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Views and Commentaries

Consideration about negative controls for LC3 and expression vectors for four colored fluorescent protein-LC3 negative controls

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Abbreviations: CFP, a cyan colored variant of GFP; GFP, an enhanced green fluorescent protein; HcRed, a far-red chromoprotein derived from *H. crispa*; LC3ΔG, a mutant human LC3, in which the Gly^{120} is deleted; LC3, wild type human microtubule-associated protein 1 light chain 3; LC3-I, soluble unlipidated form of LC3; LC3-II, LC3-phospholipid conjugate; YFP, a yellow colored variant of GFP

Key words: ubiquitylation-like modification, Atg8/Apg8/Aut7, LC3, lipidation, Atg7, Atg3

A cytosolic form of LC3 is conjugated to phosphatidylethanolamine by Atg7, an E1-like enzyme, and Atg3, an E2-like enzyme, during autophagy. To monitor intracellular autophagosomes and autolysosomes, GFP-LC3 is a useful tool. However, GFP-LC3 can aggregate without being conjugated to phosphatidylethanolamine, especially when GFP-LC3 is transiently expressed (Kuma A, Matsui M, Mizushima N. Autophagy 2007; 3:323–8). Therefore, as a negative control, we investigated a mutant human LC3ΔG protein in which the C-terminal Gly^{120} essential for LC3-lipidation is deleted, and generated a set of expression plasmids for wild-type human LC3 and mutant LC3ΔG fused to either CFP, GFP, YFP, or HcRed at the N terminus. We found that the mutant LC3ΔG protein does not react with human Atg7 and Atg3, indicating that LC3-lipidation does not occur, and few puncta containing mutant LC3ΔG form under starvation conditions. As observed with wild-type HcRed-LC3, mutant HcRed-LC3ΔG also co-localizes with polyQ150-aggregates suggesting that the colocalization of HcRed-LC3 to polyQ150-aggregates is independent of LC3-lipidation. These mutant LC3ΔG proteins will be useful negative controls in recognizing non-specific fluorescent protein-LC3 aggregates.

Fluorescent protein-LC3ΔG as a negative control

It is well-recognized that GFP-LC3 is a convenient tool to monitor intracellular autophagosomes and autolysosomes in cultured cell lines and mouse tissues.[1-7] However, caution must be taken with regard to the puncta of LC3.[8] In particular, when the protein is overexpressed, GFP-LC3 can aggregate independent of lipidation. To recognize lipidation-independent LC3-puncta, usage of a negative control should be considered. A candidate negative control is the mutant LC3ΔG, in which the C-terminal Gly essential for lipidation has been deleted.[9] This mutant has defects in the E1-like reaction mediated by Atg7 and the E2-like reaction mediated by Atg3, which results in a lack of LC3-II formation.[9-11] Based on these results and for better versatility, we have generated a set of vectors based on a modified pCXN2 plasmid to express four fluorescent proteins (CFP, GFP, YFP, and HcRed) fused to either wild-type human LC3 (LC3) or mutant LC3ΔG in order to monitor autophagy and lipidation-independent aggregation of LC3 (Fig. 1).[12,13] We used human LC3 because its cleavage pattern and all the enzymatic reactions used for its lipidation are well characterized in vivo and in vitro, and its C terminus after the essential Gly is shorter than that from rat.[9-11,14,15]

