THE
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JOURNAL

The WD40 domain of ATG16L1 is required for its non-canonical role in lipidation of LC3 at single membranes

Katherine Fletcher, Rachel Ulferts, Elise Jacquin, Talitha Veith, Noor Gammoh, Julia M. Arasteh, Ulrike Mayer, Simon R. Carding, Thomas Wileman, Rupert Beale & Oliver Florey

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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 9 August 2017

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

As you will see, the referees appreciate your data. However, they also think that the study needs to be extended to warrant publication in The EMBO Journal.

Should you be able to extend your work as outlined by the referees, I would like to invite you to submit a revised version of the manuscript. Importantly, the revision should provide strong data to offer:
- further reaching insight into how the WD40 domain mediates recruitment of ATG16L1 (see also report from referee #2), and this is also crucial to clearly elevate your findings above the work from Boada-Romero et al, 2016
- insight into the functional consequences of WD40 mediated ATG16L1 activity (see also report from referee #3).

All other points raised by the referees should also be addressed. Should you not be able to address the comments of all three reviewers, please get in touch with me. Please also contact me in case you would like to discuss the revision further. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

Fletcher et al. investigated the role of ATG16L1 in what they term "non-canonical autophagy", which is defined by single membrane endolysosomal vesicles labeled with LC3. While ATG16L1 WD repeat containing C-terminal domain (WD40 CTD) is dispensable for canonical autophagy the authors provide evidence that this region of the protein is essential for targeting to single-membrane vesicles and consequently promotion of LC3 lipidation. Employing truncated ATG16L1, the authors successfully distinguished between canonical autophagy that requires VPS34 and WIPI2b and non-canonical autophagy defined by ATG16L1 recruitment and LC3 lipidation independently of PI3P and WIPI2b. The requirement of WD40 CTD is clearly demonstrated under physiological conditions such as LC3-associated phagocytosis (LAP), exposure to the bacterial toxin VacA and infection by influenza A virus.
This research provides a solid and straightforward data evaluating a specific role for WD40 CTD of ATG16L1. The authors also provided a genetic tool that clearly enables to distinguish between macroautophagy and non-canonical usage of autophagic machinery. These findings contribute to better understanding of cellular pathways utilizing parts of the autophagic system.
Specific comments
1. The effect of the sodium/proton ionophore monensin described by the authors in this and in their previous study is rather unclear. Accordingly, the fact that it leads to LC3 lipidation may be explained not only by induction of osmotic imbalances within endosomal compartments, but an inhibition of ATG4 de-lipidation activity, which in principle will lead to increased LC3 lipidation. This should be better addressed textually and experimentally.
2. Figure 2e - high LC3 lipidation in ΔFBD mutant is not in correlation with that data presented in Figure 2g for this mutant under starvation.
3. Figure 2e and Figure 3a - no explanation is given for the elevated LC3 lipidation in the control of ΔFBD mutant.
4. The term non-canonical autophagy in this context is somewhat confusion and the authors are encouraged to better define this process.

Referee #2:

The manuscript by Fletcher et al. reports the interesting finding that the C-terminal WD40 domain of human ATG16L1 is required for macroautophagy independent lipidation of LC3.

ATG16L1 is part of the ATG5-12-16 complex, which acts in a E3-like manner to promote the conjugation of LC3 proteins to the membrane lipid PE. This process is canonically associated with macroautophagy, where cells conjugate LC3 proteins to the nascent autophagosomal membrane. However, it has become evident that LC3 proteins are also lipidated in processes that do not involve double membranes (such as the autophagosome). The ATG16L1 protein is required for all these LC3 lipidation events and the authors here show, using transduced cell lines, that the C-terminal WD40 domain of ATG16L1 is required for macroautophagy independent LC3 lipidation but not for lipidation during macroautophagy. Mechanistic insights into how the WD40 domain mediates the recruitment of ATG16L1 in the macroautophagy independent processes are not provided. Does it bind lipids, other proteins or both? In my opinion, the manuscript will be of interest for the autophagy community but in its current form it is too limited in its scope to be a strong candidate for the EMBO Journal.

Specific comments

1. I think the term non-canonical autophagy is misleading and not ideal to describe the processes studies in the paper. In reality, these are not autophagic processes as no "self" is degraded. Instead, most processes studied in the manuscript are phagocytic or endocytic in nature. For a detailed discussion please see for example a recent review in the EMBOJ (Galluzzi, 2017, EMBOJ (PMID: 28596378)).

