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Autophagy modulates miRNA-mediated gene silencing and selectively degrades AIN-1/GW182 in C. elegans

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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 21 December 2012

Thank you for the submission of your research manuscript to EMBO reports. It has been sent to three referees, and so far we have received reports from two of them, which are copied below. As both referees feel that the manuscript is interesting and recommend that you should be given a chance to revise it, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this. As soon as we will receive the third report, it will be forwarded to you as well.

As you will see, while the referees acknowledge that the findings are novel and potentially interesting, they also make several suggestions for how the study could be further improved and strengthened. Both referees indicate that it should be analyzed how conserved/significant the region of AIN-1 that interacts with LGG-1 is between worms and mammals. In general, the differences between the findings reported here and in previous, related papers should be clearly pointed out and discussed. Referee 1 further remarks that it should be investigated whether the suppression of miRNA phenotypes depends on AIN-1 activity, and that it would be better to analyze miRNA target silencing directly (instead of reporter assays) in the autophagy mutants. Referee 3 also indicates that other, commonly used autophagy-inducers should be used, and that it would be interesting to know

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how AIN-1 is degraded in wild type worms. Both referees further pinpoint missing quantifications and statistical analyses, as well as several issues that need further discussion.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Also, the revised manuscript may not exceed 30,000 characters (including spaces and references, please note that the current character count exceeds our limit) and 5 figures plus 5 supplementary figures, which should directly relate to their corresponding main figure. Please also include scale bars in the microscope images and please specify the number (n) of experiments and the error bars as well as the statistical tests used to calculate p-values for all quantifications in the corresponding figure legends. This information is currently incomplete.

We also recently decided to offer the authors the possibility to submit "source data" with their revised manuscript that will be published in a separate supplemental file online along with the accepted manuscript. If you would like to use this opportunity, please submit the source data (for example entire gels or blots, data points of graphs, additional images, etc.) of your key experiments together with the revised manuscript.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready.

REFEREE REPORTS:

Referee #1:

In this strong, well-written manuscript, Zhang and Zhang, show that autophagy activity functions to regulate a component of the microRNA silencing complex (miRISC), AIN-1. The authors find that loss of autophagy can suppress defects associated with loss of miRNA biogenesis genes (dcr-1, alg-1, ain-1) , heterochronic pathway genes (sea-2), and the lsy-6 miRNA. In addition, suppression of the let-60gf Muv phenotype is observed in autophagy mutants. These phenotypes are all associated with miRNA activity, suggesting that autophagy may function normally to repress or modulate miRNA activity. Two miRNA targets, hbl-1 and lin-41, show enhanced repression in autophagy mutants. AIN-1 was identified as a candidate target for autophagy-regulated degradation. Cytoplasmic P bodies were not found to be regulated through autophagy. These results provide new insight on the mechanisms involved in regulating miRNA activity and will be of interest to a broad audience interested in miRNA biology.

These findings using in vivo analysis of genetic mutants along with biochemical characterization complement and extend recent work of Gibbing et al., (NCB 14: 1314-1321, 2012) and Derrien et al

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(PNAS 109: 15942-15946, 2012), work that is cited by the authors in the Discussion.

Comment 1. While the observed increase in AIN-1 protein may contribute to the suppression of miRNA-dependent phenotypes, the authors find that loss of epg-6 and atg-7 can also suppress ain-1 mutant phenotypes. This suggests that there are additional targets that may function to modulate miRNA activity. This should be addressed in the discussion section. Does the suppression of the let-60gf Muv phenotype of the lsy-6 phenotype depend on ain-1 activity? This would help to show the significance in the observed changes in AIN-1 protein levels.

Comment 2. The authors state that "Autophagy mutants show enhanced miRNA-mediated silencing of target genes." However, the data to support this is limited to reporter analysis of two miRNA targets. The conclusion should clarify that the observations are limited to two targets using the indirect method of reporter gene analysis.