Fluorescent protein-LC3ΔG fusions were expressed in HEK293 cells using these new plasmids (Figs. 2A–C). We first investigated whether these mutant fusions have defects in the E1- and E2-reactions mediated by Atg7 and Atg3 respectively. When an active-site mutant $Atg7^{C567S}$ was expressed simultaneously with wild-type fluorescent protein-LC3 fusions, stable E1-substrate intermediates were formed (Fig. 2A).[10,16] However, when fluorescent protein-LC3ΔG fusions were expressed, fewer E1-substrate intermediates were formed (Fig. 2A). When human $Atg3^{C264S}$, in which the active site Cys^{264} has been changed to Ser, was expressed together with wild-type LC3 fusions and wild-type human Atg7, stable E2-substrate intermediates were formed (Fig. 2B).[11] In contrast, when mutant LC3ΔG fusions were expressed, only low levels of E2-substrate intermediates were detected (Fig. 2B). These results indicate that fluorescent protein-LC3ΔG fusions have defects in E1- and E2-reactions. LC3-II, a LC3-phosphatidylethanolamine conjugate, fractionates into the pellet, while LC3-I remains in the supernatant.[14,15,17] The majority of GFP-LC3ΔG and HcRed-LC3ΔG proteins remained in the supernatant following subcellular fractionation, while their respective wild-type proteins were pelleted (Fig. 2C).

We further investigated the intracellular localization of fluorescent protein-LC3ΔG fusions under starvation conditions. After each CFP-, GFP-, YFP- and HcRed-LC3ΔG construct was transiently expressed in CHO cells, the cells were transferred to starvation medium in the presence of E64d and pepstatin A, and incubated for

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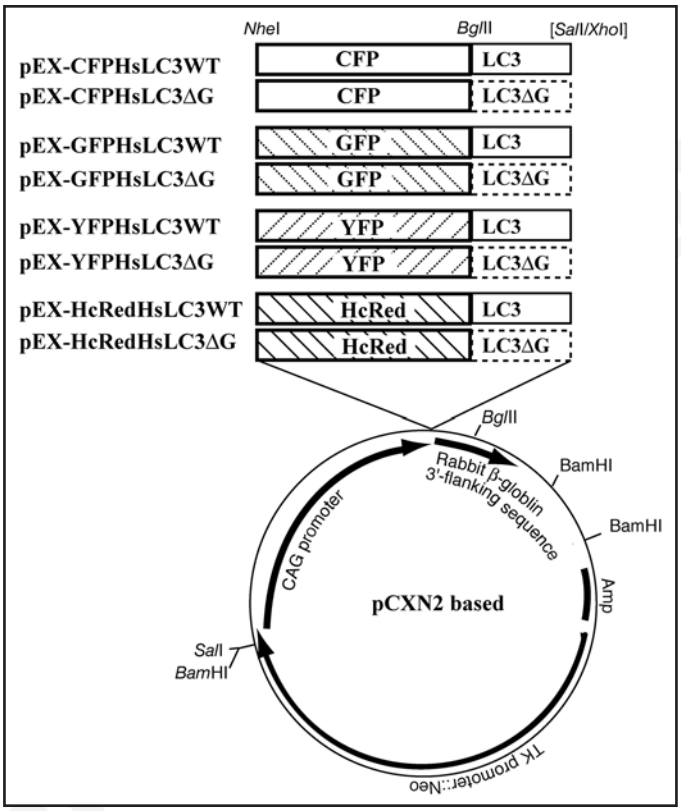

Figure 1. Schematic representation of the expression vectors for wild LC3 and mutant LC3ΔG fused to fluorescent proteins at the N terminus. The name designated to each plasmid is shown in the left panel. The cloning of human LC3/MAP1LC3B and construction of mutant LC3ΔG have been (previously described in ref. 9). The pECFP-C1, pEYFP-C1, pEGFP-C1, and pHcRed-C1 vectors were purchased from Takara (Tokyo, Japan). *Nhe*I-*Bgl*II fragments encoding CFP, YFP, GFP, and HcRed were excised from pECFP-C1, pEYFP-C1, pEGFP-C1, and pHcRed-C1, respectively. Each *Nhe*I-*Bgl*II fragment encoding fluorescent protein, and *Bgl*II-*Sal*I fragment encoding wild-type or mutant human LC3, were introduced into the *Nhe*I-*Xho*I site of a modified pCXN2 vector.[12,13] CAG promoter, chicken β-actin promoter; TK promoter:: Neo, neomycin/G418-resistant gene under the control of the herpes simplex virus thymidine kinase promoter.

