THE EMBO JOURNAL

Manuscript EMBO-2016-95063

Fluorescence-based ATG8 sensors monitor localization and function of LC3/GABARAP proteins

Alexandra Stolz, Mateusz Putyrski, Ivana Kutle, Jessica Huber, Chunxin Wang, Viktória Major, Sachdev Sidhu, Richard Youle, Vladimir Rogov, Volker Dötsch, Andreas Ernst and Ivan Dikic

Corresponding authors: Ivan Dikic and Andreas Ernst, Goethe University Medical School

Review timeline:

Submission date:	20 June 2016
Editorial Decision:	08 July 2016
Revision received:	21 October 2016
Editorial Decision:	08 November 2016
Revision received:	23 November 2016
Accepted:	27 November 2016

Editor: Andrea Leibfried

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 08 July 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 will see, the referees appreciate the development of LC3/GABARAP sensors and think that these could be of interest to the field. However, they think that your analyses should be extended to better show the value of these sensors. Furthermore, throughout the manuscript the data need to be quantified. Both referee reports are very constructive and clear and I trust that you will be able to address all issues raised. I would thus like to invite you to submit a revised version of the manuscript. Please remember that we offer scooping protection and that it is EMBO Journal policy to allow only a single round of revision. Please contact me in case you would like to discuss the revision further.

REFEREE REPORTS

Referee #1:

The article by Stolz et al. reports the development of sensors for ATG8 proteins in human cells. ATG8 proteins are crucial proteins during macroautophagy and there are 6 ATG8 proteins in mammalian cells. Their functions during autophagy are only incompletely understood. Therefore, the development of sensors to specifically localize the endogenous ATG8 proteins is potentially of

wider interest to the autophagy community. In particular, the study by Stolz and colleagues describes the development of sensors for the ATG8 proteins LC3A/B, LC3C and GABL2.
My main concern with the current study is that the sensors are only incompletely characterized and it therefore unclear how useful the developed tools could be for the community.

Major points:
1) The authors correctly write in their introduction: Genome editing and tagging of endogenous genes is becoming easier with recent developments in the CRISPR technique (Kaulich et al, 2015), but this technique needs to be applied to each studied cell line individually. ... We therefore aimed to create a set of fluorescence-based sensors that could target individual mATG8s and are applicable in all kinds of cell lines.

While these are very valid points the relevant characterization of the sensors for their interference with autophagic flux have been conducted in cells that had the sensors stably integrated into the genome (Fig 4). In order to offer the above described flexibility the sensors will have to be used in transient overexpression systems. Otherwise, researchers would still have to generate individual cell lines. The authors should thus show that the sensors do not disturb autophagic activity when expressed by transient transfection.

2) The effect of the sensors on autophagic activity and flux as measured by LC3B lipidation and p62 degradation should be quantified from at least 3 independent experiments. Especially, sensor AS3_67 seems to have an effect on p62 degradation.

3) The specificity of the sensors has to be tested more thoroughly (Fig 5). It should for example be tested if the sensors for the individual ATG8 proteins (i.e the LC3C specific AS3_67) loses its punctate staining and recruitment to Salmonella and mitochondria when LC3C is knocked-down by RNAi or knocked out using CRISPR/Cas9. The experiment in figure 3C does not rule out that the sensor was recruited to autophagosomes due to other non-specific effects (for example the interaction with other ATG8 proteins), since no autophagosomes are formed in Atg5 KO cells and ATG8 lipidation is blocked in general.

4) To the reviewer's knowledge specific antibodies against LC3C do exist. Does their staining signal overlap with that of the sensor in immunofluorescence?

5) In general, the degree of co-localization of the sensors with ATG8 proteins should be quantified. In addition, the cell pictures shown are too small to see any details.

6) The authors write on page 12 lines 295-6 that the sensor AS2_10M30 was specifically recruited to GABARAPL2 (Fig S3A). The pictures shown in this figure seem to suggest that LC3A and LC3B are also targeted to some degree. In this case it would be particularly important to quantify the degree of co-localization.

Minor points:
7) The authors write that none of the sensors had a significant effect on mitophagy flux (page 14, lines 334-5, Fig. 4D) but the figure does not show any error bars and the quantification is derived from only two experiment. How can the authors know if the effect is significant?

8) The title is slightly misleading as "autophagy sensors" imply that the degradation of substances within lysosomes is monitored. More appropriate would be "ATG8 sensors".

9) The authors describe the PB1 domain as dimerization domain (Abstract, line 34) but to the reviewer's knowledge this is an oligomerization domain (see for example Ciuffa et al, 2015, Cell Rep). Also, the PB1 domain is shown as PD1 in Fig. 6 and Fig. S5C.

10) The expression levels of the sensors should be shown.