minor comments:
1. p. 1, Abstract. "Micro RNAs" should be corrected.
2. p. 1 and 2, Abstract and Introduction. Change "(miRISC), which contains miRNA, Argonaute..." to "(miRISC), which includes miRNA, Argonaute..." This indicates that there are many other proteins also in the miRISC.
3. p. 1, Abstract. Add comma after "Degradation of AIN-1 requires the adaptor protein"
4. Introduction. The recent papers showing a role for autophagy activity in the regulation of miRNA activity (Gibbing, Derrien) should be included in the Introduction to establish the rationale for this study.
5. p. 14 Methods. "gift from the Han Min lab." Should be corrected to "Min Han" lab.
6. p. 18, information should be provided for the scm::gfp transgene.
7. Table 1. Statistical analysis should be included for seam cell and alae data.
8. Figure S3. The title of this figure only describes I-K in this figure. A-D and E-H are unrelated to the interaction between ALG-1/2 and LGG-1. Could this be separated into different supplemental figures?
9. Can the authors speculate about the significance of the identified region of AIN-1 that interacts with LGG-1, amino acids 343-490?

Referee #3:

According to the manuscript, autophagy regulates miRNA activity in C. elegans, through degradation of a component of the miRISC complex - AIN-1 (GW182/ TNRC6 homologue in C. elegans). This as an interesting, novel and important finding. The authors should further discuss the overlap and/or discrepancy between their own results and the findings recently described in Arabidopsis (Derrien et al., 2012) and mammalian cells (Gibbings et at., 2012). Besides, I would suggest that the authors address the points described below.
- In fig. 1L, the noise regarding fluorescence intensity/ unit area is high. To better appreciate the differences in HBL-1 expression in the different strains, we suggest the authors to perform a western-blot against GFP.
- In table 1, the authors analyzed the genetic interaction between heterochronic mutants and autophagy, by crossing the heterochronic mutants with different autophagy mutants. Could the authors comment on this? Why weren't the same authophagy mutants crossed with all heterochronic mutants? Would the authors expect to observe similar results regardless of the autophagy mutants crossed?
- In fig. 3 and throughout the manuscript, a quantification of embryos with diffuse GFP signal versus embryos with different number of aggregates (for example, less than 10, in between 10 and 20, etc, should be presented) and the number of embryos analyzed (n) should be reported.
- Throughout the manuscript, absence of water was used by the authors as a stress condition. According to the manuscript, absence of water leads to a dramatic increase in ALG-1 and ALG-2 aggregates in autophagy mutants:
o The protocol used should be described in the Methods section
o Were the embryos viable upon this stress condition?
o There is no clear link between water absence and induction of autophagy. Can the authors to use commonly reported autophagy inducers, like rapamycin/ or inhibitors, like chloroquine, in the stress

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experiments.
- In figs. 4 and S2 co-localization index should be presented, as well as the number of embryos analyzed (n).
- The authors underline the association of AIN-1 with SQST-1 aggregates in autophagy mutants, but no clear functional correlation between SQST-1 and AIN-1 seem to be present. This aspect should be emphasized in the discussion of the results, page 9 of the manuscript.
- The authors show that C. elegans AIN-1 (aa 343-490) directly interacts with LGG-1. In contrast, in mammalian cells GW182 (AIN-1 homologue) is not an autophagy substrate (Gibbings et at., 2012) . How conserved is LGG-1 region of interaction among AIN-1 and GW182? Is there a AIM/LIR-like motif in AIN-1? The authors should further discuss the differences between their own results and the findings in mammalian cells.
- Accumulation of AIN-1 is present in aggregates that are exclusively formed in absence of autophagy, and not under physiological conditions, in wild-type embryos. How is the AIN-1 degradation regulated in wild-type embryos? Is there a steady turnover of AIN-1? Does it differ in different stages of development, etc? it is recommended that a paragraph is added where this topic is discussed.
- the last part of the Results section, regarding P-bodies, is out of the scope of the manuscript and we would recommend its removal.

Correspondence – Editorial Staff 21 December 2012

Please, find below the comments of the final referee, we had been waiting for. Please also consider this referee's comments in your revision. Many thanks for your patience and we look forward to receiving your revised manuscript in due time.