4 h. While puncta of each CFP-, GFP-, YFP- and HcRed-labeled wild-type LC3 was observed in CHO cells under these conditions, fewer puncta of each respective LC3ΔG mutant fusion protein were observed (Fig. 3). These results indicate that these fluorescent protein-LC3ΔG fusions are appropriate negative controls when the fluorescent protein-LC3 wild-type fusions are used for monitoring autophagy.

Puncta of HcRed-LC3ΔG Co-localize with PolyQ150-aggregates in a Similar Manner as Wild-type HcRed-LC3

GFP-LC3 co-localizes with polyQ72 independent of its lipidation state. Therefore, we investigated whether LC3ΔG can be used to recognize lipidation-independent co-localization of LC3 with polyQ aggregates (Fig. 4). YFP-polyQ150 and HcRed-LC3 or HcRed-LC3ΔG were transiently expressed in CHO cells, and YFP and HcRed fluorescence were subsequently visualized. Wild-type LC3 and HcRed-LC3ΔG puncta both co-localized with YFP-polyQ150 puncta in CHO cells. Quantification of the fluorescent images indicated that about 60 ± 5% (mean ± SE) of mutant HcRed-LC3ΔG puncta co-localized with YFP-polyQ150-aggregates, and about 68 ± 11% (mean±SE) of wild-type HcRed-LC3 puncta co-localized with YFP-polyQ150-aggregates (Fig. 4). The p-value from the student t-test between polyQ150-puncta co-localized with wild-type HcRed-LC3 and HcRed-LC3ΔG is greater than 0.5, indicating that there is no statistical difference. Since HcRed-LC3ΔG is not lipidated, the colocalization of LC3ΔG with polyQ150 is independent of lipidation. Therefore, these results suggest that fluorescent protein-LC3ΔG fusions are appropriate negative controls for fluorescent protein-LC3 in order to determine whether the colocalization of puncta with aggregates is dependent on LC3-lipidation.