11) Details of how the ELISA assay was conducted should be included in the methods. In general, more experimental details should be included in the methods to allow reproduction.

12) Some of the labelling of the figures is inconsistent as LC3B is sometimes written as Lc3b (Fig. 3).

13) Scale bars are always missing. Also, the indication of how many times the experiments have been performed is often missing. Where the experiments have been performed more than once (e.g. figure 4D) error bars are generally missing.

14) Figure S4A, lowest panel: the labelling "mCh-3xp6AS3_ΔLIRp62" is not explained.

15) Figure S5B: Individual panels are not labelled. Also, the legend cumulatively refers to figure S5B-D, but applies only to panels C and D.

Referee #2:

In this study Stolz and co-workers have used peptide phage display to select peptides that bind specifically to different members of the LC3 and GABARAP subfamilies (mATG8s) after pre-adsorption to other ATG8 family members and several rounds of selection. The in vitro binding specificities of the identified peptides were analyzed using ELISA and mATG8-GST pulldown assays. In vivo binding specificities were tested using an elegant annexin A4-driven membrane co-translocation assay where the identified peptides are fused to A4-mCherry and binding to EGFP-mATG8 is analyzed by their colocalization. To increase the affinity/avidity of the selected peptides they tested several approached, as generation of sensors (fusions proteins) containing peptide triplicates, having more acidic residues and/or by fusion of the peptides to membrane binding (FYVE) or polymerization (PB1) domains. To show that the mATG8 sensors (AS) can be used to detect their respective mATG8, the peptide sensors (fused to mCherry) were expressed in cells together with different EGFP-tagged mATG8s and their specificity and degree of colocalization analyzed. Finally, the authors show for a few peptide sensors that they can be used used to monitor autophagy processes (starvation-induced autophagosome formation, xenophagy and mitophagy) in cells.

This is a very extensive, well performed and important study. The identification of specific mATG8 sensors opens up for detailed studies of the role of the individual mATG8 proteins in autophagy and other cellular processes. The authors should however address a few major and minor concerns, as detailed below, before acceptance of the paper.

Major concerns:
The authors nicely show in vivo binding specificities of their different ATG8 sensors by their colocalization with corresponding overexpressed EGFP-mATG8 proteins (and not with non-binding mATG8s). The main use of these probes would however be labeling of cells where mATG8s are not overexpressed and it would therefore be important to show that the localization of the different probes are specific when used to label endogenous mATG8 proteins. This should be done for all probes in cells lacking the corresponding mATG8 (e.g. use siRNA or CRISPR). E.g. show that the staining of mCh-PB1-AS3_67 to mitochondria in Fig. 6B is lost in cells lacking LC3C.

In Figure 2 they use the A4-mCh translocation assay to investigate binding of the different sensors to different mATG8s and conclude in the text about differences in colocalization/membrane recruitment. Was this quantified in any way (no such data included)? If not, one should be careful to conclude about differences which seem subtle based on the images (e.g. in Fig 2C it is difficult to see differences between AS_67 and AS3-67). In this assay, why are some A4-mCh_AS recruited to the nuclear membrane and others to the plasma membrane?

Figure 3: it is nicely shown that AS3_p62 is recruited to EGFP- and endogenous LC3B spots. Is this LIR-dependent? Is the colocalization stronger in cells lacking p62 (competition of sensor with endogenous p62)??

In Figure 4 the authors conclude that there is no difference in the autophagy flux when cells are transfected with the AS3_p62 or AS3_67. Also, they say that p62 degradation is normal - this is not so evident from the figures. These data should all be quantified from several experiments.

In Figure 4D they should include a control of something that will block mitophagy (e.g. BafA1 or siULK1). How is the transfection efficiency in these cells?

Figure 5; are the other LC3C probes also recruited to Salmonella? Is the recruitment of mCh-PB1-AS3_67 specific, i.e. still recruited in LC3C depleted cells?

The title should be changed to read: "Fluorescence-based autophagy sensors monitor localization and function of LC3/GABARAP protein. " What are LC3/GABARAP modifiers referring to? This term is not defined or explained in the text.

Minor concerns:
Abstract: mATG8 should be indicated after "Central components of the autophagosomal biogenesis are six 30 members of the LC3 and GABARAP family of ubiquitin-like proteins (mATG8s)"

Introduction; the autophagy process should be explained better - now isolation membrane is mentioned (bottom p3) with no further introduction.

P4: "Consistent with acting at later stages of autophagy, GABARAPs seem to be
88 involved in facilitating membrane fusion (Landajuela et al, 2016; McEwan et
89 al, 2015; Wang et al, 2015).» It shoul also be mentioned that several early core components (e.g. ULK1) binds GABARAPs, so this is not so clear...

