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Development of LC3/GABARAP sensors containing a LIR and a hydrophobic domain to monitor autophagy

You-Kyung Lee, Yong-Woo Jun, Ha-Eun Choi, Yang Hoon Huh, Bong-Kiun Kaang, Deok-Jin Jang and Jin-A Lee

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 31 December 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are shown below.

As you can see, both referees appreciate the development of LC3 sensors but share concerns regarding the current analyses performed: the sensors are not sufficiently tested for interference with autophagic flux and for their specificity towards the different LC3/GABARAP proteins.

You might have noticed that a related resource has just been published online in our journal. Should you be able to address the referees' concerns - and especially the ones noted above - within the next 8 weeks, I can offer to invite you to submit a revised version of the manuscript. So please consider your options carefully as I won't be able to extend the revision time. I should add that acceptance of your manuscript will depend on the completeness of your responses in this revised version.

REFEREE REPORTS

Referee #1:

The manuscript by Lee et al. describes cleverly designed sensors for LC3/GABARAP proteins in mammalian cells. LC3/GABARAP proteins are conjugated to nascent autophagosomal membranes

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and stay associated with autophagosomes until they are degraded within lysosomes. So far, the field largely relies on expression of fluorescently tagged LC3/GABARAP proteins for detection of autophagosomes, a technique that is prone to artifacts. The sensors described here could therefore be very valuable for the field. However, as outlined below the sensors should be more rigorously and consistently characterized with respect to their interference with autophagic flux and their binding specificity for different for LC3/GABARAP proteins. In addition, the text would profit from proofreading and editing by a native speaker.

Major comments:

1. I understand that the assays showing the specific targeting of the LC3 probes were conducted with mouse embryonic fibroblasts (MEFs). However, the crucial tests for the interference of the probes with autophagic flux were conducted with HEK293T cells (Figure 5). Why did the authors choose a different cell line for this test? The experiments shown in Figure 5 should be repeated with MEFs and in addition it would be helpful to see how the sensors behave in cell lines other than MEFs (for example in HEK293T cells). Furthermore, the figure legend mentions that the cells were serum starved for these experiments rather than nutrient starved as for the other experiments. In summary, the conditions for the experiments shown in Figure 5 were so different from all the other experiments that no solid conclusion can be drawn at this point.

2. Related to the comments above, the p62 degradation assay shown in Figure 5 is not very convincing as no degradation of p62 is detected in the GFP expressing cells (lane 1 vs. 2). Since this is a crucial test for the interference of the sensors with autophagic flux the characterization should be backed up with other assays. Following mitophagy would be one possible way.

3. The authors should provide some sort of characterization of the binding specificity for different LC3s/GABARAPs. This could for example be done by pull downs from using cell lysates or purified proteins.

4. The authors should explain in more detail how exactly the ratios for the fluorescent images were quantified (i.e. in Figure 1).

5. The data in Figure 3 should be quantified.

6. The immuno-EM pictures shown in Figure 3 are somewhat small and the contrast is not ideal to see the double membrane of the autophagosomes and to clearly see the gold particles. Can the authors provide larger images and with better contrast?

7. The authors should provide the full sequences of the HyD-LIR(TP) and HyD-LIR(Fy) sensors.

Minor comments:

9. Page 4, last paragraph: the sentence "Among the core autophagy proteins, ATG8, a mammalian homolog of light chain 3 (LC3), is involved in autophagosome formation..." is wrong. There is one Atg8 in yeast and several homologs in human and mouse, which are subdivided into the LC3 and GABARAP families. The authors mention this themselves later in the introduction.

10. Scale bars should be added to all microscopy pictures.

11. Page 13, bottom: the sentence "These results indicate that continuing induction of autophagosome formation further recruited the probes from the cytosol to the autophagosomal membrane." is somewhat unclear. Can the authors please explain better what exactly they mean?

12. In the discussion the authors mention that "the new sensor, however, did not affect the morphology of cultured cortical neurons in our experiments (our unpublished data)". It would be good to show the data in the paper.