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3. In Figure 2e, was Bafilomycin used? Also, the quantification does not accurately reflect what can be seen in in the blot (Figure 2e, f).

4. The statement "The structure of the WD40 CTD has recently been solved, but its biological function remains unclear 20." is not entirely correct (see Boada-Romero, 2016, Nat Comms (PMID: 27273576)).

Referee #3:

Autophagy is characterized by the lipidation of the ubiquitin-like molecule LC3, which is mediated by the ATG16L1 E3 ligase-like complex. This event contributes to the formation of the double-membrane autophagosome. Recent studies indicate that lipidated LC3 can also insert into single membrane vesicles to mediate autophagy-related processes such as LC3-associated phagocytosis (LAP). The mechanisms that distinguish these disparate functions of LC3 are incompletely understood. Previous studies have shown that the WD40 domain of ATG16L1 is dispensable for conventional starvation-induced autophagy. In this manuscript, the authors examine whether the WD40 domain is necessary for other functions associated with LC3 lipidation by ATG16L1.

The authors use LAP and monensin-induced LC3 targeting as models of "non-canonical autophagy". They first show that recruitment of LC3 and/or ATG16L1 to single membranes occurs independent of VPS34 and WIPI2b, factors typically required for targeting of LC3 to double membranes during autophagy. Consistent with this finding, they show that the ATG16L1 truncation mutant lacking the WIPI2b and FIP200 binding region (delta FBD) can mediate LC3 targeting to single membranes when introduced into cell lines in which endogenous ATG16L1 is removed by CRISPR/Cas9. In contrast, ATG16L1 lacking the WD40 domain (delta WD40) are deficient in LC3 targeting to single membranes, but retain the ability to mediate autophagy. The inability to mediate LC3 targeting to single membranes was not due to failure to form the E3 ligase-like complex (ATG5-ATG12-ATG16L1), but was associated with lack of recruitment of ATG16L1 delta WD40 to the phagosome. Finally, they show that the same pathway is triggered by influenza M2 protein, which inserts into membranes to act as a proton channel and can have a similar effect as monensin. In a manner dependent on the proton channel activity of M2, influenza infection induces relocalization of LC3 in cells harboring ATG16L1 delta FBD but not ATG16L1 delta WD40.

The role of the WD40 domain in mediating LC3 targeting is novel and the observation that autophagy and this autophagy-related function of ATG16L1 can be decoupled through mutagenesis is exciting. The experiments include validation through multiple techniques with appropriate quantification and controls. The use of several cell lines and results with influenza infection are additional strengths. Thus, the conclusions are generally supported well by the data. The manuscript could be strengthened further if the authors can address the following concerns:

1. The terminology used in this manuscript is confusing. As articulated in a recent comprehensive review article (Galluzi et al, EMBO J. 2017 36(13):1811), the term "non-canonical autophagy" is ambiguous and misleading.

2. A related issue is that the relationship between LAP, monensin treatment, and M2 activity is obscure. Are these the same events at the molecular level? For instance, does LC3 targeting to single membranes in the presence of monensin or M2 require rubicon (an essential LAP protein)? Unifying these disparate models would significantly improve this manuscript and allow the authors to use the umbrella term LAP. It would also justify switching between models. Alternatively, in some experiments it appears that monensin enhances LAP. Is this enhancement or induction?

3. Most experiments rely on transformed cell lines, which typically display dysregulated autophagy. The exception is the MEFs. Are the ATG16L1 knockout MEFs transformed? Information on passage number would be helpful.

4. Although LC3 lipidation and trafficking is clearly affected by ATG16L1 mutagenesis, the authors do not provide evidence demonstrating a functional consequence. For instance, LAP has clear

functions in pathogen control and cytokine production. Are these dependent on the WD40 domain? In the example of influenza M2, does the WD40 domain affect viral replication or immune responses to the virus? The authors refer to controversies in the field regarding the relationship between M2 and autophagy. They appear to have an opportunity to address this through functional studies. The authors are not expected to chase every downstream function of LC3 targeting that could be important, but certainly some evidence should be provided to show that the WD40 domain matters beyond LC3 Western blots and immuno-fluorescence.