Figure 1: IB is used in legend, but neither explained or shown in the figure.
Figure 2 legend; text say that tandem repeats are used. This should be changed to indicate single or triplicate peptides.
It would be nice to show insets for all images in Figure 3.
In Figure 3D AS3_AB2, what is the difference between upper and lower panels (both show LC3A).
The cartoon presented in Figure 6C should be included earlier and the terminology used for the different AS variants should be indicated (e.g. AS3,...). This would make it easier to read the text.
P7: "Such a mechanism to mediate specificity to LC3C has been described for ALFY (Lystad et al, 2014)." LC3C should be changed to GABARAP.

Table 1: should be less colorful.
Methods: should explain the function of KU-0063784 and BafA1.

1st Revision - authors' response 21 October 2016

Referee #1:

The article by Stolz et al. reports the development of sensors for ATG8 proteins in human cells. ATG8 proteins are crucial proteins during macroautophagy and there are 6 ATG8 proteins in mammalian cells. Their functions during autophagy are only incompletely understood. Therefore, the development of sensors to specifically localize the endogenous ATG8 proteins is potentially of wider interest to the autophagy community. In particular, the study by Stolz and colleagues describes the development of sensors for the ATG8 proteins LC3A/B, LC3C and GABL2.
My main concern with the current study is that the sensors are only incompletely characterized and it therefore unclear how useful the developed tools could be for the community.

Major points:
1) The authors correctly write in their introduction: Genome editing and tagging of endogenous genes is becoming easier with recent developments in the CRISPR technique (Kaulich et al, 2015), but this technique needs to be applied to each studied cell line individually. ... We therefore aimed to create a set of fluorescence-based sensors that could target individual mATG8s and are applicable in all kinds of cell lines.

While these are very valid points the relevant characterization of the sensors for their interference with autophagic flux have been conducted in cells that had the sensors stably integrated into the genome (Fig 4). In order to offer the above described flexibility the sensors will have to be used in

transient overexpression systems. Otherwise, researchers would still have to generate individual cell lines. The authors should thus show that the sensors do not disturb autophagic activity when expressed by transient transfection.

We thank the reviewer to raise this point and to give us the chance to explain the choice of our experimental setup better.
While we have tested our sensors extensively upon transient transfection, certain experiments are more reliable and therefore more meaningful with stable cell lines due to the following technical reasons:

1) Upon transient transfection a (considerable) amount of cells might not express the sensor. This is the case for many cell lines used in IF (HeLa, U2OS) and could mask a potential inhibitory effects. We therefore performed in this publication autophagy flux experiments in stable cell lines with close to 100% of cells expressing the sensor in a level suitable for IF, thereby ensuring the correct representation of the influence of the sensor.
2) Transient transfection usually leads to a broad spectrum of expression levels in individual cells in the same dish. Our data indicate, and several microscopy experts stated in personal communications, that the researcher should select cells with low expression levels. This applies in general to all localization / IF experiments with (initially) cytosolic proteins, as strong overexpression (i.e. cytosolic background) will mask recruitment events to distinct structures. Choosing cells with high expression for such IF experiments, are in general prone to false negative results. We chose a setup where the majority of cells show a moderate expression level, i.e. cells which would be suitable for IF.

To still address the concern of the reviewer, we have performed autophagy flux experiments upon transient transfection of the sensor AS3_67. For this experiment, we chose HEK293T cells as a cell line known to be easily transfectable (new figure EV4A+B). The effect of the sensor upon transient transfection did not differ from the results observed in the previously established stable cell lines (see also next point: quantification of autophagy flux).

Regarding the reviewer's comment about the CRISPR technique, we would like to respond that tagging of endogenous proteins is time consuming. Furthermore, in CRISPR mediated endogenous tagging, several cell lines derived from individual clones have to be analyzed for each experiment in order to evaluate damages originating from loss of cell-cell contacts over a long time period and local interference with the genome (e.g. loss of expression). Also, integration of bulky tags (e.g. GFP) is hardly feasible with the current techniques available, as integration cassettes for small tags (e.g. HA) have already very bad statistics of successful integration. This limitation complicates application in IF in general and live cell imaging in particular.
In contrast, viral infection of cell lines with subsequent selection is feasible within weeks and potential off-target effects are largely neutralized by avoidance of single clone selection. Other widely used approach relies on the use of predefined genomic loci for integration (TREx system). In addition, all tags - including larger fluorophores - can be implemented.

Last but not least, ATG8 proteins cannot be tagged at their C-terminus due to processing by ATG4 and differ specifically in their N-terminal domain. Up to now, potential effects on functional impairment via introduction of N-teminal tags have been largely ignored by the field. Certainly, functional aspects of these N-terminal regions will be reported in the near future.

2) The effect of the sensors on autophagic activity and flux as measured by LC3B lipidation and p62 degradation should be quantified from at least 3 independent experiments. Especially, sensor AS3_67 seems to have an effect on p62 degradation.