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Referee #2:

In this manuscript Lee and co-authors have developed specific GFP-tagged sensors to detect LC3. They LIR domain of 34 different proteins was fused to a N-terminal hydrophobic domain (HyD) and a C-terminal GFP tag and the resulting constructs tested for colocalization with overexpressed or endogenous LC3 in cells where autophagy was induced (by starvation or rapamycin) or blocked (with chloroquine or genetically). The LIR motif from TP53INP2 (HyD-LIR(TP)-GFP) was found to be the best LC3 sensor and its localization to endogenous LC3-positive autophagosome was specific (no colocalization in Atg5 or Atg7 KO MEFs or of the LIR mutant construct). Expression of the HyD-LIR(TP)-GFP protein did not seem to affect autophagic flux and could be used to monitor autophagosome formation in live cells.

This study is well performed and the data are nicely presented. However, as the authors have only tested the colocalization of their LIR probes with LC3B (?) (overexpressed and endogenous) the study is somewhat limited. There are six ATG8 proteins in mammalian cells (LC3-A,B,C and GABARAP, -L1, -L2). Their functions during autophagy are only incompletely understood and development of sensors to detect the different endogenous ATG8 proteins would therefore be interesting and useful to the autophagy community.

Specific comments:
- the authors should specify which LC3 is used (for RFP-LC3)
- they should test the specificity of their sensors, i.e. look for their colocalization and binding (pulldown experiments) to all LC3s (A,B,C) and GABARAPs
- all immunofluorescence data are done in MEFs while the western blot analysis is done in HEK cells. They should also include images from HEK cells and show flux western blots from MEFs. It would be nice to confirm that the autophagic flux is unaffected by using the long-lived protein degradation assay, which measure turnover of an autophagic substrate in the lysosome.
- is selective autophagy normal in cells expressing the HyD-LIR(TP)-GFP sensor?
- does the sensor bind to LC3 in both the inner and outer membrane of the forming autophagosome? They should perform proteinase K protection experiments.
- the cells are starved (for serum or amino acids) for 8 hrs to induce autophagy. Why so long? Autophagy is normally induced for 2 or 4 hrs in medium lacking both serum and amino acids. They should also show images taken after shorter time point starvation in EBSS.
- the western blot experiments are not described in the methods
- the quantification of the ratios of sensor-positive RFP-LC3 spots and autophagosomal/cytosol (A/C) fluorescent intensity should be better described. E.g. in Fig.2 B, C - they state that they have used 20 cells for quantification and have done three experiments. Is this 20 cells from each experiment or 20 in total? How were the cells selected? How is the significance value calculated - between the averages of the 3 independent experiments or between all cells? The authors state: "***P < 0.001 (comparison with all other groups according to one-way ANOVA followed by the Tukey's post-hoc test." -- does this mean that ATG13, BNIP3 and FUNDC1 (in B) are significantly different from each other and all other groups?

1st Revision - authors' response 23 February 2017

Referee #1:

The manuscript by Lee et al. describes cleverly designed sensors for LC3/GABARAP proteins in mammalian cells. LC3/GABARAP proteins are conjugated to nascent autophagosomal membranes and stay associated with autophagosomes until they are degraded within lysosomes. So far, the field largely relies on expression of fluorescently tagged LC3/GABARAP proteins for detection of autophagosomes, a technique that is prone to artifacts. The sensors described here could therefore be very valuable for the field. However, as outlined below the sensors should be more rigorously and consistently characterized with respect to their interference with autophagic flux and their binding specificity for different for LC3/GABARAP proteins. In addition, the text would profit from proofreading and editing by a native speaker.

Response: We thank you for your positive and valuable comments, which helped to improve our manuscript.

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Major comments:

1. I understand that the assays showing the specific targeting of the LC3 probes were conducted with mouse embryonic fibroblasts (MEFs). However, the crucial tests for the interference of the probes with autophagic flux were conducted with HEK293T cells (Figure 5). Why did the authors choose a different cell line for this test? The experiments shown in Figure 5 should be repeated with MEFs and in addition it would be helpful to see how the sensors behave in cell lines other than MEFs (for example in HEK293T cells).

Response: To examine whether expression of our sensor affects autophagic flux, we used HEK293T cells because of their higher transfection efficiency compared to MEFs for our sensor plasmids (HyD-LIR(TP or Fy)-GFP).

