A Drosophila toolkit for HA-tagged proteins unveiled a block in autophagy flux in the last instar larval fat body

Tadayoshi Murakawa, Tsuyoshi Nakamura, Kohei Kawaguchi, Futoshi Murayama, Ning Zhao, Timothy J. Stasevich, Hiroshi Kimura and Naonobu Fujita
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Review timeline

Submission to Review Commons:	18 May 2021
Submission to Development:	30 September 2021
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First revision received:	16 December 2021
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Reviewer 1

Evidence, reproducibility and clarity

Murakawa and co-workers describe an innovative labeling procedure for use in Drosophila, and they document its use to characterize lysosomal dynamics in larval and pre-pupal fat bodies. These authors previously developed and characterized the HA frankenbody in mammalian cells; here, its adaptation for the fly system will greatly increase the utility of the several thousand available transgenic lines expressing HA-tagged open reading frames. The authors show clearly that HA frankenbody-GFP localizes to expected compartments in multiple cell types when co-expressed with HA-tagged protein residents of the nucleus, Golgi, mitochondria and microtubules. They also highlight how this technology can be used to for live imaging of fragile structures that are disrupted by fixation. Altogether, the reagents and methodology will be of great use to the fly community, all the more as other frankenbody fusions are developed.

As proof of principle, the authors co-express a GFP/mCherry tandem-tagged frankenbody with the autophagy substrate Ref(2)P to characterize lysosomal acidity and size during late larval and early pupal development. Previous studies have described a developmental induction of autophagy and a marked expansion of the lysosomal compartment at this stage. Here, the authors argue that this lysosomal expansion is not due primarily to an increase in autophagy induction, but rather to reduced lysosomal activity. Although this analysis demonstrates the utility of the frankenbody reagents for labeling autolysosomes and assessing their relative pH, this aspect of the manuscript is less developed and would benefit from a more thorough investigation of these issues.

Specific comments

Changes in lysosomal size reflect an imbalance between growth due to fusion with autophagic and/or endocytic vesicles on the one hand, and shrinkage due to digestion and efflux of cargo on the other. The authors argue that reduced activity/acidification, rather than increased autophagy, is the dominant cause of the lysosomal enlargement observed in pre- pupal fat bodies. This is based on their findings that blocking autophagy with an

Atg5 mutation inhibits lysosomal enlargement only partially (~40%), and that blocking acidification with chloroquine causes only a mild (~40%) further increase in lysosome size. The latter suggests that lysosomal activity is already low, in agreement with their findings using the TA- frankenbody as well as lysosensor staining. However, the observation that lysosomes continue to grow even when autophagy is blocked implies that some other factor is driving this growth; an obvious candidate here is the massive endocytic uptake of larval storage proteins into the fat body, which coincides with this developmental period. As autophagosomes are known to fuse with the endocytic pathway en route to the lysosome, it would be expected that autophagy cargo such as Ref(2)P would be merged into this large influx. Thus, increased endocytosis may be the major driver of autolysosomal enlargement, along with contributions from increased autophagy induction and reduced lysosomal activity. This could be examined by experimentally manipulating endocytic uptake, and should be more evenly discussed.

The authors propose a developmentally regulated decrease in lysosomal activity, potentially through changes in expression of v-ATPase subunits or other lysosomal regulators. Consistent with this idea, overexpression of the transcriptional regulator MITF was found to suppress the lysosomal enlargement of pre-pupal fat body. Additional evidence is needed to support this model, however, as reduced lysosomal acidity could instead result passively from greater fusion with the increased autophagic and endocytic activity at this stage. The authors should compare expression of lysosomal genes during 3rd larval and prepupal fat bodies, and measure whether MITF overexpression leads to increased lysosomal acidity.

The description of the experiments shown in Figures 4B and 4C and the labeling of 4C are unclear and confusing, for a number of reasons.

First, 4B shows enlarged structures that are positive for GFP and mCherry, along with some that are mCherry-positive and GFP-negative. The double-positive structures are described in the text as "GFP-positive", which implies that they are mCherry-negative. This is all the more confusing because in the figure these structures indeed look to be GFP+ and mCh-, that is, they look green rather than white. To remedy, please call these GFP and mCherry double- positive in the text and on panel C; perhaps explain (in the figure legends?) that the green- looking lysosomes are actually double positive.

In Figure 4B, the insets show the mCh and GFP channels as negative images; this should be mentioned in the legend.

Also in Figure 4B, localization of CK2beta-3xHA is shown, but the relevance or reason for selecting this protein is not mentioned in the text.

In Figures 5A-D, the authors compare the ratio of lysosomal area to autophagosome number between starved larval fat body and white pre-pupal fat body; a higher ratio in the white pre- pupae is used to that autophagy is not the sole contributor to lysosomal enlargement. This comparison is problematic, in that the number of autophagosomes is not a reliable proxy for autophagy rate or flux; and that the timescales of these samples are not really comparable. The larvae are starved for an arbitrary amount of time, and the white pre-pupae have been undergoing increased developmental autophagy for several hours. Even a small increase in autophagy could lead to lysosomal enlargement given sufficient time. A direct comparison of autophagy flux at these two developmental stages should be made instead.

Significance

Major technical advance that will be of significant interest to the Drosophila community. The scientific findings represent a more minor advance.

Referees cross-commenting

I agree with the comments of reviewer #2. To address point 2, the authors could examine the number of punctae formed by these markers when autophagy is blocked.