We agree with the reviewer, that quantification of the autophagy flux will offer easier access to experiments for readers.
Since previous experiments were analyzed via films, which are not optimal for quantification (oversaturation not easily noticeable and subsequent errors upon digitalization of the signal), we repeated all experiments in biological triplicates and detected p62 and LC3 levels with the help of a ChemiDOC MP (BioRad). Representative western blots and respective quantifications can be found in the newly added figure EV4A+B.

We have quantified basal autophagy flux as well as autophagy flux during induction by starvation or Torin1 in the absence and presence of sensors as well as a control construct (mCh-AS3_ΔLIRp62). The construct mCh-AS3_ΔLIRp62 is identical to the p62 derived sensor mCh-AS3_p62, but with alanine substitutions of the two hydrophobic residues within the LIR motif, which were reported in numerous publications to be critical for LC3 interaction. Additionally, the control construct mCh-AS3_ΔLIRp62 does not co-localize in our setup with overexpressed LC3B (new figure 3D and new figure EV3B).

In brief, we found that expression of the control construct revealed slightly elevated levels of basal autophagy upon DOX treatment and subsequent overexpression of the construct. This was mirrored in a slightly enhanced turnover rate of p62 (new figure EV4A+B). Hence, p62 levels at time point 1h were higher compared to non-expressing control cells. Notably, degradation rates of p62 and turnover of LC3B were comparable in the presence and absence of the construct, reflected in a comparable trend of the two graphs.

Taking this into account, we could - if at all - observe a marginal effect of sensor mCh-AS3_67 on p62 degradation. In contrast, sensor mCh-AS3_p62 had a clear effect on p62 degradation, which was already mentioned in the first manuscript. We included all quantification results in the revised version of the main text.

3) The specificity of the sensors has to be tested more thoroughly (Fig 5). It should for example be tested if the sensors for the individual ATG8 proteins (i.e the LC3C specific AS3_67) loses its punctate staining and recruitment to Salmonella and mitochondria when LC3C is knocked-down by RNAi or knocked out using CRISPR/Cas9. The experiment in figure 3C does not rule out that the sensor was recruited to autophagosomes due to other non-specific effects (for example the interaction with other ATG8 proteins), since no autophagosomes are formed in Atg5 KO cells and ATG8 lipidation is blocked in general

To address the concern of the reviewer, we aimed to generate ATG8 KO cell lines. Three guide-RNAs for each ATG8 gene were chosen and cloned into respective vectors. To generate respective cell lines within the given time frame of the revision, we did not aim for single clones, but decided for pooled selection, which was already successfully used in our lab. In addition we used GFP-tagged CAS9 to use FACS sorting as an additional selection option.

We infected U2OS cells with respective viruses and selected for Puromycin. After completed selection, we sorted for GFP positive cells. Except for LC3A, we successfully established qPCR primers for 5 ATG8 genes to validate the status of the generated cell lines.

Unfortunately, based on our qPCR results, our attempts to create clean KO cells were unsuccessful, as up to 80% of wild type ATG8 levels remained present in the pooled cell population.

As an alternative approach to address the reviewer's concern, we have performed ITC measurements to further validate specificity of peptide 67 for LC3C. ITC experiments showed significant preference of Ub19-AS_67 fusion construct and free peptide 67 bound LC3C with K_D 2.4 µM and 2.2µM, respectively. These values are comparable to p62 and NBR1 LIR interactions to LC3B (Rozenknop et al., 2011). Importantly, titration of all others human ATG8-proteins with Ub19-AS_67 fusion construct revealed only weak enthalpy changes that did not differ significantly from Ub19-AS_67 dilution heat. Furthermore, neither LC3A nor LC3B substantially interact with untagged peptide 67 *in vitro*. Results of these experiments are presented in the new figure EV5F-H.

Together with the quantitative data presented in figure EV3B from *in vivo* experiments (see also upcoming point 5) and the IF data of endogenous LC3C staining (figure 5,6 and EV5; see also next point 4), we hope to have convinced the reviewer that our sensor based on peptide 67 is specific for LC3C and that crosstalk with remaining mATG8s is negligible.

4) To the reviewer's knowledge specific antibodies against LC3C do exist. Does their staining signal overlap with that of the sensor in immunofluorescence?

During our studies we tested three different antibodies for LC3C (abcam ab168813; viva VB2882, **α**-LC3C described in Stadel et al. 2015). We had tested specificity of respective antibodies via their ability to pull down purified, recombinant ATG8s. The LC3C antibody from abcam cross-reacted with other ATG8s in our hands. The LC3C antibody from viva was very specific, but with low affinity. The LC3C antibody described in Stadel at al., 2015 worked best in our hands and showed high specificity towards LC3C (new figure EV5E). Unfortunately this LC3C antibody is not commercially available and we had only limited amount available for our experiments.