We also confirmed efficient co-localization of our sensors (HyD-LIR(TP or Fy)-GFP) to LC3/GABARAP-positive autophagosome in HEK293T cells (revised Figure EV2). As the reviewer suggested, we performed an autophagic flux assay in MEFs expressing our sensors (HyD-LIR(TP or Fy)-GFP) and included the new data in the revised manuscript (Revised Figure 5F-H)

Furthermore, the figure legend mentions that the cells were serum starved for these experiments rather than nutrient starved as for the other experiments. In summary, the conditions for the experiments shown in Figure 5 were so different from all the other experiments that no solid conclusion can be drawn at this point.

Response: In our experimental setting, we initially used serum starvation because we could induce serum-starvation in MEFs and HEK293T cells without using EBSS media. Because we confirmed the presence of mRFP-LC3B-positive autophagosomes and accumulation of LC3-II, or p62 as autophagy substrates in the presence of CQ under serum starvation in MEFs and HEK293T cells, we considered that autophagy was induced and autophagic flux was normal.

As the reviewer pointed out, to evaluate whether the expression of our sensors affects autophagic flux in MEFs starved with EBSS, we performed an autophagic flux assay and added the new data to Figure. 5F-H of the revised manuscript. Furthermore, we examined the cellular localization of our sensors (HyD-LIR(TP or Fy)-GFP) to autophagosome in EBSS-starved MEFs (revised Figure EV3)

2. Related to the comments above, the p62 degradation assay shown in Figure 5 is not very convincing as no degradation of p62 is detected in the GFP expressing cells (lane 1 vs. 2). Since this is a crucial test for the interference of the sensors with autophagic flux the characterization should be backed up with other assays. Following mitophagy would be one possible way.

Response: As the reviewer suggested, we performed the autophagic flux assay in MEFs starved with EBSS and added new data to Figure. 5F-H of the revised manuscript. In MEFs starved with EBSS, we showed that protein level of p62 was reduced upon starvation and there was no dramatic difference on autophagic flux in cells expressing GFP, HyD-LIR(Fy)-GFP or HyD-LIR(TP)-GFP (revised Figure 5F-H).

In our experimental setting, in HEK293T cells upon serum starvation without CQ, we couldn't get dramatic reduction of level of p62 (revised Figure EV2C-E). According to our personal communication with expertise in autophagy field (Dr. Yongkeun Jung and Dr. Myungshik Lee), level of p62 seems to be dependent on cell lines under various conditions of autophagy induction. We examined whether our sensors could be used for monitoring a selective autophagy. As a result, we found that HyD-LIR(Fy)-GFP or HyD-LIR(TP)-GFP was efficiently localized to MitoTracker-positive damaged mitochondria spots in CCCP- or phenanthroline-induced mitophagy in MEFs or HeLa cells, respectively (revised Figure 8A-D). Moreover, HyD-LIR(Fy)-GFP- or HyD-LIR(TP)-GFP-positive damaged mitochondria accumulated in the presence of CQ. There is no dramatic difference on the numbers of sensor-positive MitoTracker spot in cells expressing GFP-LC3B and our HyD-LIR sensors, suggesting that damaged mitochondria were degraded in cells expressing GFP-LC3B or our HyD-LIR sensors (revised Figure 8B and D). These results raise its potential use to detect mitophagy. However, more detailed analysis such as the flux assay using mtKeima or the flux assay using mitochondria targeting proteins is needed for its practical use on mitophagy. Due to

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time limitation for the revision (8 weeks), we couldn't perform all experiments regarding the evaluation of our sensors on mitophagy. Therefore, as a further study, we are going to characterize our sensor as a marker for a selective autophagy such as mitophagy or pexophagy.

3. The authors should provide some sort of characterization of the binding specificity for different LC3s/GABARAPs. This could for example be done by pull downs from using cell lysates or purified proteins.

Response: We thank you for pointing this out. As the reviewer suggested, to examine the binding preference for different LC3s/GABARAPs of our sensors (HyD-LIR(TP)-GFP or HyD-LIR(Fy)-GFP), we performed a pulldown assay of our GFP-sensors in HEK293T cells expressing each different LCs/GABARAP and analyzed their cellular colocalization in MEFs or HEK293T cells. We added the new data to the revised manuscript (revised Figure 2–4, Figure EV2 and Table 1).