Reviewer 2

Evidence, reproducibility and clarity

The manuscript by Murakawa et al. applied the recently developed tool- the HA frankenbody in Drosophila for imaging nascent and mature HA-tagged proteins in vivo. Using the GFP-mCherry tandem fluorescent-tagged HA frankenbody, they found an autophagic flux blockage and accumulation of enlarged autolysosomes in the late larval and prepupal fat body, indicating that downregulated lysosomal function in the fat body is important during larval-pupa transition in Drosophila. Overall the HA frankenbody is a potentially useful tool for the fly community.

However, there are a few issues should be addressed:

1.The authors use the TF-tagged HA frankenbody to show the lysosomal degradation of HA-tagged POI in Fig 3. However, without proper lysosomal marker, the conclusion remains premature.

2.In Fig3B, there are mCh+ only signals in starvation groups of GAPDH and even lacZ. These results indicate that frankenbody-GFP-mCh itself could be degraded by lysosome. It also raises the question that whether binding of frankenbody-GFP-mCh and POI-HA protein may trigger degradation in a POI independent manner. Moreover, frankenbody itself is ~25kDa, however, it constitutes over 70kDa protein when it fused with GFP and mCherry. Is it possible that it may interferes the function of target protein and forces the protein to be diminished by autophagy?

3. Authors utilized lysotracker or lysosensor to show that lysosomal function is downregulated in late stage of larvae. However, the intensity of lysotracker or sensor alone may not precisely reflect lysosomal activity. For example, Fig4E show lower intensity of lysosensor in white pupal stage, but the size of lysosome is much bigger. Actually, other groups have demonstrated that the enlarged autolysosome would affect the penetration of microscopic light thus we observe the weaker signal of marker such as Atg8 or lysotracker. To verify whether lysosomal function is downregulated, authors should also perform cathepsin cleavage assay or MagicRed to confirm the activity of lysosomes.

4. Fig5G, authors try to use CQ treatment and observe lysosomal size to clarify lysosomal activity. Cathepsin cleavage assay or MagicRed staining should be included.

5. The authors further showed that Mitf overexpression suppressed the enlargement of lysosomes in the white prepupal fat body and pupa lethality. It is not convinced that this phenomenon is caused by lysosomal over-activation. Mitf not only contributes to lysosomal protein but also autophagic regulatory protein transcription. It has been known that

excessive autophagy could cause cell death. The pupa lethality may be resulted from hyper- activated autophagy. Additional studies should be included to support their claims.
Significance

Overall the HA frankenbody is a potentially useful tool for the fly community.

Author response to reviewers' comments

1. General Statements [optional]

We are excited by our work that establishes a versatile Drosophila toolkit for the functional analysis of HA-tagged protein in live tissues. This system allows various analyses of HA-tagged proteins of interest (POI) in Drosophila tissues by a simple crossing of an HA Frankenbody fly with an HA-tagged POI fly. Here we demonstrate for the first time that there is a block in autophagy flux in the last instar larval and prepupal fat body using the HA Frankenbody system. We also show the block plays a role in the metamorphosis of Drosophila. Accordingly, this paper will have considerable impact and appeal to a broad readership in cell biology and development, and contribute to the fly community. Therefore, we have already donated all HA Frankenflies listed in our paper to the Vienna Drosophila Resource Center (VDRC).

Thank you for the review. We sincerely appreciate the reviewers' constructive and insightful comments.

2. Description of the planned revisions

Below is a point-by-point reply that explains additional experiments are planned to address the points raised by the reviewers.

Reviewer #1 *(Evidence, reproducibility and clarity (Required)):*
Murakawa and co-workers describe an innovative labeling procedure for use in Drosophila, and they document its use to characterize lysosomal dynamics in larval and pre-pupal fat bodies. These authors previously developed and characterized the HA frankenbody in mammalian cells; here, its adaptation for the fly system will greatly increase the utility of the several thousand available transgenic lines expressing HA-tagged open reading frames. The authors show clearly that HA frankenbody-GFP localizes to expected compartments in multiple cell types when co-expressed with HA-tagged protein residents of the nucleus, Golgi, mitochondria and microtubules. They also highlight how this technology can be used to for live imaging of fragile structures that are disrupted by fixation. Altogether, the reagents and methodology will be of great use to the fly community, all the more as other frankenbody fusions are developed.

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Specific comments

Changes in lysosomal size reflect an imbalance between growth due to fusion with autophagic and/or endocytic vesicles on the one hand, and shrinkage due to digestion and efflux of cargo on the other. The authors argue that reduced activity/acidification, rather than increased autophagy, is the dominant cause of the lysosomal enlargement observed in pre-pupal fat bodies. This is based on their findings that blocking autophagy with an Atg5 mutation inhibits lysosomal enlargement only partially (~40%), and that blocking acidification with chloroquine causes only a mild (~40%) further increase in lysosome size. The latter suggests that lysosomal activity is already low, in agreement with their findings using the TA- frankenbody as well as lysosensor staining.