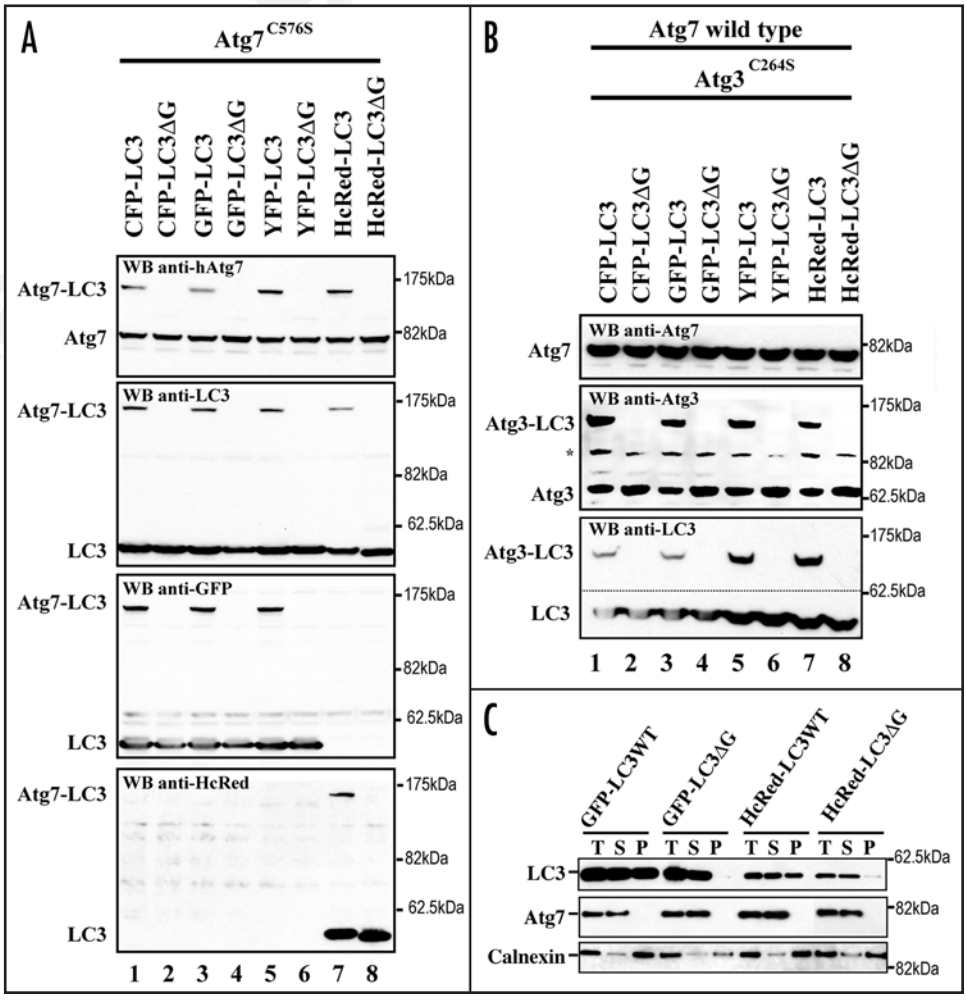

Figure 2. See legend, page 3.

Figure 2 (Previous page). Fluorescent protein-LC3ΔG fusions have defects in E1- and E2-reactions. A) Formation of E1-substrate intermediates of human LC3 fusion proteins and Atg7. Wild-type LC3 (odd lanes) or mutant LC3ΔG (even lanes) fused with CFP (lanes 1 and 2), GFP (lanes 3 and 4), YFP (lanes 5 and 6), or HcRed (lanes 7 and 8) were transiently expressed together with the human Atg7^{C576S} protein in HEK293 cells. After preparation of cell lysates, total protein was separated by SDS-PAGE. Human Atg7^{C576S} (Atg7) and E1-substrate intermediates of human Atg7^{C576S}-LC3 fusion proteins (Atg7-LC3) were recognized by immunoblotting with an anti-human Atg7 IgG (WB anti-Atg7) (as described in ref. 10). Human LC3 fused with fluorescent proteins and E1-substrate intermediates were recognized with an anti-human LC3 IgG (WB anti-LC3) (as described in ref. 11). GFP and its variants were recognized with an anti-GFP antibody (WB anti-GFP) (TAKARA, Tokyo, Japan), and HcRed-fusion proteins were detected with an anti-HcRed antibody (WB anti-HcRed) (TAKARA). Note that E1-substrate intermediates were not observed in the even lanes. (B) Formation of E2-substrate intermediates of human LC3 fusion proteins and Atg3. Wild-type LC3 (odd lanes) or mutant LC3ΔG (even lanes) fused with CFP (lanes 1 and 2), GFP (lanes 3 and 4), YFP (lanes 5 and 6), or HcRed (lanes 7 and 8) was transiently expressed together with human Atg7 and GFP-hAtg3^{C264S} in HEK293 cells. After cell lysate preparation, total proteins were separated by SDS-PAGE. Human Atg7 was recognized with an anti-human Atg7 IgG (WB anti-hAtg7). Human Atg3^{C264S} and E2-substrate intermediates of LC3 and Atg3^{C264S} were recognized with an anti-human Atg3 serum (WB anti-Atg3) (as described in Ref. 11). LC3 fusion proteins and E2-substrate intermediates were observed with an anti-human LC3 IgG (WB anti-LC3). The asterisk indicates a non-specific band. Note that E2-substrate intermediates were not formed in the even lanes. (C) Subcellular fractionation of cells expressing GFP-LC3, GFP-LC3ΔG, HcRed-LC3, and HcRed-LC3ΔG. Cells expressing fluorescent protein-LC3 were lysed by sonication, and the lysates (T) were centrifuged at 100,000 x g for 1 h. Proteins in the supernatant (S) and pellet (P) fractions were separated on a 4–12% NuPAGE gel. LC3-fusions were recognized by immunoblotting with an anti-LC3 IgG. The blot was also probed with an antibody against Atg7, a cytosolic protein marker, and an antibody against the endoplasmic reticulum resident protein calnexin, as a membrane-bound protein marker.