We had used this antibody to stain for endogenous LC3C in some of the presented Salmonella experiments. The staining for endogenous LC3C did overlap with the sensor signal (updated figure 5A+B), however with low intensity in the case of Salmonella escaping into the cytosol.

We now extended this set of data by staining U2OS cells expressing sensor mCH-PB1-AS3_67 and undergoing mitophagy (treatment with CCCP+Baf for 2h). In accordance with our original data presented in figure 6A+B, the sensor signal overlapped with the staining for endogenous LC3C to a great extent (new figure 6D).

In accordance with our discussion about potentially different functions of LC3C vs. LC3B in mitophagy and xenophagy, a subpopulation of cytosolic Salmonella was coated completely with endogenous LC3C while endogenous LC3B accumulated in puncta around the escaped Salmonella (new figure 5E). We also speculated that a LC3C coat is necessary and sufficient for Salmonella to be delivered to the lysosome. However, with our current set of data we can neither confirm nor dismiss this hypothesis (new figure EV5D).

We analyzed the distribution of endogenous LC3C upon induction of mitophagy. In accordance with our presented data on sensor AS3_67, endogenous LC3C is localized to mitochondria upon depolarization with CCCP (new figure 6C).
Of note, events with high intensity of endogenous LC3C in combination with a nearly complete coat around mitochondria as presented in the upper panels are rather rare. Respective cells have an extremely deformed nucleus and are probably undergoing apoptosis at late stage. We still decided to include such an image in the figure to make this point clear for readers with little or no experience in microscopy. We have mentioned this point in the text and in addition included a representative image of LC3C distribution upon CCCP treatment in the lower panels of figure 6C. As it can be seen, in accordance with our data presented in figure 6A+B, endogenous LC3C is covering large areas of the mitochondrial surface upon CCCP treatment.

5) In general, the degree of co-localization of the sensors with ATG8 proteins should be quantified. In addition, the cell pictures shown are too small to see any details.
The authors write on page 12 lines 295-6 that the sensor AS2_10M30 was specifically recruited to GABARAPL2 (Fig S3A). The pictures shown in this figure seem to suggest that LC3A and LC3B are also targeted to some degree. In this case it would be particularly important to quantify the degree of co-localization.

We are grateful to the reviewer to point out this flaw in our initial submission. We have now enlarged sections of respective images to provide more details to the reader (updated figure 3). Additionally, we kept the larger overview, as we want to give potential users an impression of the expected image quality when using our sensors.

Finding a good setting to quantify recruitment of sensors in an unbiased and meaningful approach was more challenging. Taking several options into account, we decided to quantify recruitment with the help of the analysis software provided by Leica. A customized quantification program was designed in close collaboration with the Leica customer service. In brief, the program identifies ATG8 spots based on high local intensity above background signal. This is repeated in the channel detecting the sensor. Subsequently, an overlay of both channels is created and the number of ATG8 spots recognized by the sensor is given for each image. We included a more detailed description of the technical settings in the material and method part.
We have analyzed between 50-100 cells representing between 700-4500 individual events for each combination of the six ATG8s with our most characterized sensors (mCh-AS3_p62, mCh-AS3_67 and mCh-AS2_10M30; figure EV3A+B).

The results show that our sensors based on the peptides 67 and 10M30 are highly specific for LC3C and GABARAPL2, respectively. Of note, the degree of specificity of the presented sensors is most probably even higher than presented, as e.g. sensor mCh-AS2_10M30 could be recruited to structures positive for overexpressed GFP-LC3B by endogenous GABARAPL2.

Minor points:
7) The authors write that none of the sensors had a significant effect on mitophagy flux (page 14, lines 334-5, Fig. 4D) but the figure does not show any error bars and the quantification is derived from only two experiment. How can the authors know if the effect is significant?

We have added error bars and indicated significant changes in the updated figure 4D. In addition we have included FIP200 KO as a positive control for mitophagy inhibition in our data set (new figure EV4D).

8) The title is slightly misleading as "autophagy sensors" imply that the degradation of substances within lysosomes is monitored. More appropriate would be "ATG8 sensors".

The reviewer has a point there. We have renamed the sensors into mATG8 sensors and changed the title accordingly.

9) The authors describe the PB1 domain as dimerization domain (Abstract, line 34) but to the reviewer's knowledge this is an oligomerization domain (see for example Ciuffa et al, 2015, Cell Rep). Also, the PB1 domain is shown as PD1 in Fig. 6 and Fig. S5C.

We thank the reviewer for this correction. Indeed the PB1 domain of p62 is an oligomerization domain. We have updated the abstract accordingly and also corrected the spelling mistakes in our figures.

10) The expression levels of the sensors should be shown.