1st Revision - authors' response 10 November 2017

Referee #1:

Fletcher et al. investigated the role of ATG16L1 in what they term "non-canonical autophagy", which is defined by single membrane endolysosomal vesicles labeled with LC3. While ATG16L1 WD repeat containing C-terminal domain (WD40 CTD) is dispensable for canonical autophagy the authors provide evidence that this region of the protein is essential for targeting to single-membrane vesicles and consequently promotion of LC3 lipidation. Employing truncated ATG16L1, the authors successfully distinguished between canonical autophagy that requires VPS34 and WIPI2b and non-canonical autophagy defined by ATG16L1 recruitment and LC3 lipidation independently of PI3P and WIPI2b. The requirement of WD40 CTD is clearly demonstrated under physiological conditions such as LC3-associated phagocytosis (LAP), exposure to the bacterial toxin VacA and infection by influenza A virus.
This research provides a solid and straightforward data evaluating a specific role for WD40 CTD of ATG16L1. The authors also provided a genetic tool that clearly enables to distinguish between macroautophagy and non-canonical usage of autophagic machinery. These findings contribute to better understanding of cellular pathways utilizing parts of the autophagic system.

We thank the reviewer for their positive comments.

Specific comments
1. The effect of the sodium/proton ionophore monensin described by the authors in this and in their previous study is rather unclear. Accordingly, the fact that it leads to LC3 lipidation may be explained not only by induction of osmotic imbalances within endosomal compartments, but an inhibition of ATG4 de-lipidation activity, which in principle will lead to increased LC3 lipidation. This should be better addressed textually and experimentally.

We understand the reviewer's point, that revealing LC3 lipidation could in principle be due to induction of lipidation or prevention of ATG4 de-lipidation. However we do not believe the LC3 lipidation we observe is related to inhibition of ATG4 activity. We are able to induce endolysosomal LC3 lipidation with a wide variety of lysosomotropic drugs in addition to monensin (Florey et al, 2015; Jacquin et al, 2017). Indeed, we are able to induce lipidation simply by altering the osmotic properties of the media. Under these osmotic conditions LC3 is only seen to lipidate to single-membrane endolysosomal compartments rather than early endosomes or the plasma membrane. If there was an inhibition of ATG4 activity we may expect to see indiscriminate LC3 relocalization to membranes. We have now included extra experimental FRAP data (Fig EV2 C and D), which shows that monensin treatment induces the prolonged recruitment of ATG16L1 to lysosomal membranes, which we believe is the driver of LC3 lipidation under this system.

2. Figure 2e - high LC3 lipidation in ΔFBD mutant is not in correlation with that data presented in Figure 2g for this mutant under starvation.

Data from Figure 2E is from HCT116 cells, while 2G come from MEF cells. The corresponding confocal images for HCT116 cells are found in Fig EV 1A.

3. Figure 2e and Figure 3a - no explanation is given for the elevated LC3 lipidation in the control of ΔFBD mutant.

It has previously been shown (Gammoh et al, 2013) that deletion of the FBD does not eliminate basal autophagy, or glucose starvation induced autophagy. Hence, we would not expect to see a complete loss of LC3-II in these cell lines. The quantification data shown was obtained from blots from three independent experiments, which – like all biological samples- showed some variability. The blot shown here is one of these blots.

4. The term non-canonical autophagy in this context is somewhat confusion and the authors are encouraged to better define this process.

We thank the reviewers for raising this interesting point. We agree that the term "non-canonical" autophagy is not perfect, as it does not fit within narrow definitions of what can be termed an autophagic process. However, there exists a growing body of work in which the term non-canonical autophagy has already been applied to the lipidation of LC3 to single-membrane compartments (Henault et al, 2012; Kim et al, 2013; Martinez et al, 2016). Indeed, many reviews and guidelines on autophagy have termed the process "non-canonical autophagy" or a "noncanonical autophagy process" (Cadwell, 2016; Klionsky et al, 2016). LC3 associated phagocytosis, or LAP, is commonly used to describe these events. However, many of the processes we study are not phagocytosis, and there are some molecular mechanisms that appear to be specific to phagocytosis rather than the non-canonical autophagy process in general (see response to reviewer 3 point 2). We considered introducing another term to describe LC3 lipidation at single membranes that is dependent on the WD40 domain of ATG16L1, but decided that yet another acronym to describe this set of processes would if anything lead to more confusion. We have therefore altered our text in the introduction to make this point as clear as possible, and define precisely what we mean when we use the term non-canonical autophagy (pages 4-5). We have also altered the title to make it clearer on what processes we are studying.