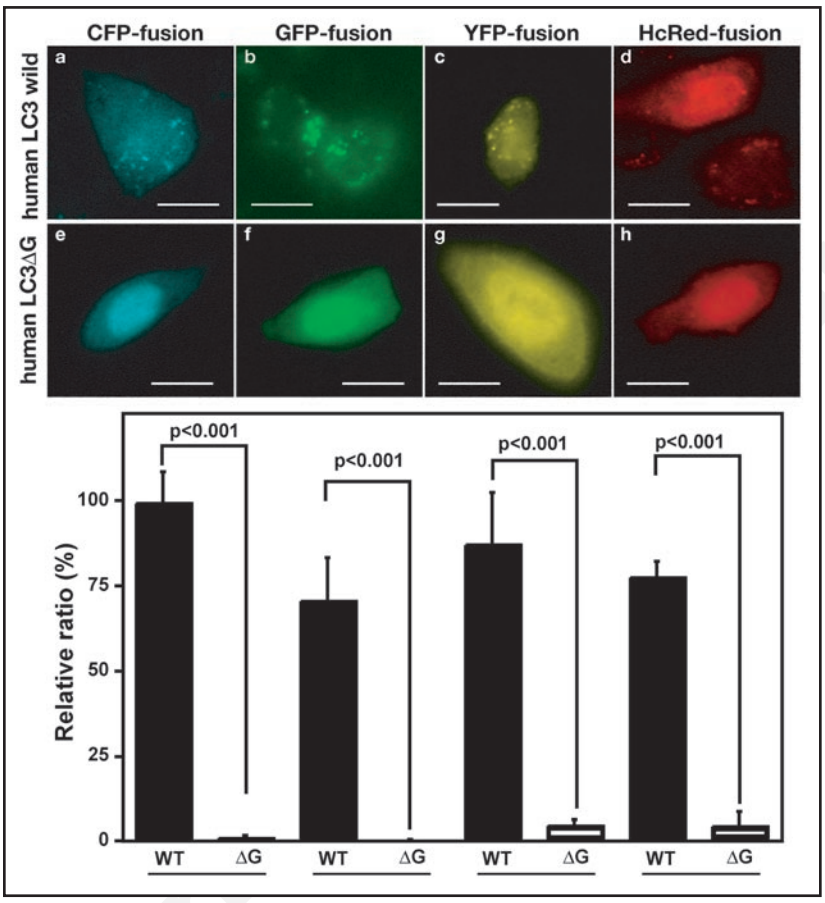

Figure 3. LC3ΔG fusion proteins are defective in puncta formation in starvation conditions. Fewer puncta of mutant LC3ΔG fusion proteins were visible under starvation conditions in the presence of lysosomal protease inhibitors. We transfected each plasmid [pEX-CFPHsLC3WT (a), pEX-GFPHsLC3WT (b), pEX-YFPHsLC3WT (c), pEX-HcRedHsLC3WT (d), pEX-CFPHsLC3ΔG (e), pEX-GFPHsLC3ΔG (f), pEX-YFPHsLC3ΔG (g), pEX-HcRedHsLC3ΔG (h)] into CHO cells using the lipofection reagent HilyMax (Dojindo, Tokyo, Japan). Following transfection, the CHO cells were cultured in nutrient-rich medium for 48 h, transferred to Krebs-Ringer buffered medium containing E64d (10 μg/ml) and pepstatin A (10 μg/ml), and incubated for an additional 4 h (as described in Ref. 18). The fluorescence within each cell was visualized with a Biozero BZ-8000 microscope (Keyence, Tokyo, Japan) using an appropriate filter set. The numbers of puncta observed were counted, and the relative percentage of puncta is represented graphically, where an average number of wild-type GFP-LC3-puncta is regarded as 100%. p, p-value of student t-test.