REFEREE REPORT:

Referee #2:

The manuscript entitled "Autophagy modulates miRNA-mediated gene silencing and selectively degrades the GW182 homolog AIN-1 in C. elegans" by Zhang and Zhang reports a very important finding. Namely, autophagy in C. elegans selectively degrades a component of the miRNA-induced silencing complex, AIN-1/GW182, thereby regulating miRNA-mediated RNA interference. A paper has just appeared in Nat Cell Biol (Gibbins et al. 14: 1314, 2012), showing a similar phenomenon from mammalian cell lines: autophagy degrades Dicer and AGO2, two components of the siRNA and miRNA pathways, to control miRNA activity. Since the miRNA pathway mediates various developmental events and cellular functions, findings presented here have strong developmental and medical significance. In addition, these data reveal a novel cellular function for autophagy, and show that this regulatory relationship between autophagy and RNAi is evolutionary conserved as it operates in divergent animal taxa ranging f
rom worms to mammals. Thus, the manuscript is potentially suitable for publication in EMBO Rep. However, before making the final decision, the authors should change the manuscript according to the comments listed below.

1, The introduction section is rather brief. At least the function of those atg genes should be mentioned that are used in the study.

2, The authors should prove that the extra number of seam cells in dcr-1(-); atg-2(-) double mutant background results from extra cell division ("reiteration of the L2-specific seam cell proliferation programs") rather than misspecification of cell fate. In addition, the authors should show scm:gfp-positive (scm-?) seam cells in dcr-1(-) single mutant versus dcr-1; atg-2 double mutant animals to convincingly demonstrate the suppression phenomena. In Table 1, the number of seam cells in dcr-1(-); atg-7(-) double mutant, but not in atg-7(-) single mutants, is indicated. This should be corrected.

3, "The fluorescence intensity of HBL-1:GFP in VNC was significantly reduced in atg-2 and epg-6 autophagy mutants (Fig 1I-L)." Since down-regulation of HBL-1:GFP in VNC is mediated by let-7

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miRNA, the authors should see GFP expression in atg2; let-7 double mutant background (if the proposed regulatory relationship is correct, gfp expression should be as strong as in let-7 single mutants). Also, HBL-1 accumulation should be demonstrated in let-7 single mutants. Furthermore, the authors should test reporter expression in an autophagy hyperactive background (induced by TOR deficiency or starvation).

4, Demonstration of the suppressive effect of atg gene mutations on a let-60(gf) phenotype (%Muv) is clear. let-60 is a known target of let-7, which in turn is regulated - as the authors show here - by autophagy. mpk-1, which acts downstream of let-60 in the same signaling axis to control vulval development, is not. The authors should test that autophagy deficiency is not able to suppress the Muv phenotype in mpk-1 hyperactive background.

5, Autophagic activity influences cell fate specification in lsy-6 hypomorph mutant background (Fig. 2G). It would be interesting to know whether starvation elevating autophagic activity can also modify neuronal fate specification in this sensitized background.

Minor comments:
1, On figure 1, panels I-K show the reporter construct (hbl-1:gfp), but panels C and E do not. The authors should consistently label the panels.
2, some references should be corrected; e.g., on page 6, "...(4,5,6)." should be changed to "...(4-6)." "Fig" should be Fig.
3, page 4: "dcr-1(bp132) young adults ..." should be changed to dcr-1(bp132) mutant young adults ...
4, Nomenclature: "In wild type embryos," should be In wild-type embryos,

1st Revision - authors' response 18 March 2013

Responses to the reviewer's comments:

We thank the reviewers for their constructive comments. We conducted additional experiments to strengthen our conclusion that autophagy modulates miRNA-mediated gene silencing and selectively degrades AIN-1 in *C. elegans*.

Here are the reviewers' comments with our point-by-point responses:

Referee #1:
1. While the observed increase in AIN-1 protein may contribute to the suppression of miRNA-dependent phenotypes, the authors find that loss of epg-6 and atg-7 can also suppress ain-1 mutant phenotypes. This suggests that there are additional targets that may function to modulate miRNA activity. This should be addressed in the discussion section. Does the suppression of the let-60gf Muv phenotype of the lsy-6 phenotype depend on ain-1 activity? This would help to show the significance in the observed changes in AIN-1 protein levels.

As suggested by the reviewer, we performed additional experiments and found that the suppression of the *let-60(n1046gf)* Muv phenotype by loss of autophagy activity partially depends on *ain-1* activity. We also examined the role of *ain-1* in the specification of ASEL fate in *lsy-6(ot150)* mutants. *ain-1(ku322)* exacerbates the ASEL specification defect in *lsy-6(ot150)* mutants. *ain-1(ku322)* also reduces the suppression effect of loss of autophagy activity on *lsy-6(ot150)*. These results have been included in the revised manuscript (Page 7, lines 14-15; Fig 2D,G).