Referee #2:

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it has become evident that LC3 proteins are also lipidated in processes that do not involve double membranes (such as the autophagosome). The ATG16L1 protein is required for all these LC3 lipidation events and the authors here show, using transduced cell lines, that the C-terminal WD40 domain of ATG16L1 is required for macroautophagy independent LC3 lipidation but not for lipidation during macroautophagy. Mechanistic insights into how the WD40 domain mediates the recruitment of ATG16L1 in the macroautophagy independent processes are not provided. Does it bind lipids, other proteins or both? In my opinion, the manuscript will be of interest for the autophagy community but in its current form it is too limited in its scope to be a strong candidate for the EMBO Journal.

We thank the reviewer for their positive comments. We have included new data (Fig 6) to increase our mechanistic understanding how ATG16L1 functions during non-canonical autophagy. We now identify key amino acid sites within the WD40 CTD that are required for non-canonical autophagy. This is the first report of such sites and increases our understanding of how the WD40 CTD orchestrates LC3 lipidation.

Specific comments

1. I think the term non-canonical autophagy is misleading and not ideal to describe the processes studies in the paper. In reality, these are not autophagic processes as no "self" is degraded. Instead, most processes studied in the manuscript are phagocytic or endocytic in nature. For a detailed discussion please see for example a recent review in the EMBOJ (Galluzzi, 2017, EMBOJ (PMID: 28596378)).

We thank the reviewer for this comment and refer them to our answer to referee 1 point 4.

2. Are there other assays than LC3 lipidation to asses "non-canonical" autophagy? For example, the delivery of material into lysosomes and/or its degradation? Are the processes studied actually dependent on LC3 lipidation and blocked by deletion of the WD40 domain of ATG16L1?

We thank the reviewer for this suggestion. The functions of non-canonical autophagy are still being determined. However, a number of immune cell functions have been proposed to be dependent on it. We have utilized a new mouse model where the C-terminal WD40 domain of ATG16L1 has been truncated. This renders the mouse deficient for non-canonical autophagy, while remaining competent for canonical autophagy. Using this system we now demonstrate a requirement for non-canonical autophagy in dendritic cell MHC class II antigen presentation (Fig 7).

3. In Figure 2e, was Bafilomycin used? Also, the quantification does not accurately reflect what can be seen in in the blot (Figure 2e, f).

Bafilomycin was not included in the experiment. For the quantification, blots of three independently experiments were used. Only one of these

blots can unfortunately be included in the paper. The main point of the experiment was to demonstrate that the ΔFBD cells show a defect in canonical autophagy responses to mTor inhibition, while the ΔWD cell do not. We have adjusted the analysis to show fold induction of LC3II/LC3I ratios over control (Fig 2 F). This shows that ΔFBD cells respond significantly less to PP242 than full length expressing cells, where as there is no difference between FL and ΔWD cells. These western blots are just one assay to test the autophagic response, as we have also performed LC3 and WIPI2b puncta counts. All data support our conclusions, and these are in line with previous publications that show the same result.

4. The statement "The structure of the WD40 CTD has recently been solved, but its biological function remains unclear 20." is not entirely correct (see Boada-Romero, 2016, Nat Comms (PMID: 27273576)).

We agree with the reviewer that recent work has demonstrated some role for the WD40 CTD and have altered our text accordingly to reference this, Page 7 line 131.

Further to this we now include data, which identifies key residues within the WD40 CTD that are required for non-canonical autophagy (Fig 6). This is the first report of residues within the WD40 CTD of ATG16L1 that affect its function. This further distinguishes our work from that of Boada-Romero et al, and extends our understanding of how the WD40 CTD controls LC3 lipidation to single membranes.

Referee #3:

Autophagy is characterized by the lipidation of the ubiquitin-like molecule LC3, which is mediated by the ATG16L1 E3 ligase-like complex. This event contributes to the formation of the double-membrane autophagosome. Recent studies indicate that lipidated LC3 can also insert into single membrane vesicles to mediate autophagy-related processes such as LC3-associated phagocytosis (LAP). The mechanisms that distinguish these disparate functions of LC3 are incompletely understood. Previous studies have shown that the WD40 domain of ATG16L1 is dispensable for conventional starvation-induced autophagy. In this manuscript, the authors examine whether the WD40 domain is necessary for other functions associated with LC3 lipidation by ATG16L1.