In the future, it will be advantageous to isolate a cell line that stably expresses fluorescent protein-LC3. However, when analyzing autophagy in cells that transiently express LC3, usage of mutant LC3ΔG as a negative control will minimize an overestimation of LC3-puncta aggregates.

Acknowledgments

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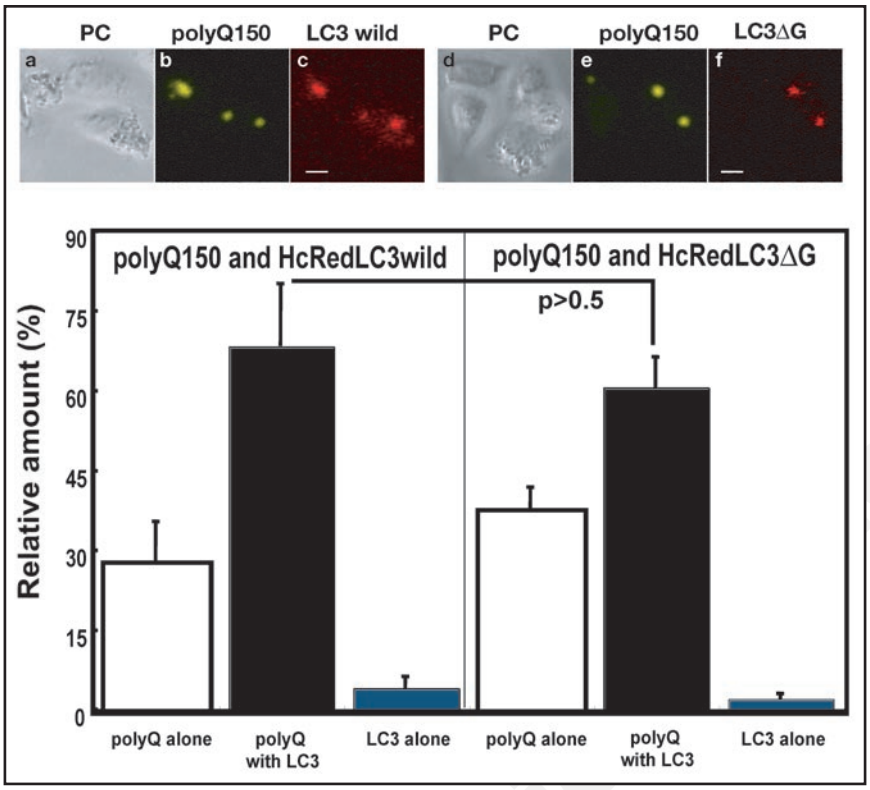

Figure 4. Colocalization of LC3 with polyQ150-aggregates. YFP-polyQ150 and wild-type HcRed-LC3 (a–c) or mutant HcRed-LC3ΔG (d–f) were transiently expressed in CHO cells. Panels a and d are phase contrast images (PC). Panels b and e are fluorescence images of YFP-polyQ150 (polyQ150). (c and f) are fluorescence images of wild-type (LC3 wild) or mutant HcRed-LC3 (LC3ΔG). Based on these images (n = 300), the relative percentage of puncta of YFP-polyQ150 alone (polyQ alone), YFP-polyQ150 co-localized with wild-type or mutant HcRed-LC3 (polyQ with LC3), and wild-type or mutant HcRed-LC3 alone (LC3 alone) per total number of puncta are represented graphically.

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