C. elegans contains two GW182 homologs, encoded by *ain-1* and *ain-2*, which are involved in miRNA-mediated gene silencing. *ain-1* and *ain-2* function redundantly. *ain-1* single mutants exhibit retarded heterochronic defects, while *ain-2* single mutants have no evident defects. *ain-1/-2* double mutants display much stronger defects than either single mutants. The defects in *ain-1; ain-2* double mutants are weaker than those in *dcr-1* mutants and *alg-1; alg-2* double mutants. Loss of function of *nhl-2*, encoding a cofactor of miRISC, dramatically enhances the heterochronic defect associated with *ain-1* null mutants. These results indicate that miRNA-mediated gene silencing still occurs in

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the absence of AIN-1. Additional targets of autophagy may function to modulate miRNA activity. This has been discussed in the revised manuscript (Page 5, lines 20-22).

2. The authors state that "Autophagy mutants show enhanced miRNA-mediated silencing of target genes." However, the data to support this is limited to reporter analysis of two miRNA targets. The conclusion should clarify that the observations are limited to two targets using the indirect method of reporter gene analysis.

In the revised manuscript, we clearly stated that loss of autophagy activity reduces the expression of reporters for two genetically verified targets of *let-7* miRNA.

We further showed that loss of autophagy activity does not affect the elevated expression of *hbl-1::gfp::hbl-1* in VNC and *col-10::gfp::lin-41(3'UTR)* in hypodermal cells in *let-7* mutants. Activating autophagy by inactivation of Tor signaling or starvation increases the expression of *hbl-1::gfp::hbl-1* in VNC and *col-10::gfp::lin-41 (3'UTR)* in hypodermal cells. These results have been included in the revised manuscript (Page 6, lines 21-22; Page 7, lines 1-2; Fig 1S,T; supplementary Fig S1).

3. p. 1, Abstract. "Micro RNAs" should be corrected.

This has been corrected in the revised manuscript (Page 1, line 14).

4. p. 1 and 2, Abstract and Introduction. Change "(miRISC), which contains miRNA, Argonaute..." to "(miRISC), which includes miRNA, Argonaute..." This indicates that there are many other proteins also in the miRISC.

These have been changed in the revised manuscript (Page 1, line 15; Page 2, line 9).

5. p. 1, Abstract. Add comma after "Degradation of AIN-1 requires the adaptor protein"

This sentence has been removed from the abstract in the revised manuscript.

6. Introduction. The recent papers showing a role for autophagy activity in the regulation of miRNA activity (Gibbing, Derrien) should be included in the Introduction to establish the rationale for this study.

As suggested by the reviewer, a role of autophagy activity in the regulation of miRNA activity has been included in the Introduction section in the revised manuscript (Page 4, lines 4-9).

7. p. 14 Methods. "gift from the Han Min lab." Should be corrected to "Min Han" lab.

This has been corrected in the revised manuscript (supplementary information, Page 1, line 20).

8. p. 18, information should be provided for the scm::gfp transgene.

Details on the *scm::gfp* reporter, including the strain name, have been included in the revised manuscript (Page 16, line 11; supplementary information, Page 1, line 11).

9. Table 1. Statistical analysis should be included for seam cell and alae data.

p values for seam cell and alae data have been included in Table 1 in the revised manuscript.

10. Figure S3. The title of this figure only describes I-K in this figure. A-D and E-H are unrelated to the interaction between ALG-1/2 and LGG-1. Could this be separated into different supplemental figures?

As suggested by the reviewer, Figure S3 has been separated into two supplemental figures in the revised manuscript.

11. Can the authors speculate about the significance of the identified region of AIN-1 that interacts with LGG-1, amino acids 343-490?

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We further analyzed the interaction between AIN-1 and LGG-1. The LGG-1 interacting fragment in AIN-1(aa 343-490) contains two AIM/LIR motifs (^{437}WGEL^{440} and ^{462}WNDL^{465}). However, mutating either one or both LIR motifs did not alter LGG-1 binding, indicating that the LIR motif is not essential for binding of AIN-1 to LGG-1. These results have been included in the revised manuscript (Page 12, lines 4-7; supplementary Fig S4G-I). We have shown previously that the LIR motif is also not essential for binding of other substrates, including EPG-7 or SQST-1, to LGG-1. Thus, the LGG-1/Atg8 interacting motif has yet to be determined in *C. elegans*.