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LC3 targeting to single membranes, but retain the ability to mediate autophagy. The inability to mediate LC3 targeting to single membranes was not due to failure to form the E3 ligase-like complex (ATG5-ATG12-ATG16L1), but was associated with lack of recruitment of ATG16L1 delta WD40 to the phagosome. Finally, they show that the same pathway is triggered by influenza M2 protein, which inserts into membranes to act as a proton channel and can have a similar effect as monensin. In a manner dependent on the proton channel activity of M2, influenza infection induces relocalization of LC3 in cells harboring ATG16L1 delta FBD but not ATG16L1 delta WD40.

The role of the WD40 domain in mediating LC3 targeting is novel and the observation that autophagy and this autophagy-related function of ATG16L1 can be decoupled through mutagenesis is exciting. The experiments include validation through multiple techniques with appropriate quantification and controls. The use of several cell lines and results with influenza infection are additional strengths. Thus, the conclusions are generally supported well by the data. The manuscript could be strengthened further if the authors can address the following concerns:

We thank the reviewer for their encouraging comments. We have performed new experiments to address the questions regarding the consequences of non-canonical autophagy.

1. The terminology used in this manuscript is confusing. As articulated in a recent comprehensive review article (Galluzi et al, EMBO J. 2017 36(13):1811), the term "non-canonical autophagy" is ambiguous and misleading.

We than the reviewer for this comment and refer them to our response to referee 1 point 4.

2. A related issue is that the relationship between LAP, monensin treatment, and M2 activity is obscure. Are these the same events at the molecular level? For instance, does LC3 targeting to single membranes in the presence of monensin or M2 require rubicon (an essential LAP protein)? Unifying these disparate models would significantly improve this manuscript and allow the authors to use the umbrella term LAP. It would also justify switching between models. Alternatively, in some experiments it appears that monensin enhances LAP. Is this enhancement or induction?

This is an interesting point which requires clarification within the field. Data from our laboratories show that all tested processes that activate non-canonical autophagy (phagocytosis, macropinocytosis, entosis, influenza infection, ionophore and lysosomotropic drugs) require V-ATPase activity and the LC3 lipidation machinery – ATG5, ATG7, ATG12 and ATG16L1 (specifically now the WD40 CTD). The requirement for Rubicon and ROS appears to be more specific for LAP, rather than being essential for non-canonical autophagy in general. Indeed, in unpublished work we are able to induce LC3 lipidation to phagosomes from cells lacking Rubicon, or where NADPH oxidase has been

inhibited, by treating the cells with monensin. It is possible that during LAP, Rubicon and ROS act to generate another "signal" that activates non-canonical autophagy. This "signal" may be generated through different mechanisms in other processes. As such we do not believe LAP can be used as an umbrella term.

3. Most experiments rely on transformed cell lines, which typically display dysregulated autophagy. The exception is the MEFs. Are the ATG16L1 knockout MEFs transformed? Information on passage number would be helpful.

We share the reviewer's concern – indeed we decided to demonstrate the same dependence on the WD40 CTD in three separate knock-out cell lines to avoid drawing conclusions that might be specific to dysregulated autophagy peculiar to a particular cell type. To exclude the possibility that all cell lines tested are similarly dysregulated we now include data from primary mouse dendritic cells that lack the WD40 CTD (Fig 7).

4. Although LC3 lipidation and trafficking is clearly affected by ATG16L1 mutagenesis, the authors do not provide evidence demonstrating a functional consequence. For instance, LAP has clear functions in pathogen control and cytokine production. Are these dependent on the WD40 domain? In the example of influenza M2, does the WD40 domain affect viral replication or immune responses to the virus? The authors refer to controversies in the field regarding the relationship between M2 and autophagy. They appear to have an opportunity to address this through functional studies. The authors are not expected to chase every downstream function of LC3 targeting that could be important, but certainly some evidence should be provided to show that the WD40 domain matters beyond LC3 Western blots and immuno-fluorescence.

This is an important point. What are the functions of non-canonical autophagy? We very much appreciate the reviewer's comment that we are not expected to chase every downstream function of LC3 targeting. To address the issue of functional consequences of inhibiting non-canonical autophagy through deletion of the WD40 CTD, we used primary dendritic cells generated from a mouse which lacks the C-terminal domain of ATG16L1. We show that presentation of exogenous antigen on MHC class II is deficient in cells from this mouse, demonstrating an important functional role for non-canonical autophagy dependent on ATG16L1 WD40 CTD in a non-transformed cell (Fig 7).