We have compared levels of different sensors (stable and transient expression) by Western Blot. Presented data consists of samples derived from experiments shown in figure EV4A+B. The levels of the further validated sensors are comparable in stable expressing cell lines and higher in transiently transfected cells.
Equal, high expression levels of sensors in cells, used for the mitophagy flux experiments (figure 4D), were ensured by gating the detection window for high GFP values (see also Methods).

11) Details of how the ELISA assay was conducted should be included in the methods. In general, more experimental details should be included in the methods to allow reproduction.

We have updated the description of methods and added more technical details to allow easier reproduction.

12) Some of the labelling of the figures is inconsistent as LC3B is sometimes written as Lc3b (Fig. 3).

We went once again through the figures to correct inconsistent labeling. In the case mentioned by the reviewer, the difference is based on the cell line used (human vs. mice). The correct nomenclature for the mouse homologue is Lc3b.

13) Scale bars are always missing. Also, the indication of how many times the experiments have been performed is often missing. Where the experiments have been performed more than once (e.g. figure 4D) error bars are generally missing.

We thank the reviewer for this point and have now included the error bars in figure 4D and the new associated figure EV4D.

14) Figure S4A, lowest panel: the labelling "mCh-3xp6AS3_ΔLIRp62" is not explained.

A proper description of this control construct was indeed missing. We have now described the composition and usage of the control construct mCh-3xp6AS3_ΔLIRp62 in the main text and included the sequence in the table EV1 (former supplementary table 1).

15) Figure S5B: Individual panels are not labelled. Also, the legend cumulatively refers to figure S5B-D, but applies only to panels C and D.

We updated figure EV5 (former figure S5B) and corresponding figure legends.

Referee #2:

In this study Stolz and co-workers have used peptide phage display to select peptides that bind specifically to different members of the LC3 and GABARAP subfamilies (mATG8s) after pre-adsorption to other ATG8 family members and several rounds of selection. The in vitro binding specificities of the identified peptides were analyzed using ELISA and mATG8-GST pulldown assays. In vivo binding specificities were tested using an elegant annexin A4-driven membrane co-translocation assay where the identified peptides are fused to A4-mCherry and binding to EGFP-mATG8 is analyzed by their colocalization. To increase the affinity/avidity of the selected peptides they tested several approached, as generation of sensors (fusions proteins) containing peptide triplicates, having more acidic residues and/or by fusion of the peptides to membrane binding (FYVE) or polymerization (PB1) domains. To show that the mATG8 sensors (AS) can be used to detect their respective mATG8, the peptide sensors (fused to mCherry) were expressed in cells together with different EGFP-tagged mATG8s and their specificity and degree of colocalization analyzed. Finally, the authors show for a few peptide sensors that they can be used used to monitor autophagy processes (starvation-induced autophagosome formation, xenophagy and mitophagy) in cells.
This is a very extensive, well performed and important study. The identification of specific mATG8 sensors opens up for detailed studies of the role of the individual mATG8 proteins in autophagy and other cellular processes. The authors should however address a few major and minor concerns, as detailed below, before acceptance of the paper.

Major concerns:

1) The authors nicely show in vivo binding specificities of their different ATG8 sensors by their colocalizaJusttion with corresponding overexpressed EGFP-mATG8 proteins (and not with non-binding mATG8s). The main use of these probes would however be labeling of cells where mATG8s are not overexpressed and it would therefore be important to show that the localization of the different probes are specific when used to label endogenous mATG8 proteins.
This should be done for all probes in cells lacking the corresponding mATG8 (e.g. use siRNA or CRISPR). E.g. show that the staining of mCh-PB1-AS3_67 to mitochondria in Fig. 6B is lost in cells lacking LC3C.

We agree with the reviewer and our main aim (vision) when starting this project was to have tools to monitor all 6 individual mATG8 isoforms (LC3s and GABARAPs) at endogenous levels. Three years later we have proven the applicability of the concept by showing recruitment of sensors to endogenous LC3B and LC3C (figure 3B and figure 5 and 6) And provided first data for their potential in live cell imaging (e.g to monitor mitophagy; new movie EV6).

Functionality of developed sensors for remaining ATG8s on the endogenous levels has still to be proven. At the moment we are missing ATG8 specific antibodies as well as knowledge, in which processes these proteins are involved. As discussed in the manuscript, sensors do need high local concentration of ATG8 – like on autophagosomes – to proceed. This characteristic on endogenous level has not been proven to be the case for all ATG8s. To validate other sensors we have even established phage display development of antibodies again specific mATG8 (data not shown) but have not been successful in creating antibodies with high specificity and affinity.
Another way to validate remaining sensors would have been a comparison of staining patterns between WT and KO cell lines under basal or stress conditions. However, establishment of respective cell lines within the time of revision has failed.