The LGG-1/Atg8 interacting domain is mapped to the GAGH domain of AIN-1, which is conserved in the GW182 family. No LIR motifs are present in the GAGH domain of GW182. In mammalian cells, DICER and AGO2, but not GW182 (also known as TNRC6), are targeted for selective autophagic degradation. The degradation of DICER and AGO2 is mediated by the selective autophagy receptor NDP52, which associates with the cargoes and also with Atg8/LC3. No identifiable NDP52 homologs are present in *C. elegans*. In addition to the Atg8/LC3 interacting receptor, a family of scaffold proteins is required for conferring cargo selectivity and efficient autophagic degradation. The presence of distinct autophagy receptor and scaffold proteins may determine different components of the RISC complex are degraded by autophagy in *C. elegans* and mammals. This has been clearly discussed in the revised manuscript (Page 12, lines 9-15).

Referee #2:

1. The introduction section is rather brief. At least the function of those atg genes should be mentioned that are used in the study.

The role of *atg* and *epg* genes in the autophagy pathway has been described in the Introduction section in the revised manuscript (Page 3, lines 8-22).

2. The authors should prove that the extra number of seam cells in dcr-1(-); atg-2(-) double mutant background results from extra cell division ("reiteration of the L2-specific seam cell proliferation programs") rather than misspecification of cell fate. In addition, the authors should show scm:gfp-positive (scm-?) seam cells in dcr-1(-) single mutant versus dcr-1; atg-2 double mutant animals to convincingly demonstrate the suppression phenomena. In Table 1, the number of seam cells in dcr-1(-); atg-7(-) double mutant, but not in atg-7(-) single mutants, is indicated. This should be corrected.

Images showing *scm::gfp*-positive seam cells in *dcr-1* and *dcr-1; atg-2* mutants have been included (Fig 1G,I). The number of seam cells in *atg-7* and other autophagy single mutants has been included in Table 1 in the revised manuscript.

The extra number of seam cells in *dcr-1; atg-2* double mutant background results from extra cell divisions rather than misspecification of cell fate. First, the number of seam cells is 16 in *dcr-1; atg-2* double mutants at the late L2 larval stage and increases at the L3 stage. Second, we observed that some seam cells underwent the L2-prolifereative division pattern at the L3 stage in the double mutants. Third, the seam cell fate is not mis-specified in autophagy mutants.

3. "The fluorescence intensity of HBL-1:GFP in VNC was significantly reduced in atg-2 and epg-6 autophagy mutants (Fig 1I-L)." Since down-regulation of HBL-1:GFP in VNC is mediated by let-7 miRNA, the authors should see GFP expression in atg2; let-7 double mutant background (if the proposed regulatory relationship is correct, gfp expression should be as strong as in let-7 single mutants). Also, HBL-1 accumulation should be demonstrated in let-7 single mutants. Furthermore, the authors should test reporter expression in an autophagy hyperactive background (induced by TOR deficiency or starvation).

As suggested by the reviewer, these experiments have been performed. Expression of *hbl-1::gfp::hbl-1* in VNC and *col-10::gfp::lin-41(3'UTR)* in hypodermal cells is greatly elevated in *let-7* mutants at the young adult stage. Loss of autophagy activity causes no effect on the expression of these reporters in *let-7* mutants. Activating autophagy by inactivation of Tor signaling or starvation increases the expression of *hbl-1::gfp::hbl-1* in VNC and *col-10::gfp::lin-41 (3'UTR)* in hypodermal cells. These results have been included in the revised manuscript (Page 6, lines 21-22; Page 7, lines 1-2; Fig 1S,T; supplementary Fig S1).

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4. Demonstration of the suppressive effect of atg gene mutations on a let-60(gf) phenotype (%Muv) is clear. let-60 is a known target of let-7, which in turn is regulated - as the authors show here - by autophagy. mpk-1, which acts downstream of let-60 in the same signaling axis to control vulval development, is not. The authors should test that autophagy deficiency is not able to suppress the Muv phenotype in mpk-1 hyperactive background.