To exclude the possibility that the effect of ATG16L1 WD40 CTD on non-canonical autophagy during influenza infection is due to a replication deficiency, we measured the kinetics of influenza infection in ATG16L1 deficient HCT116 cells and found no difference between uncomplemented or FL and ∆WD expressing cells (Fig EV4). Cell lines do not model the whole life cycle of influenza and laboratory adapted influenza strains for which reverse genetic systems are readily available (such as PR8) are not efficient inducers of cell-autonomous immune responses in cell culture. It is likely there will be important functional

consequences for influenza infection in vivo as the virus encodes an evolutionarily conserved LC3 interacting region: it would be very interesting to study non-canonical autophagy and immune responses to influenza in a whole organism context (though mice are not ideal for such studies, a ferret model is preferred). These therefore are important questions which go beyond the scope of this paper. We believe that our demonstration of the importance of the WD40 domain of ATG16L1 will allow such questions to be interrogated with much greater precision in the future.

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Martinez J, Cunha LD, Park S, Yang M, Lu Q, Orchard R, Li QZ, Yan M, Janke L, Guy C, Linkermann A, Virgin HW, Green DR (2016) Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. *Nature* **533:** 115-11

2nd Editorial Decision 22 November 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by the three original referees again whose comments are enclosed. As you will see, all three referees express interest in your manuscript and are broadly in favour of publication, pending satisfactory minor revision.

I would thus like to ask you to address referee #2 and #3's remaining concerns and to provide a final version of your manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The authors addressed my concerns and the manuscript now meets EMBO J scientific merit.

Referee #2:

The authors have addressed all my comments and have added substantial mechanistic insights. In my opinion, the manuscript has become very strong. One thing the authors should still do is to add a loading control for the blot shown in Figure 6D.

Referee #3:

In this revised manuscript, the authors provide new data demonstrating that the WD40 domain of ATG16L1 mediates autophagy-related processes that are distinct from starvation-induced autophagy. The authors were generally responsive to previous critiques, and the manuscript is much improved. In particular, they include key data with mice deficient in the WD40 domain (ATG16L1 E230 mice) demonstrating physiological relevance of their findings. Their observations are consistent with the literature indicating that LAP or a similar pathway is necessary for presentation of exogenous antigens by dendritic cells.

The authors should include information on the origin of the ATG16L1 E230 mice and how they were generated. The manuscript is otherwise appropriate for publication.

2nd Revision - authors' response 6 December 2017

Referee #2:
The authors have addressed all my comments and have added substantial mechanistic insights. In my opinion, the manuscript has become very strong. One thing the authors should still do is to add a loading control for the blot shown in Figure 6D.

We have now included a loading control for figure 6D.

Referee #3:
In this revised manuscript, the authors provide new data demonstrating that the WD40 domain of ATG16L1 mediates autophagy-related processes that are distinct from starvation-induced autophagy. The authors were generally responsive to previous critiques, and the manuscript is much improved. In particular, they include key data with mice deficient in the WD40 domain (ATG16L1 E230 mice) demonstrating physiological relevance of their findings. Their observations are

consistent with the literature indicating that LAP or a similar pathway is necessary for presentation of exogenous antigens by dendritic cells.

The authors should include information on the origin of the ATG16L1 E230 mice and how they were generated. The manuscript is otherwise appropriate for publication.

As we stated in our previous point-by point response, the generation of this mouse is being published in another manuscript from the labs of Dr Thomas Wileman and Dr Ulrike Mayer. We added basic information on the how the ATG16L1 gene was targeted. We hope the reviewer understands why we cannot provide more information at this time.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Oliver Florey
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-97840R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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?	sample size was chosen empirically.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Animal numbers used were predetermined based on experience that this sample size is siufficient to distinguish significant differences between conditions.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All measures were included in analysis.
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.	Animals were chosen for experimentation as they were bred.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used.
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.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
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.	Yes
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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-tun

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/

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 have provided source and catalogue number in the methods section for all antibodies used.
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 routinely test for mycoplasma.

* 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.	Male 8-13 week C57/BL6 mice. For E230 mice, The translation of the WD domain and linker region of Atg16L1 were prevented by inserting two stop codons in exon 6 after the E230 residue necessary for WIPI2b binding.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal use in this study was conducted under the project license (70/8177 and 70/8332) authorized by the UK home office. All experiments were performed in accordance with the Animals (Scientific Procedures) Act, UK, 1986.
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.	Confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n/a
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 a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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. 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

22. 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