2) In Figure 2 they use the A4-mCh translocation assay to investigate binding of the different sensors to different mATG8s and conclude in the text about differences in colocalization/membrane recruitment.
-Was this quantified in any way (no such data included)?
-If not, one should be careful to conclude about differences which seem subtle based on the images (e.g. in Fig 2C it is difficult to see differences between AS_67 and AS3-67).
-In this assay, why are some A4-mCh_AS recruited to the nuclear membrane and others to the plasma membrane?

We thank the reviewer for making this point. The recruitment of A4-fused substrates to the nuclear and/or plasma membrane depends on their subcellular localization at the time of ionomycin treatment, i.e. annexin A4 fusions, which are also expressed in the nucleus will translocate upon ionomycin treatment to the nuclear envelope.

We had validated the co-recruitment in annexin assays by assigning the range of "no / little / good / very good" co-recruitment to single cells. A minimum of 50 cells per experiment, and three biological independent experiments per condition were rated. We have included this data in the new figure 2F and EV2F.

3) Figure 3: it is nicely shown that AS3_p62 is recruited to EGFP- and endogenous LC3B spots. Is this LIR-dependent?

We have performed a respective experiment in a stable inducible cell line expressing a LIR mutant of AS3_p62 (AS3_ΔLIRp62) and could not see recruitment to autophagosomes positive for overexpressed LC3B (updated figure 3D). The respective quantification can be found in the new figure EV3B.

4) Is the colocalization stronger in cells lacking p62 (competition of sensor with endogenous p62)??

From our autophagy flux data presented in figure 4 and EV4, a competition with endogenous p62 can be observed to a certain extent. The overexpression of the sensor based on the p62 sequence did lead to an increase of endogenous p62 levels, indicating decreased turnover of p62 by the basal autophagy flux.

5) In Figure 4 the authors conclude that there is no difference in the autophagy flux when cells are transfected with the AS3_p62 or AS3_67. Also, they say that p62 degradation is normal - this is not so evident from the figures. These data should all be quantified from several experiments.

We have quantified the autophagy flux experiments and included respective results in figure EV4. Please see also point 2 of reviewer 1.
We have stated in the original version of our manuscript, that the overexpression of mCh-AS3_p62 minimally affected p62 levels. We removed the word minimally in the revised version of the manuscript, as the influence of this sensor on p62 levels is now convincingly shown in the quantification data.

6) In Figure 4D they should include a control of something that will block mitophagy (e.g. BafA1 or siULK1). How is the transfection efficiency in these cells?

We agree with the reviewer that a positive control is needed to show the range of the presented assay. We have used a FIP200 KO to show the effect of a potent defect in mitophagy on our assay (new figure EV4 D).
In this assay, stable cell lines expressing the respective construct were used. In addition, the detection gate of the FACS machine was set to sort for high expressing cells (high GFP signal) to ensure equal expression levels between sensors and to detect the maximum of a potential inhibitory effect.

7) Figure 5; are the other LC3C probes also recruited to Salmonella? Is the recruitment of mCh-PB1-AS3_67 specific, i.e. still recruited in LC3C depleted cells?

We have not further tested the PB1/FYVE-domain containing variants of sensor 67 for recruitment to Salmonella. However, we started a collaboration with a lab specialized on xenophagy and hope to report further on this topic in the near future.

As mentioned above, our attempts to generate LC3C KO cells failed. However, we included IF staining of endogenous LC3C localized to cytosolic Salmonella in our presented data set to provide further evidence for the specificity of our sensor (updated figure 5 and EV5).

8) The title should be changed to read: "Fluorescence-based autophagy sensors monitor localization and function of LC3/GABARAP protein. " What are LC3/GABARAP modifiers referring to? This term is not defined or explained in the text.

According to the suggestions of reviewer 2 and reviewer 1, we have changed the title of the manuscript into
'Fluorescence-based mammalian ATG8 sensors monitor localization and function of LC3/GABARP proteins'.

Minor concerns:

9) Abstract: mATG8 should be indicated after "Central components of the autophagosomal biogenesis are six 30 members of the LC3 and GABARAP family of ubiquitin-like proteins (mATG8s)"

Along the reviewer's suggestion, we have introduced the term mATG8 in the abstract.

10) Introduction; the autophagy process should be explained better - now isolation membrane is mentioned (bottom p3) with no further introduction.

We have introduced the term 'isolation membrane' in the text.

11) P4: "Consistent with acting at later stages of autophagy, GABARAPs seem to be
88 involved in facilitating membrane fusion (Landajuela et al, 2016; McEwan et
89 al, 2015; Wang et al, 2015).» It shoul also be mentioned that several early core components (e.g. ULK1) binds GABARAPs, so this is not so clear...

We have included the reviewer's comment in the main text of the manuscript.