However, the observation that lysosomes continue to grow even when autophagy is blocked implies that some other factor is driving this growth; an obvious candidate here is the massive endocytic uptake of larval storage proteins into the fat body, which coincides with this developmental period. As autophagosomes are known to fuse with the endocytic pathway en route to the lysosome, it would be expected that autophagy cargo such as Ref(2)P would be merged into this large influx. Thus, increased endocytosis may be the major driver of autolysosomal enlargement, along with contributions from increased autophagy induction and reduced lysosomal activity. This could be examined by experimentally manipulating endocytic uptake, and should be more evenly discussed.
To examine the contribution of endocytic uptake to the developmentally regulated lysosome enlargement, we will conditionally disrupt *shibire*, the sole fly ortholog of dynamin, which is involved in endocytic uptake for a significant portion of cell surface and extracellular cargoes delivered to lysosomes. *shibire* temperature-sensitive (*shibire*^{ts}) mutant will be expressed in the fat body by the GAL4-UAS system because the blockade of *shibire* in whole-body would block the larval development. As a backup, Rab5 RNAi will also be tested. We will analyze the effect of blocking the endocytic pathway on the size change of lysosomes and discuss the contributions of both autophagy and endocytosis to the enlargement of lysosomes.

The authors propose a developmentally regulated decrease in lysosomal activity, potentially through changes in expression of v-ATPase subunits or other lysosomal regulators. Consistent with this idea, overexpression of the transcriptional regulator MITF was found to suppress the lysosomal enlargement of pre-pupal fat body. Additional evidence is needed to support this model, however, as reduced lysosomal acidity could instead result passively from greater fusion with the increased autophagic and endocytic activity at this stage. The authors should compare expression of lysosomal genes during 3rd larval and prepupal fat bodies, and measure whether MITF overexpression leads to increased lysosomal acidity.
We agree with the reviewer that the mechanism of downregulation of lysosomes is an important question raised by our novel findings. To examine the expression level of v-ATPase subunits and other lysosomal regulators extensively, we will perform mRNA-seq of fat bodies from 1)early 3IL, 2) white prepupa, and 3) white prepupa overexpressing Mitf. When the expression level of lysosome-related genes is developmentally regulated, we will confirm the result by qPCR further.

As the reviewer mentioned, the regulation of lysosomal pH is complex, and the reduced lysosomal acidity could result from other mechanisms, such as excess influx from autophagic and/or endocytic pathways. However, Mitf overexpression suppressed the developmentally regulated enlargement of lysosomes. Thus, our transcriptome analysis should give important clues for the mechanisms of lysosomal enlargement in any case.

In Figures 5A-D, the authors compare the ratio of lysosomal area to autophagosome number between starved larval fat body and white pre-pupal fat body; a higher ratio in the white pre-pupae is used to that autophagy is not the sole contributor to lysosomal enlargement. This comparison is problematic, in that the number of autophagosomes is not a reliable proxy for autophagy rate or flux; and that the timescales of these samples are not really comparable. The larvae are starved for an arbitrary amount of time, and the white pre-pupae have been undergoing increased developmental autophagy for several hours. Even a small increase in autophagy could lead to lysosomal enlargement given sufficient time. A direct comparison of autophagy flux at these two developmental stages should be made instead.
To compare the flux in starved early 3IL and prepupal fat bodies at the same time scale, we will perform a flux assay using bafilomycin A1. Baf.A1 is a specific inhibitor for v-ATPase and is widely used for autophagy flux assays in mammalian culture cells. Since Baf.A1 blocks the lysosomal degradation, the fold change of lysosome size with or without Baf.A1 indicates the lysosomal flux at each time point. In the experiment, we compare the impact of Baf.A1 injection or soaking on the size of Lamp1-3xRFP-positive lysosomes in both starved early 3IL and prepupal fat bodies. Together with the data in Fig. 5E-H, the flux assay data would strengthen our model in which lysosomal activity is developmentally downregulated in the last instar larval fat body.

Reviewer #1 (Significance (Required)):
Major technical advance that will be of significant interest to the Drosophila community. The scientific findings represent a more minor advance.

Referees cross-commenting

I agree with the comments of reviewer #2. To address point 2, the authors could examine the number of punctae formed by these markers when autophagy is blocked.

***Reviewer #2** (Evidence, reproducibility and clarity (Required)):*
The manuscript by Murakawa et al. applied the recently developed tool- the HA frankenbody in Drosophila for imaging nascent and mature HA-tagged proteins in vivo. Using the GFP-mCherry tandem fluorescent-tagged HA frankenbody, they found an autophagic flux blockage and accumulation ofenlarged autolysosomes in the late larval and prepupal fat body, indicating that downregulated lysosomal function in the fat body is important during larval-pupa transition in Drosophila. Overall the HAfrankenbody is a potentially useful tool for the fly community.

However, there are a few issues should be addressed:

2. In Fig3B, there are mCh+ only signals in starvation groups of GAPDH and even lacZ. These results indicate that frankenbody-GFP-mCh itself could be degraded by lysosome. It also raises the question that whether binding of frankenbody-GFP-mCh and POI-HA protein may trigger degradation in a POI independent manner. Moreover, frankenbody itself is ~25kDa, however, it constitutes over 70kDa protein when it fused with GFP and mCherry. Is it possible that it may interferes the function of target protein and forces the protein to be diminished by autophagy?
Thank you for this comment. We think Frankenbody-TF is also degraded in the lysosomes via autophagy, even in the absence of HA-tagged autophagy cargo. In response to the concern, we monitor lysosomal degradation of Frankenbody-TF itself in wildtype and *ATG* null conditions.

Since most transgenic flies expressing Frankenbody-TF and POI-HA are alive and fully developed, we think any perturbation to protein levels is caused by the expression of Frankenbody-TF and POI-HA. As the reviewer pointed, we have not shown whether binding of Frankenbody-TF and POI-HA trigger degradation of POI-HA. To test the possibility, the protein level of POIs-HA (GAPDH1-3xHA, TOM20-3xHA, and Ref(2)p-HA) in the fat body will be checked by WB in the presence or absence of Frankenbody-TF. If the binding triggersdegradation of POI-HA, the protein level of POI-HA would be reduced by Frankenbody-TF expression.