4. The authors should explain in more detail how exactly the ratios for the fluorescent images were quantified (i.e. in Figure 1).

Response: As the reviewer suggested, we added a more detailed description to the Methods section of the revised manuscript.

"Quantitative analysis of the number of sensor- and Mitotracker-positive dots, ratios of sensor-positive LC3/GABARAPs autophagosome dots, and A/C ratio of fluorescence intensities of the sensor-positive spots

For quantitative analysis of co-localization spots including both sensor and MitoTracker-positive dots and sensor-positive LC3/GABARAPs autophagosomes dots, at least 20 cells were randomly selected and excluded from selection if they were directly adjacent to a previously selected cell. The number of both sensor and MitoTracker-positive dots or sensor-positive LC3/GABARAP autophagosomes recognized by the respective probe in a valid cell was counted by overlaying the two channels (co-localization of objects in confocal images) using ZEN imaging software (Carl Zeiss). Ratios of sensor-positive LC3/GABARAP autophagosome dots was calculated as the fold of number of co-localization events divided by total number of LC3/GABARAP-positive spots. To determine the quantitative ratio of autophagosomal/cytosol (A/C) fluorescent intensities, an average value of autophagosomal or cytosol fluorescent intensities was measured for at least randomly selected 5 points on the autophagosome and cytosol in single cell using ZEN software. In the same manner, the quantitative A/C ratio of at least randomly selected 20 cells was analyzed. All statistical data were calculated and graphed using GraphPad Prism 5 (GraphPad, Inc., La Jolla, CA, USA)."

5. The data in Figure 3 should be quantified.

Response: In the revised manuscript, we tested the cellular localization of our candidate sensors into LC3/GABARAP-positive autophagosomes. As a result, we found that HyD-LIR(Fy)-GFP was more efficiently localized to LC3A-positive autophagosomes, while HyD-LIR(TP)-GFP was more preferentially GABARAPL1-positive autophagosomes (Revised Fig. 2–4, Table 1). We quantified their co-localization efficiency in MEFs expressing each sensor together with either mRFP-LC3(A, B, or C) or mRFP-GABARAP/GABARAP(L1 or L2) and presented the new data in Figure 2 - 4 of the revised manuscript.
We also performed immunostaining in MEFs expressing HyD-LIR(Fy)-GFP or HyD-LIR(TP)-GFP using anti-LC3(A, B) or anti-GABARAPL1 antibody. We included this data in revised Figure 5A, B. We also quantified the co-localization of our sensors into LC3A/B- or GABARAPL1-positive dots (revised Figure 5C).

6. The immuno-EM pictures shown in Figure 3 are somewhat small and the contrast is not ideal to see the double membrane of the autophagosomes and to clearly see the gold particles. Can the authors provide larger images and with better contrast?

Response: As the reviewer suggested, we provide larger images with better contrast in Figure. 5D-E of the revised manuscript. In our immunoEM analysis, a clear double membrane structure was not observed. Cellular membranes did not appear to be intact compared to those in the EM images. To further characterize cellular localization of our sensor (HyD-LIR(TP)-GFP), we performed *proteinase K protection experiments* and added the new data to Figure 6G of the revised manuscript.

7. The authors should provide the full sequences of the HyD-LIR(TP) and HyD-LIR(Fy) sensors.

Response: As the reviewer pointed out, we included the full amino acid sequences of the HyD-LIR(TP) and HyD-LIR(Fy) sensors in Appendix Figure S2 in the revised manuscript.

Minor comments:

9. Page 4, last paragraph: the sentence "Among the core autophagy proteins, ATG8, a mammalian homolog of light chain 3 (LC3), is involved in autophagosome formation..." is wrong. There is one Atg8 in yeast and several homologs in human and mouse, which are subdivided into the LC3 and GABARAP families. The authors mention this themselves later in the introduction.
Response: We thank you for pointing this out. We revised the Introduction accordingly.

10. Scale bars should be added to all microscopy pictures.
Response: We added scale bars to all microscopy images in the Figures of the revised manuscript.