As suggested by the reviewer, we conducted the experiment and found that the Muv phenotype in *mpk-1* hyperactive mutants is not suppressed by loss of autophagy activity. This result has been included in the revised manuscript (Page 7, lines 15-19).

5. Autophagic activity influences cell fate specification in lsy-6 hypomorph mutant background (Fig. 2G). It would be interesting to know whether starvation elevating autophagic activity can also modify neuronal fate specification in this sensitized background.

Activating autophagy by inactivation of components of Tor signaling, including *let-363* and *rheb-1*, exacerbates the AESL fate specification defect in *lsy-6(ot150)* mutants. This result has been included in the revised manuscript (Page 8, lines 8-10; Fig 2G).

Referee #3:

1. In fig. 1L, the noise regarding fluorescence intensity/ unit area is high. To better appreciate the differences in HBL-1 expression in the different strains, we suggest the authors to perform a western-blot against GFP.

hbl-1::gfp is expressed in multiple tissues at larval stages. Its expression in some tissues is not temporally regulated. *hbl-1::gfp* is strongly expressed in many neurons of the anterior nerve ring, the dorsal nerve cord and several neurons in the tail. Expression of *hbl-1::gfp* in these tissues persists throughout the development. In addition to VNC, *hbl-1::gfp* also shows temporal expression pattern in hypodermal cells and muscle cells, which is regulated by miRNA and also the transcription factor SEL-7. In these tissues, *hbl-1::gfp* is strongly expressed at early larval stages and becomes weak from L3 stage onwards. Thus, HBL-1::GFP in VNC only constitutes a small portion of total levels of HBL-1::GFP. The immunoblotting assay is unlikely to capture the downregulation of *hbl-1* in VNC in autophagy mutants. In the revised manuscript, we further demonstrated that activating autophagy by inactivation of Tor signaling increases the expression of *hbl-1::gfp* in VNC. The elevated expression of *hbl-1::gfp* in *let-7* mutants is not affected by loss of autophagy activity. These results have been included in the revised manuscript (Page 6, lines 21-22; Page 7, lines 1-2; Fig 1S; supplementary Fig S1). Taken together, loss of autophagy activity enhances *let-7*-mediated down-regulation of target genes.

2. In table 1, the authors analyzed the genetic interaction between heterochronic mutants and autophagy, by crossing the heterochronic mutants with different autophagy mutants. Could the authors comment on this? Why weren't the same autophagy mutants crossed with all heterochronic mutants? Would the authors expect to observe similar results regardless of the autophagy mutants crossed?

In the revised manuscript, we crossed more autophagy mutants with heterochronic mutants, including *atg-7; ain-1(ku322)*, *epg-5; ain-1(ku322)* and *epg-1; ain-1(ku322)*. The retarded heterochronic phenotypes in *ain-1(ku322)* mutants are partially suppressed by loss of function of *atg-7*, *epg-5* and *epg-1* (Table 1). In this study, we have been analyzed the interaction of heterochronic mutants with autophagy genes that act at discrete steps of the autophagy pathway, including induction, expansion and maturation of autophagosome. Similar results are observed regardless of the autophagy mutants analyzed.

3. In fig. 3 and throughout the manuscript, a quantification of embryos with diffuse GFP signal versus embryos with different number of aggregates (for example, less than 10, in between 10 and 20, etc, should be presented) and the number of embryos analyzed (n) should be reported.

The number of AIN-1::GFP, ALG-2::GFP and GFP::ALG-1 aggregates in autophagy mutants have been quantified and included in the revised manuscript. The number of embryos analyzed has

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also been clearly indicated in the revised manuscript (Page 18, lines 16-18; Fig 3G,H; supplementary Fig S2H).

4. Throughout the manuscript, absence of water was used by the authors as a stress condition. According to the manuscript, absence of water leads to a dramatic increase in ALG-1 and ALG-2 aggregates in autophagy mutants: The protocol used should be described in the Methods section. Were the embryos viable upon this stress condition? There is no clear link between water absence and induction of autophagy. Can the authors to use commonly reported autophagy inducers, like rapamycin/ or inhibitors, like chloroquine, in the stress experiments.