We found that HA Frankenbody expression interferes with the function of Amph-HA in muscle cells. Amph is a BAR domain protein and functions in both endocytosis and T-tubule formation in muscle cells. Since we have not tested whether Frankenbody triggers the degradation of Amph-HA in the muscle, we will test the possibility by WB as described above.

3. Authors utilized lysotracker or lysosensor to show that lysosomal function is downregulated in late stage of larvae. However, the intensity of lysotracker or sensor alone may not precisely reflect lysosomal activity. For example, Fig4E show lower intensity of lysosensor in white pupal stage, but the size of lysosome is much bigger. Actually, other groups have demonstrated that the enlarged autolysosome would affect the penetration of microscopic light thus we observe the weaker signal of marker such as Atg8 or lysotracker. To verify whether lysosomal function is downregulated, authors should also perform cathepsin cleavage assay or MagicRed to confirm the activity of lysosomes.
We will compare lysosomal function in the fat body at the early 3IL and white prepupal stage by MagicRed and/or DQ-Red BSA, a fluorogenic substrate for proteases. Since DQ-Red BSA is transported to the lysosome via endocytosis, the signal could be affected by the endocytic activity. Thus, we will co-inject DQ-Red BSA and Alexa488 BSA into the larvae, and the signal of DQ-Red BSA is normalized by the intensity of incorporated Alexa488-BSA via endocytosis.

Cathepsin cleavage assay is an elegant strategy; however, we think the assay is difficult for the purpose. Because the lysosomal activity is downregulated temporally at the last instar larval stage, a significant amount of cathepsin is already transported and matured in the lysosomes before the time point.

4. Fig5G, authors try to use CQ treatment and observe lysosomal size to clarify lysosomal activity. Cathepsin cleavage assay or MagicRed staining should be included.
Same as above, we will perform the assays using MagicRed and/or DQ-Red BSA with or without CQ treatment.

5. The authors further showed that Mitf overexpression suppressed the enlargement of lysosomes in the white prepupal fat body and pupa lethality. It is not convinced that this phenomenon is caused by lysosomal over-activation. Mitf not only contributes to lysosomal protein but also autophagic regulatory protein transcription. It has been known that excessive autophagy could cause cell death. The pupa lethality may be resulted from hyper-activated autophagy. Additional studies should be included to support their claims.
To examine whether the lethality induced by Mitf overexpression depends on autophagy or not, we will test fat body-targeted Mitf overexpression in an *ATG* null condition. If Mitf overexpression induces the pupal lethality in the absence of the *ATG* gene, it indicates thatlysosome hyperactivation is the cause of lethality rather than excessive autophagy. We will also check the effect of Mitf overexpression on lysosomal morphology in an *ATG* null condition.

Reviewer #2 (Significance (Required)):
Overall the HA frankenbody is a potentially useful tool for the fly community.

3. Description of the revisions that have already been incorporated in the transferred manuscript

Below is a point-by-point response describing the revisions that were already carried out and included in the transferred manuscript.

Reviewer #1
The description of the experiments shown in Figures 4B and 4C and the labeling of 4C are unclear and confusing, for a number of reasons.
Thank you for the suggestions. We corrected the labeling following the reviewer's comments as below.

First, 4B shows enlarged structures that are positive for GFP and mCherry, along with some that are mCherry-positive and GFP-negative. The double-positive structures are described in the text as "GFP- positive", which implies that they are mCherry-negative. This is all the more confusing because in the figure these structures indeed look to be GFP+ and mCh-, that is, they look green rather than white. To remedy, please call these GFP and mCherry double-positive in the text and on panel C; perhaps explain (in the figure legends?) that the green-looking lysosomes are actually double positive.

We corrected the main text (page7) and the label in Fig. 4C (page 25); from "# of GFP^+ lysosomes" to "# of GFP and mCh double-positive lysosomes". Further, this sentence is added to the figure legends "Please note that the enlarged green-looking lysosomes are also positive for mCh".

In Figure 4B, the insets show the mCh and GFP channels as negative images; this should be mentioned in the legend.
We added the sentence in the figure 4 legend (page 25) "The cropped single channels are inverted images of black and white."

Also in Figure 4B, localization of CK2beta-3xHA is shown, but the relevance or reason for selecting this protein is not mentioned in the text.
Although we showed only data of CK2b-3xHA in Fig. 4B, we had obtained a similar result when 3xHA-fused GAPDH1was co-expressed. Therefore, the data of GAPDH1-3xHA was also included in Fig. 4B.

Reviewer #2, comment 1.
1.The authors use the TF-tagged HA frankenbody to show the lysosomal degradation of HA-tagged POI in Fig 3. However, without proper lysosomal marker, the conclusion remains premature.
Thank you for this comment. To confirm the lysosomal degradation of TF-tagged HA frankenbody and only mCh-positive objects represent acidic lysosomal compartments, we examined the colocalization between TF-tagged HA frankenbody and Lysotracker DeepRed or Cathepsin L. As shown in Fig. S3A, the mCh-single-positive puncta were colocalized well with both Lysotracker DeepRed and Cathepsin L. These new results clearly show that TF-tagged HA frankenbody is transported and degraded in acidic lysosomal compartments.

We added a sentence on page 6 as follows; “The mCh-single-positive puncta were colocalized well with LysoTracker dye and Cathepsin L, indicating that mCh accumulated in the acidic lysosomal compartments (Fig. S3A).”