11. Page 13, bottom: the sentence "These results indicate that continuing induction of autophagosome formation further recruited the probes from the cytosol to the autophagosomal membrane." is somewhat unclear. Can the authors please explain better what exactly they mean?
Response: As the reviewer pointed out, the meaning of sentence was not clear. We have removed this sentence from the revised manuscript.

12. In the discussion the authors mention that "the new sensor, however, did not affect the morphology of cultured cortical neurons in our experiments (our unpublished data)". It would be good to show the data in the paper.
Response: We added neuronal morphology images in neurons expressing GFP, GFP-LC3, and HyD-LIR(TP, or Fy)-GFP to Appendix Figure S1 of the revised manuscript.

Referee #2:

In this manuscript Lee and co-authors have developed specific GFP-tagged sensors to detect LC3. They LIR domain of 34 different proteins was fused to a N-terminal hydrophobic domain (HyD) and a C-terminal GFP tag and the resulting constructs tested for colocalization with overexpressed or endogenous LC3 in cells where autophagy was induced (by starvation or rapamycin) or blocked (with chloroquine or genetically). The LIR motif from TP53INP2 (HyD-LIR(TP)-GFP) was found to be the best LC3 sensor and its localization to endogenous LC3-positive autophagosome was specific (no colocalization in Atg5 or Atg7 KO MEFs or of the LIR mutant construct). Expression of the HyD-LIR(TP)-GFP protein did not seem to affect autophagic flux and could be used to monitor autophagosome formation in live cells.

This study is well performed and the data are nicely presented. However, as the authors have only tested the colocalization of their LIR probes with LC3B (?) (overexpressed and endogenous) the study is somewhat limited. There are six ATG8 proteins in mammalian cells (LC3-A,B,C and GABARAP, -L1, -L2). Their functions during autophagy are only incompletely understood and development of sensors to detect the different endogenous ATG8 proteins would therefore be interesting and useful to the autophagy community.

Response: We thank you for your positive and valuable comments, which helped to improve our manuscript. We added new data to show the binding properties of our sensors for all six LC3/GABARAP proteins in Figure 2–4 and Table 1 of the revised manuscript.

Specific comments:
- the authors should specify which LC3 is used (for RFP-LC3)

Response: We initially used mRFP-LC3B to test our sensor to detect autophagosomes. In the revised manuscript, we determined cellular colocalization of our sensors and their binding preferences to all LC3s (A, B, C) and GABARAP/GABRAPs (L1, L2) (revised Figure 2-4 and Table1).

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- they should test the specificity of their sensors, i.e. look for their colocalization and binding (pulldown experiments) to all LC3s (A,B,C) and GABARAPs

Response: As the reviewer suggested, we performed a pulldown assay for binding property of our sensors and determined the co-localization of our eight sensors with all LC3s and GABARAPs in MEFs as shown in Figure. 2–4 and Table 1 of the revised manuscript. We added cellular localization data of HyD-LIR(TP)-GFP or HyD-LIR(Fy)-LIR into LC3/GABARAP-positive autophagosome or LC3A/B positive autophagosome in HEK293T cells (revised Figure EV2).

- all immunofluorescence data are done in MEFs while the western blot analysis is done in HEK cells. They should also include images from HEK cells and show flux western blots from MEFs. It would be nice to confirm that the autophagic flux is unaffected by using the long-lived protein degradation assay, which measure turnover of an autophagic substrate in the lysosome.

Response: To examine whether the expression of our sensors affects autophagic flux, we used HEK293T cells because they showed higher transfection efficiency compared to MEFs with our sensor plasmid (HyD-LIR(TP or Fy)-EGFP).
As the reviewer suggested, we performed the autophagic flux assay in MEFs expressing our sensors and included this new data in the revised manuscript (Revised Figure 5F-H). We also show cellular localization of our sensors into LC3/GABARAP-positive autophagosome in HEK293T cells and added new cellular images to Figure EV2 of the revised manuscript.
Regarding the long-lived protein degradation assay, we apologize that we could not perform this assay in the limited time (-8 weeks) allowed for our revision. Our institution has very strict rules regarding the use of isotope-labeled amino acids because of safety issues. A protocol must be submitted and permission must be obtained to use these materials. Permission is typically granted in approximately 3 months. Although we submitted our protocol to obtain permission, this assay could not be completed before submitting the revised manuscript. We hope the reviewer understands our situation.