12) Figure 1: IB is used in legend, but neither explained or shown in the figure. Figure 2 legend; text say that tandem repeats are used. This should be changed to indicate single or triplicate peptides.

We have updated the legend of figure 1 and 2.

It would be nice to show insets for all images in Figure 3.

We present now insets for all merged images of figure 3.

In Figure 3D AS3_AB2, what is the difference between upper and lower panels (both show LC3A).

We thank the reviewer to draw our attention to this spelling mistake and have changed the labeling of the lower panel to LC3B.

14) The cartoon presented in Figure 6C should be included earlier and the terminology used for the different AS variants should be indicated (e.g. AS3,...). This would make it easier to read the text.

We relocated the cartoon for different sensor variants to figure 3 and indicated the name for the variants used in the text.

15) P7: "Such a mechanism to mediate specificity to LC3C has been described for ALFY (Lystad et al, 2014)." LC3C should be changed to GABARAP.

We have changed LC3C to GABARAP.

16) Table 1: should be less colorful.

We have substituted the background color of the table for shades of gray.

17) Methods: should explain the function of KU-0063784 and BafA1.

We have explained the function of KU and BafA1 in the method section.

2nd Editorial Decision 08 November 2016

Thank you for submitting your revised manuscript for our consideration.
Your manuscript has now been seen once more by the original referees (see comments below), and I am happy to inform you that they are both broadly in favor of publication, pending satisfactory minor revision.

I would therefore like to ask you to address referee #2's suggestion and to provide a final version of your manuscript including scale bars for the IF images.

REFEREE REPORTS

Referee #1:

The authors have addressed my main concerns and I have no further comments.

Referee #2:

The authors have addressed all my comments and concerns in a satisfactory manner. One minor issue; scale bars are missing from all immunofluorescence images and should be indicated.

2nd Revision - authors' response 23 November 2016

According to your suggestion, we have uploaded high quality images of all figures after including scale bars in IF images of figure 3, figure 5, figure 6 and figure EV5.

A single pdf file containing uncropped/-processed electrophoretic blots of figure 4 was uploaded as 'Additional Figure Data 1'.

3rd Editorial Decision 27 November 2016

Thank you for sending the final version of your manuscript to us. I appreciate the introduced changes, and I am happy to accept your manuscript for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ivan Dikic and Andreas Ernst
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-95063R1

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).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
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?	NA
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.	NA
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.	For the annexin A4-based co-translocation assay (Fig EV2;), an unblinded evaluation of the results was performed. For FACS analysis of the mitophagy assay (Fig 4D and Fig EV5C; p32), unbias was ensured by equal gating of the GFP window. For quantification of sensor recruitment to autophagosoms (Fig EV3 A+B; p30), cells for quantification have been chosen in the ATG8 channel only. Recruitment was then quantified with the help of the analysis software provided by Leica. A customized quantification program was designed in close collaboration with the Leica customer service. In brief, the program identifies ATG8 spots based on high local intensity above background signal. This is repeated in the channel detecting the sensor. Subsequently, an overlay of both channels is created and the number of ATG8 spots recognized by the sensor is given for each image.
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?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	Miitophagy assay (Fig 4D and Fig EV5C, p32): yes, using 2-way anova of one-way anova and one-way anova analysis.

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).	p26: Antibodies used for immunofluorescence: anti-dsRED (rabbit, 632496, Clontech, 1:300), anti-LC3B (mouse, M152-3, MBL, 1:300 or rabbit, PM036, MBL, 1:300), anti-LC3C (rabbit, 1:100, (Stadel et al, 2015)), anti-LAMP1 (mouse, H4A3, DSHB, 1:400) Antibodies used for Western blot: anti-GFP (mouse, B-2, sc-9996, Santa Cruz Biotechnology, 1:1000), anti-dsRED (rabbit, 632496, Clontech, 1:1000), anti-tRFP (rabbit, AB234, BioCat, 1:1000), anti-LC3B (mouse. 0231-100/LC3-5F10, Nano tools, 1:1000), anti-p62 (mouse, M162-3, MBL, 1:1000), anti-vinculin (mouse, G1160, Sigma, 1:1000).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	p26: HeLa Kyoto - Carsten Schultz, EBML Heidelberg, Germany; HEK293T and U2OS cells were acquired from ATCC. HeLa cells (mitophagy assay) were acquired from the ATCC and authenticated by the Johns Hopkins GRCF Fragment Analysis Facility using STR profiling. HeLa FRT/TO cells for the generation of stable cell lines using the Flp-In T-REx System (Invitrogen) were provided by S. Taylor (Tighe et al, 2008). All cell lines used in the study were tested for mycoplasma contamination at least monthly.

* 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	Peptide sequences were provided in Table EV1.
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).	NA
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).	NA
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	NA
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.	NA

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.	NA