4. Description of analyses that authors prefer not to carry out None

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Original submission

First decision letter

MS ID#: DEVELOP/2021/200243

MS TITLE: A Drosophila Toolkit for Imaging of HA-tagged Proteins Unveiled a Block in Autophagy Flux in the Last Instar Larval Fat Body

AUTHORS: Tadayoshi Murakawa, Tsuyoshi Nakamura, Kohei Kawaguchi, Futoshi Murayama, Ning Zhao, Timothy J Stasevich, Hiroshi Kimura, and Naonobu Fujita

Thanks for submitting your manuscript for consideration at Development after receiving reviewer reports through Review Commons. I have now looked over the manuscript, the reviews and your proposed revisions. I think your responses to the reviews are well-considered and the additional experiments proposed should improve the study. Consequently,I will be happy receive a revised version of the manuscript.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

First revision

Author response to reviewers' comments

Below is a point-by-point response to the reviewers’ comments. In the main text, the parts written in red are the parts that have been revised in this round.

***Reviewer #1** (Evidence, reproducibility and clarity (Required)):*
Murakawa and co-workers describe an innovative labeling procedure for use in Drosophila, and they document its use to characterize lysosomal dynamics in larval and pre-pupal fat bodies. These authors previously developed and characterized the HA frankenbody in mammalian cells; here, its adaptation for the fly system will greatly increase the utility of the several thousand available transgenic lines expressing HA-tagged open reading frames. The authors show clearly that HA frankenbody-GFP localizes to expected compartments in multiple cell types when co-expressed with HA-tagged protein residents of the nucleus, Golgi, mitochondria and microtubules. They also highlight how this technology can be used to for live imaging of fragile structures that are disrupted by fixation. Altogether, the reagents and methodology will be of great use to the fly community, all the more as other frankenbody fusions are developed.

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As this referee could point out, endocytosis is a promising candidate to drive the lysosomal swelling in the fat body. To examine the contribution of endocytic uptake to the lysosome enlargement, $Rab5^{S43N}$ mutant was transiently overexpressed in the fat body by the Lsp2- GAL4, which drives gene expression in the last instar larval fat body. It is established that the expression of $Rab5^{S43N}$ blocks all endocytic pathways, both clathrin-dependent and - independent mechanisms. As shown in new Fig. 5G and J, $Rab5^{S43N}$ expression drastically suppressed the enlargement of autolysosomes. Since the loss of autophagy affected the lysosomal swelling only mildly, we conclude that endocytosis plays a dominant role in the swelling of lysosomes. We revised the result section (p9, line 15-24), and the discussion section as follows (p13, line 5-10); “Rab5-mediated endocytosis was indispensable for the swelling of lysosomes (Fig. 5G,J). Therefore, it is also possible that increased endocytic flux to lysosomes is a driver of the enlargement to some extent. It should be examined whether the endocytic activity is upregulated in the white prepupal fat body than 3IL fat body.
However, it is challenging because the lysosomal activity is significantly decreased in the white prepupal fat body.”

The authors propose a developmentally regulated decrease in lysosomal activity, potentially through changes in expression of v-ATPase subunits or other lysosomal regulators. Consistent with this idea, overexpression of the transcriptional regulator MITF was found to suppress the lysosomal enlargement of pre-pupal fat body.
Additional evidence is needed to support this model, however, as reduced lysosomal acidity could instead result passively from greater fusion with the increased autophagic and endocytic activity at this stage. The authors should compare expression of lysosomal genes during 3rd larval and prepupal fat bodies, and measure whether MITF overexpression leads to increased lysosomal acidity.
To compare the expression level of v-ATPase subunits and other lysosomal regulators extensively, we performed RNA-seq of fat bodies of 1) early 3IL, 2) white prepupa, and 3) white prepupa overexpressing Mitf. Consistent with the previous report, Mitf overexpression upregulated the expression of a series of lysosome-related genes (Fig. 6F, bottom). We predict that the Mitf is dispensable for the lysosomal swelling since most of the genes regulated by Mitf were not

transcriptionally affected in the white prepupal fat body (Fig. 6F, top). Interestingly, the transcription of several lysosome-related genes was significantly attenuated at the white prepupal stage. They were Vha36-3, Vha100-2, spinster, and CG13397. As this reviewer pointed out, the mechanism of downregulation of lysosomes is a crucial question raised by our novel findings. Further studies are needed to unveil the mechanism. We added a new figure (Fig. 6F) and revised text (p10, line 12-19; p12, line 28 to p13, line 4).

To check the effect of Mitf overexpression on lysosomal acidity in the white prepupal fat body, we quantified the LysoSensor intensity in the fat body with or without Mitf overexpression. As shown in new Fig. 6A and B, the intensity of LysoSensor increased upon Mitf expression, indicating that Mitf overexpression leads to increased lysosomal acidity. We also revised the result section (p9, line 32 to p10, line 1).

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We revised the main text (p7, line 3-7) and the label in Fig. 4C, from "# of GFP^+ lysosomes" to "# of GFP and mCh double-positive lysosomes". Further, this sentence is added to the figure legends "Please note that the enlarged green-looking lysosomes are also positive for mCh" (p30, line 3-4).

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Although we showed only data of CK2b-3xHA in Fig. 4B in the previous version, we had obtained a similar result when 3xHA-fused GAPDH1 was co-expressed. Therefore, the data of GAPDH1-3xHA was also included in Fig. 4B.