Although we agree that the long-lived protein degradation assay should be performed, because autophagic flux assay in macroautophagy was not affected by our sensors based on our flux assay, we believe that autophagic degradation of substrates including long-lived proteins is normal.
We will perform long-lived protein degradation assay once we obtain permission to use isotope-labeled amino acids.

- is selective autophagy normal in cells expressing the HyD-LIR(TP)-GFP sensor?
Response: We examined whether our sensors could be used for a selective autophagy. As a result, we found that HyD-LIR(Fy)-GFP or HyD-LIR(TP)-GFP was efficiently localized to MitoTracker-positive damaged mitochondria spots in CCCP- or phenanthroline-induced mitophagy in MEFs or HeLa cells, respectively (revised Figure 8A-D). Moreover, HyD-LIR(Fy)-GFP- or HyD-LIR(TP)-GFP-positive damaged mitochondria accumulated in the presence of CQ. There is no dramatic difference on the numbers of sensor-positive MitoTracker spot in cells expressing GFP-LC3B and our HyD-LIR sensors, suggesting that damaged mitochondria are degraded in cells expressing GFP-LC3B or our HyD-LIR sensors (revised Figure 8B and D). These results raise its potential use to detect mitophagy. However, more detailed analysis such as the flux assay using mtKeima or the flux assay using mitochondria targeting proteins is needed for its practical use on mitophagy. Due to time limitation for the revision, we couldn't perform all experiments regarding the evaluation of our sensors on mitophagy. Therefore, as a further study, we are going to characterize our sensor as a marker for a selective autophagy such as mitophagy or pexophagy.

- does the sensor bind to LC3 in both the inner and outer membrane of the forming autophagosome? They should perform proteinase K protection experiments.

Response: As the reviewer suggested, we performed proteinase K (PK) protection experiments and added this new data to Fig. 6G of the revised manuscript. Based on the PK protection assay, our newly developed HyD-LIR(TP)-GFP was also present in the inner membrane of autophagic vacuoles.

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- the cells are starved (for serum or amino acids) for 8 hrs to induce autophagy. Why so long? Autophagy is normally induced for 2 or 4 hrs in medium lacking both serum and amino acids. They should also show images taken after shorter time point starvation in EBSS.

Response: We thank you for pointing this out.
In our experimental setting, we initially used serum starvation because we induced serum-starvation in MEF and HEK293T cells in DMEM in the absence of serum (10% FBS) without the requirement for EBSS media. To allow sufficient time for cell starvation for autophagy induction, we incubated the cells for up to 8 h under serum starvation conditions.
Because we confirmed the formation of mRFP-LC3B-positive autophagosomes and accumulation of LC3-II in the presence of CQ under serum starvation conditions in MEF and HEK293T cells with normal cell morphology, we concluded that autophagy was induced and autophagic flux was normal.
However, as the reviewer suggested, we also confirmed that our sensors were efficiently localized to autophagosomes under EBSS starvation in MEF (for 2 and 4 h) or HeLa cells (for 4h). We included new cellular images in Figure EV3 and Appendix Figure S3
.

- the western blot experiments are not described in the methods
Response: We thank you for pointing this out. We added a detailed description of western blot experiments to Appendix Supplementary methods.

- the quantification of the ratios of sensor-positive RFP-LC3 spots and autophagosomal/cytosol (A/C) fluorescent intensity should be better described.
E.g. in Fig.2 B, C - they state that they have used 20 cells for quantification and have done three experiments. Is this 20 cells from each experiment or 20 in total? How were the cells selected? How is the significance value calculated - between the averages of the 3 independent experiments or between all cells?

Response: Thank you for pointing this put. We added detailed descriptions of these analyses to the methods. To quantify fluorescent intensity, we randomly choose 20 cell images and averaged the fluorescent intensity ratio between the spots and cytosol. For western blotting, we conducted 3 independent experiments and averaged the results. We revised the Materials and Methods and included additional descriptions in the revised manuscript as follows.

