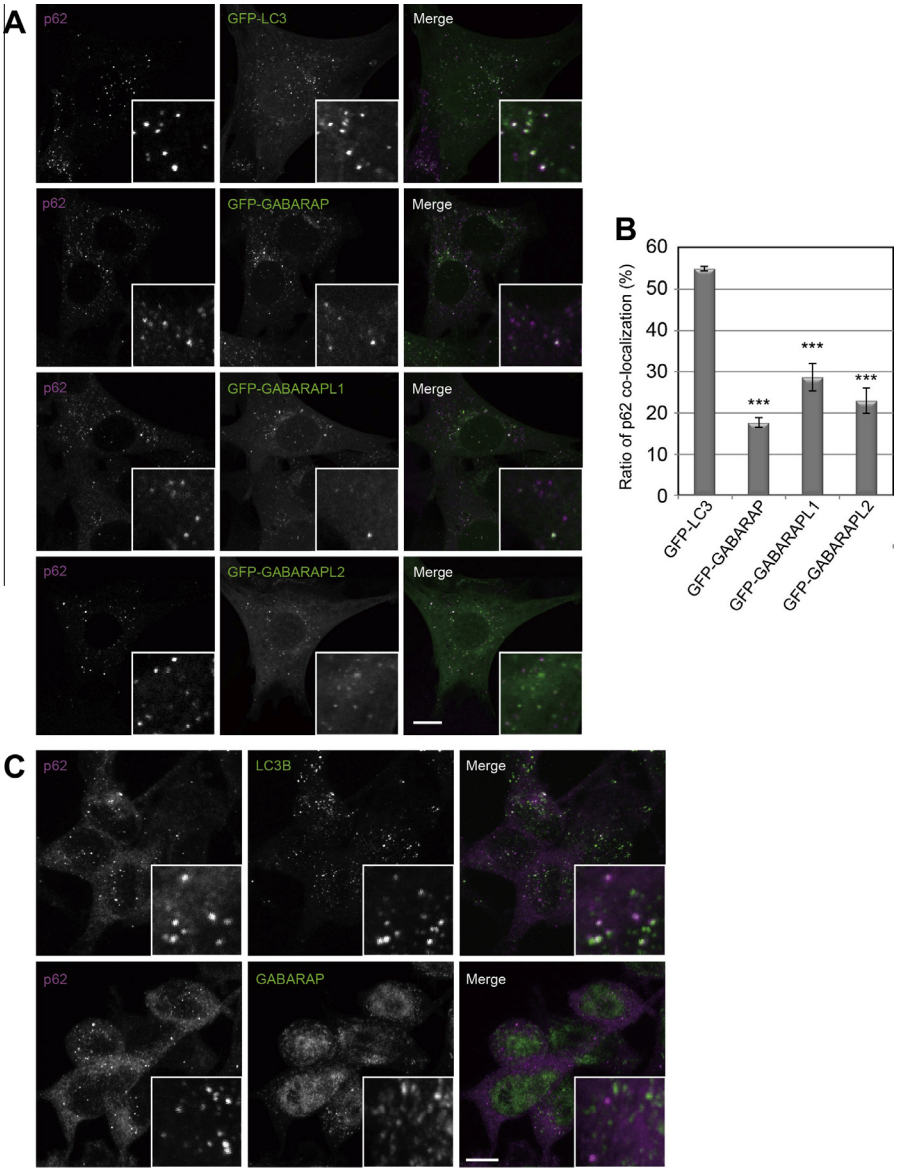


**Fig. 3.** p62 preferentially co-localizes with LC3B. (A) Immunofluorescence analysis. GFP-LC3B, GFP-GABARAP, GFP-GABARAPL1, or GFP-GABARAPL2 was introduced into MEFs, and the MEFs were immunostained with anti-p62 antibody. The right panels show the merged images of GFP (green) and p62 (magenta). Each inset is a magnified image. Scale bars, 10 μm. (B) The percentage of the p62-positive puncta associated with Atg8 homologues was determined by fluorescence microscopy shown in (A). The average ± SE is shown for three independent experiments where at least 100 puncta were counted. $^{***}P < 0.001$. (C) Double immunofluorescence analysis. HEK293T cells were immunostained with anti-p62 and anti-LC3B or anti-p62 and anti-GABARAP antibodies. The right panels show the merged images of LC3B or GABARAP (green) and p62 (magenta). Each inset is a magnified image. Scale bars, 10 μm.

specific or preferential interaction. In addition, residues outside the LIR-binding hydrophobic pockets of LC3 or GABARAP family may confer specificity to interactions with Atg8 homolog proteins. Very recently, we found that Alfy, a scaffold protein for selective autophagy interacts with GABARAP family through a LIR in the WD40 domain specifically and that 3 amino acid residues outside the LIR-binding hydrophobic pockets of GABARAP family ensure this specificity (Lystad AH et al., *EMBO reports* in press). Further, it has been reported that NDP52, an autophagy adaptor protein specifically interacts with LC3C via noncanonical LIR motif in anti-bacterial autophagy [16]. These lines of evidence suggest that autophagy and/or selective autophagy is governed by interaction of LIR-containing proteins with specific Atg8 mammalian homolog.

While the N-terminal region of LC3 or GABARAP is required for membrane fusion activity [4], the region of LC3B is also indispensable for interaction with p62 [10,11,27]. The membrane fusion activity of LC3 is mediated by homo-oligomerization of the PE-conjugated form [4] and seems to be inhibited by the interaction of autophagy receptors/cargos. Given that p62 has higher affinity for LC3-PE than free LC3 [28], LC3-PE bound to p62 should be distinct from LC3-PE involved in membrane fusion. One possibility is post-translational modification (i.e., phosphorylation) of Atg8/LC3. The LC3 proteins become phosphorylated, and specific phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC) have been identified in the N-terminal region of LC3, which is involved in the binding of LC3 to LIR-containing proteins [29]. It is therefore plausible that phosphorylation of the PKA and PKC sites might facilitate or prevent the interaction of LC3 with LIR-containing proteins such as p62. Further analysis is needed to clarify this critical issue.

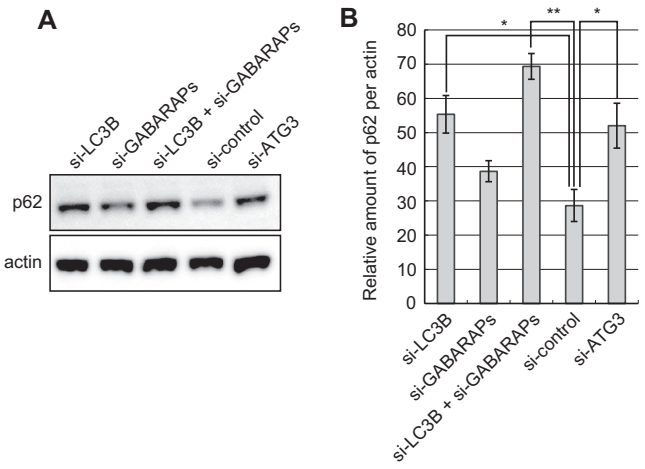


**Fig. 4.** LC3B but not GABARAPs is indispensable for turnover of p62. (A) Immunoblot analysis of p62. At 48 h after introduction of the indicated siRNA, the cell lysates were analyzed by immunoblots with anti-p62 and anti-actin antibodies. (B) Quantitative densitometry of immunoblotting data shown in (A). Graph indicates the ratios of p62 relative to actin. Data are means ± SE of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

## 5. Conflict of interest

The authors declare no competing financial interests.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.093.

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