In Figures 5A-D, the authors compare the ratio of lysosomal area to autophagosome number between starved larval fat body and white pre-pupal fat body; a higher ratio in the white pre-pupae is used to that autophagy is not the sole contributor to lysosomal enlargement. This comparison is problematic, in that the number of autophagosomes is not a reliable proxy for autophagy rate or flux; and that the timescales of these samplesare not really comparable. The larvae are starved for an arbitrary amount of time, and the white pre-pupae have been undergoing increased developmental autophagy for several hours. Even a small increase in autophagy could lead to lysosomal enlargement given sufficient time. A direct comparison of autophagy flux at these two developmental stages should be made instead.
Thank you for this comment. We agree that autophagic flux assay is important; however, there is no report using autophagic flux assay in Drosophila to date. To compare the autophagic flux in starved early 3IL and prepupal fat bodies at the same time scale, we tried a flux assay using bafilomycin A1 (Baf.A1). Baf.A1 is a specific inhibitor for v-ATPase and is widely used for autophagy flux assays in mammalian culture cells. Since Baf.A1 blocks the lysosomal degradation, the fold change of protein level of Atg8-PE with or without Baf.A1 indicates the lysosomal flux at the time scale. First, we tested the impact of Baf.A1 injection on Atg8-PE level in starved early 3IL. Early 3IL were injected with DMSO (control), 200 μM Baf.A1, or 2 mM Baf.A1 stock solution. Then, the larvae were starved for 4 h and dissected to isolate fat bodies (Figure for reviewers A). However, in contrast to our prediction, the Atg8-PE level was hardly affected by Baf.A1 injection (Figure for reviewers B). We

also tried chloroquine (CQ) injection: but, it did not work again (Figure for reviewers C). We speculate that the injected drugs would be unstable in the hemolymph or excreted from the animals. Therefore, we next tried an ex vivo experiment. Dissected fat bodies were incubated with or without 200 μM Baf.A1 (final concentration) in serum-free Schneider's medium (starvation condition). As shown in Figure for reviewers D, the Atg8-PE level was not elevated. Although the autophagic flux in the larval fat body could not be assessed as described above, we showed that autophagy was dispensable for the enlargement of lysosomes in the last instar larval fat bodies (Fig. 5E-I and S6). In this round, we confirmed that the lysosome swelling was independent of FIP200, an essential gene for autophagy, besides ATG5 (Fig. 5G and I). More importantly, we revealed that endocytosis contributed dominantly to the enlargement of lysosomes (Fig. 5G and J). Further, we showed that CQ feeding did not affect the lysosomal function and enlargement of lysosomes (Fig. S6).
Collectively, we believe our current data support the model that lysosomal function is downregulated and autophagy flux is attenuated in the last instar larval fat bodies. In a future study, we would like to establish an autophagic flux assay in Drosophila.

Reviewer #1 (Significance (Required)):
Major technical advance that will be of significant interest to the Drosophila community. The scientific findings represent a more minor advance.

Referees cross-commenting
I agree with the comments of reviewer #2. To address point 2, the authors could examine the number of punctae formed by these markers when autophagy is blocked.

***Reviewer #2** (Evidence, reproducibility and clarity (Required)):*
The manuscript by Murakawa et al. applied the recently developed tool- the HA frankenbody in Drosophila for imaging nascent and mature HA-tagged proteins in vivo. Using the GFP-mCherry tandem fluorescent- tagged HA frankenbody, they found an autophagic flux blockage and accumulation of enlarged autolysosomes in the late larval and prepupal fat body, indicating that downregulated lysosomal function in the fat body is important during larval-pupa transition in Drosophila. Overall the HA frankenbody is a potentially useful tool for the fly community.

However, there are a few issues should be addressed:

1. The authors use the TF-tagged HA frankenbody to show the lysosomal degradation of HA-tagged POI in Fig 3. However, without proper lysosomal marker, the conclusion remains premature.
Thank you for this comment. To confirm the lysosomal degradation of TF-tagged HA Frankenbody and only mCh-positive objects represent acidic lysosomal compartments, we examined the colocalization between TF-tagged HA Frankenbody and LysoTracker Deep Red or Cathepsin L. As shown in Fig. S3C, the mCh-single-positive puncta were colocalized well with both LysoTracker Deep Red and Cathepsin L. These new results clearly show that TF-tagged HA Frankenbody is transported and degraded in acidic lysosomal compartments. We added the data and revised the result section (p6 line 32 to p7 line 2).

2. In Fig3B, there are mCh+ only signals in starvation groups of GAPDH and even lacZ. These results indicate that frankenbody-GFP-mCh itself could be degraded by lysosome. It also raises the question that whether binding of frankenbody-GFP-mCh and POI-HA protein may trigger degradation in a POI independent manner. Moreover, frankenbody itself is ~25kDa, however, it constitutes over 70kDa protein when it fused with GFP and mCherry. Is it possible that it may interferes the function of target protein and forces the protein to be diminished by autophagy?
Thank you for these comments.

The data in the previous version suggested that HA Frankenbody-TF is degraded in the lysosomes even in the absence of HA-tagged construct. To confirm this further, we monitor lysosomal degradation of HA Frankenbody-TF itself in wildtype and FIP200 null conditions. As shown in new Fig. S3D and E, the degradation of HA Frankenbody-TF was totally dependent on FIP200, an essential gene for autophagy. We added the figure (Fig. S3D-E) and revised the result section (p7, line 7-9).