*"**Quantitative analysis of the number of sensor- and Mitotracker-positive dots, ratios of sensor-positive LC3/GABARAPs autophagosome dots, and A/C ratio of fluorescence intensities of the sensor-positive spots***
*For quantitative analysis of co-localization spots including both sensor and MitoTracker-positive dots and sensor-positive LC3/GABARAPs autophagosomes dots, at least 20 cells were randomly selected and excluded from selection if they were directly adjacent to a previously selected cell. The number of both sensor and MitoTracker-positive dots or sensor-positive LC3/GABARAP autophagosomes recognized by the respective probe in a valid cell was counted by overlaying the two channels (co-localization of objects in confocal images) using ZEN imaging software (Carl Zeiss). Ratios of sensor-positive LC3/GABARAP autophagosome dots was calculated as the fold of number of co-localization events divided by total number of LC3/GABARAP-positive spots. To determine the quantitative ratio of autophagosomal/cytosol (A/C) fluorescent intensities, an average value of autophagosomal or cytosol fluorescent intensities was measured for at least randomly selected 5 points on the autophagosome and cytosol in single cell using ZEN software. In the same manner, the quantitative A/C ratio of at least randomly selected 20 cells was analyzed. All statistical data were calculated and graphed using GraphPad Prism 5 (GraphPad, Inc., La Jolla, CA, USA).**"***

The authors state: "P < 0.001 (comparison with all other groups according to one-way ANOVA followed by the Tukey's post-hoc test." -- does this mean that ATG13, BNIP3 and FUNDC1 (in B) are significantly different from each other and all other groups?***

Response: We wished to express that ATG13, BNIP3, and FUNDC1 were significantly different from all other groups. To avoid confusion, we revised the sentence in the revised manuscripts.

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2nd Editorial Decision 02 March 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the referees again whose comments are enclosed. As you will see, the referees appreciate the revision of your work, and I am thus happy to accept your manuscript in principle for publication here.

REFEREE REPORTS

Referee #1:

The authors have satisfactorily addressed all my comments.

Referee #2:

The Authors have satisfactorily addressed my comments and concerns and the revised manuscript is now acceptable for publication in EMBO Journal.

The text should however undergo extensive proofreading for English grammar. The title and headings should also be revised - some of the headings are too long and extensive, e.g. "Among 34 candidate sensors, HyD-LIR(TP)-GFP was the most efficient candidate sensor for detecting all LC3/GABARAPs-positive autophagosomes but preferentially localized to GABARAPL1-positive autophagosome, while HyD-LIR(Fy)-GFP was selective for LC3A/B-positive autophagosome upon autophagy induction in MEFs".

I suggest the following title: "Development of LC3/GABARAP sensors containing a LIR and a hydrophobic domain to monitor autophagy"

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jin-A Lee

Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2016-96315

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ➔ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We performed 3 independent cultures and experiements. More than 20 cells were randomly selected and quantified the numbers of sensor-positive LC3/GABARAP dots per cell and the ratio of the fluorescence intensity of each sensor in autophagosome/cytosol from 5 regions per cell.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There was no exclusion in our experimental groups
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Normally, we performed the blind experiment to get the data
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	We performed the blind test to analyze the data in all experiments
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Two-tailed unpaired T test (Fig 1D and 7F) between gaussian distributed two groups. If not two-tailed Mann-Whitney U test (Fig 1E and 6F). For the comparision of more than three groups Kruskal-Wallis test (Fig 2C, 2F, 2I, 3C, 3F, 3I, 4C, 4F and 7B) for non-gaussian distributed or one way ANOVA (Fig 6B, 6D, 7B, 8B and 8D) for gaussia distributed
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used KS normalities to examine the normal distribution
Is there an estimate of variation within each group of data?	N/A
Is the variance similar between the groups that are being statistically compared?	N/A

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We added antibody information including catalog number in Appendix Supplementary Method
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We added the source of cell line in the Material and Method section (or Appendix Supplementary Method). HeLa, or HEK293T cells obtained from ATCC. The MEFs (WT, atg5KO) were obtained from Dr. Noboru Mizushima. The MEFs(atg7KO) wer obtained from Dr. Lee IH from EWHA womans University.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A