To address whether the binding of HA Frankenbody-TF and POI-HA triggers degradation in a POI-independent manner, we compared the protein level of GAPDH1-3xHA or TOM20- 3xHA in the presence or absence of HA Frankenbody-TF. If the binding triggers degradation of POI-HA, it is

predicted that the protein level of POI-HA is reduced by HA Frankenbody-TF expression. As shown in new Fig. S5A and B, the protein level of GAPDH1-3xHA or TOM20- 3xHA were hardly affected by HA Frankenbody-TF expression, indicating that the binding of HA Frankenbody-TF does not trigger degradation of POI-HA non-specifically. In contrast, the level of HA-ref(2)p was elevated by HA Frankenbody-TF expression. This data suggests that HA Frankenbody-TF stabilizes HA-ref(2)p. To test whether the stabilization depends on autophagy, we examined the loss of Atg5 on HA-ref(2)p level in the presence or absence of HA Franken-TF. The elevation of HA-ref(2)p induced by HA Frankenbody-TF was not dependent on Atg5 (Fig. S5D), indicating that HA-ref(2)p is stabilized in an autophagy- independent mechanism. Since it has been known that p62/ref(2)p is also degraded by the ubiquitin-proteasome system (Song et al., 2016). Therefore, it is plausible that HA Frankenbody-TF binds and stabilizes HA-ref(2)p through protection from the ubiquitin-proteasome system, and almost all of HA Frankenbody-TF form a complex with HA-ref(2)p in our experimental condition. Altogether, we conclude that the HA Frankenbody-TF visualizes the lysosomal degradation of HA-tagged protein of interest in Drosophila tissues. We added a new supplemental figure (Fig. S5), and revised text (p7, line 18-32).

We found that HA Frankenbody-TF expression interferes with the function of Amph-3xHA in larval muscle cells at 29˚C but not at 25˚C. Please note that their expression level was significantly higher at 29˚C than 25˚C because of the temperature dependence of the GAL4/UAS system. Amph is a BAR domain protein and functions in endocytosis andT-tubule formation. Co-expression of Amph-3xHA and HA Frankenbody-TF affected the organization of muscle cells (Figure for reviewers E). However, the protein level of Amph- 3xHA was hardly affected by HA Frankenbody-TF expression (Figure for reviewers F). This result suggests again that the binding of HA Frankenbody-TF does not trigger degradation of POI-HAnon-specifically.

3. Authors utilized lysotracker or lysosensor to show that lysosomal function is downregulated in late stage of larvae. However, the intensity of lysotracker or sensor alone may not precisely reflect lysosomal activity. For example, Fig4E show lower intensity of lysosensor in white pupal stage, but the size of lysosome is much bigger. Actually, other groups have demonstrated that the enlarged autolysosome would affect the penetration of microscopic light thus we observe the weaker signal of marker such as Atg8 or lysotracker. To verify whether lysosomal function is downregulated, authors should also perform cathepsin cleavage assay or MagicRed to confirm the activity of lysosomes.

Thank you very much for this comment. The cathepsin cleavage assay is an elegant strategy; however, we think the assay is difficult for the purpose. Because the lysosomal activity is downregulated temporally at the last instar larval stage, a significant amount of cathepsin is already transported and matured in the lysosomes before the time point. Thus, to compare lysosomal function, we measured the intensities of MagicRed staining in the fat body at the early 3IL and white prepupal stage. As shown in new Fig. 4G and H, the intensities of Magic Red also decreased in the white prepupal fat body than the starved 3IL fat body. This result strengthened our conclusion that lysosomal function is developmentally downregulated in the white prepupal fat body. Therefore, we added new Fig. 4G,H and revised the result section (p8, line 21-24).

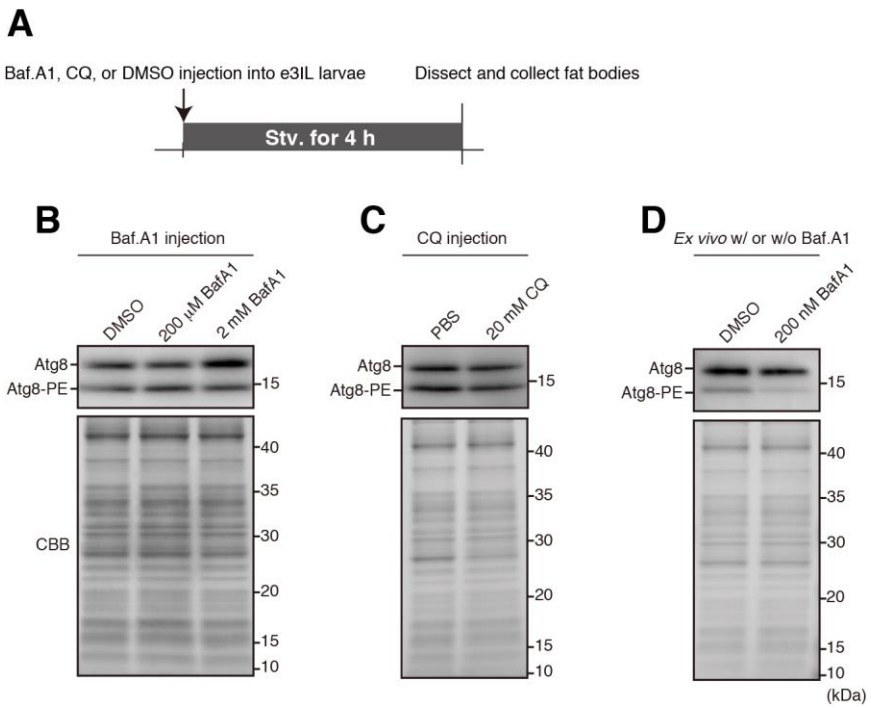
4. Fig5G, authors try to use CQ treatment and observe lysosomal size to clarify lysosomal activity. Cathepsin cleavage assay or MagicRed staining should be included.

Same as above, we compared the intensities of MagicRed staining in the white prepupal fat bodies with or without CQ feeding. Consistent with the data of lysosome size (Fig. S6A and D), CQ feeding did not affect the intensity of MagicRed staining (Fig. S6E,F), indicating that lysosomal function is downregulated in the white prepupal fat body even in the absence of CQ feeding.

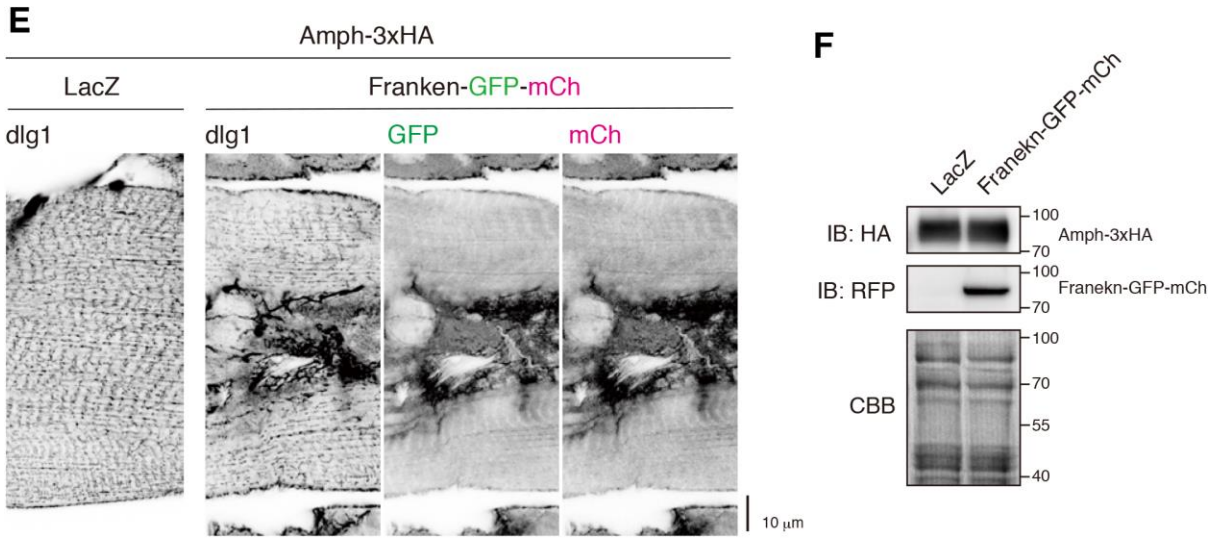
5. The authors further showed that Mitf overexpression suppressed the enlargement of lysosomes in the white prepupal fat body and pupa lethality. It is not convinced that this phenomenon is caused by lysosomal over- activation. Mitf not only contributes to lysosomal protein but also autophagic regulatory protein transcription. It has been known that excessive autophagy could cause cell death. The pupa lethality may be resulted from hyper-activated autophagy. Additional studies should be included to support their claims.

Thank you so much for your comment. To examine whether the lethality induced by Mitf overexpression depends on autophagy, we tested fat body-targeted Mitf overexpression in Atg9 null condition. Mitf overexpression induced the pupal lethality even in the absence of the Atg9 (Fig. S7), indicating that lysosome hyperactivation is the cause of lethality rather than excessive autophagy. Therefore, we included the revised Fig. S7 and revised the result section (p10, line 6-9).

Reviewer #2 (Significance (Required)):
Overall the HA frankenbody is a potentially useful tool for the fly community.



(A) Schematics of autophagy flux assay in 3IL fat body. (B-C) Atg8-PE flux assays in Drosophila. (B) Baf.A1 injection into larvae. (C) CQ injection into larvae. (D) Ex vivo experiments w/ or w/o Baf.A1 in the medium.



(E) Coexpression of Amph-3xHA and Franken-TF disrupted Dlg1-positive T-tubule organization.

(F) Franken-TF expression on the protein level of Amph-3xHA in larval body wall muscles.

Second decision letter

MS ID#: DEVELOP/2021/200243

MS TITLE: A Drosophila Toolkit for HA-tagged Proteins Unveiled a Block in Autophagy Flux in the Last Instar Larval Fat Body

AUTHORS: Tadayoshi Murakawa, Tsuyoshi Nakamura, Kohei Kawaguchi, Futoshi Murayama, Ning Zhao, Timothy J Stasevich, Hiroshi Kimura, and Naonobu Fujita
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Development through Review Commons.

I am happy to tell you that the referees are happy with your revisions and your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This manuscript introduces the HA-Frankenbody as a new reagent and methodology for the Drosophila field.
HA-Frankenbodies will substantially increase the utility of existing HA-tagged transgenic lines. This report also increases our mechanistic understanding of developmental changes in the fat body, including an increase in lysosomal size and decrease in lysosomal activity in the white prepupal stage of development.

Comments for the author

I am satisfied with the revisions made in response to previous reviewer comments, and I congratulate the authors on an interesting study.

Reviewer 2

Advance summary and potential significance to field

useful tool and significant interest for the fly community

Comments for the author

The authors have addressed all my comments.