The protocol used has been described in the Methods section in the revised manuscript (supplementary information Page 2, lines 14-18). Embryos were placed on 3% agarose pad with a drop of M9 buffer and then covered with a coverslip. GFP::ALG-1 and ALG-2::GFP were diffusely localized in the cytoplasm in autophagy mutants when the slides were viewed immediately, while they gradually accumulated into aggregates when the slides were viewed 10 minutes later. The embryos were still viable upon this stress condition.

We identified a mutation that causes accumulation of SQST-1::GFP aggregates in the intestine at larval stages. When mutant animals are placed on the slide and viewed 10 minutes later, SQST-1::GFP aggregates gradually disappear due to autophagic removal, indicating that larval animals placed on slides experience certain stresses such as starvation that activate the autophagy activity. Here we showed that GFP::ALG-1 and ALG-2::GFP form aggregates in autophagy mutants, indicating that the formation of aggregates is unlikely due to elevated autophagy activity. The nature of the stress remains to be determined. We are performing genetic screens to identify mutants that result in accumulation of GFP::ALG-1 and ALG-2::GFP aggregates in autophagy mutants, which will provide insights into how the formation of these aggregates is regulated.

5. In figs. 4 and S2 co-localization index should be presented, as well as the number of embryos analyzed (n).

% of AIN-1::GFP, ALG-2::GFP and GFP::ALG-1 aggregates colocalized with SQST-1 aggregates, SEPA-1 aggregates and LGG-1 puncta has been included. The number of embryos analyzed has also been indicated in the revised manuscript (Page 19; lines 13-15; Fig 4Q; supplementary Fig S3Y,Z).

6. The authors underline the association of AIN-1 with SQST-1 aggregates in autophagy mutants, but no clear functional correlation between SQST-1 and AIN-1 seem to be present. This aspect should be emphasized in the discussion of the results, page 9 of the manuscript.

AIN-1 is localized in SQST-1 aggregates in autophagy mutants. SQST-1 is not required for the autophagic removal of AIN-1 and its formation of aggregates in autophagy mutants. SQST-1 aggregates contain several other unrelated self-oligomerized proteins, including C35E7.6 and ZK1053.4, which are removed by autophagy in a *sqst-1*-independent manner (Long Lin et al., JCB, in press), suggesting that AIN-1 can be recruited into SQST-1 aggregates by other components. Autophagic degradation of SQST-1 aggregates requires EPG-7, which functions as a scaffold protein linking the SQST-1 aggregate with the autophagosome assembly machinery. EPG-7 itself forms aggregates and is removed by autophagy in a *sqst-1* independent manner. We found that EPG-7 is required for degradation of AIN-1. In the revised manuscript, we also examined the role of *sqst-1* and *epg-7* in the specification of the ASEL fate in *lsy-6* hypomorphic mutants. Consistent with the role of *epg-7* in degradation of AIN-1, the ASEL specification defect in *lsy-6(ot150)* mutants was suppressed by loss of activity of *epg-7*, but not *sqst-1*. These results have been included in the revised manuscript (Page 11, lines 14-16; Fig 2G; Fig 3E; supplementary Fig S2P).

7. The authors show that C. elegans AIN-1 (aa 343-490) directly interacts with LGG-1. In contrast, in mammalian cells GW182 (AIN-1 homologue) is not an autophagy substrate (Gibbings et at., 2012). How conserved is LGG-1 region of interaction among AIN-1 and GW182? Is there a AIM/LIR-like motif in AIN-1? The authors should further discuss the differences between their own results and the findings in mammalian cells.

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As suggested by the reviewer, we further analyzed the interaction between AIN-1 and LGG-1. The LGG-1 interacting fragment in AIN-1(aa 343-490) contains two AIM/LIR motifs (^{437}WGEL^{440} and ^{462}WNDL^{465}). However, mutating either one or both LIR motifs did not alter LGG-1 binding, indicating that the LIR motif is not essential for binding of AIN-1 to LGG-1. These results have been included in the revised manuscript (Page 12, lines 4-7; supplementary Fig S4G-I). We have shown previously that the LIR motif is also not essential for binding of other substrates, including EPG-7 or SQST-1, to LGG-1. Thus, the LGG-1/Atg8 interacting motif has yet to be determined in *C. elegans*.

The LGG-1/Atg8 interacting domain is mapped to the GAGH domain of AIN-1, which is conserved in the GW182 family. No LIR motifs are present in the GAGH domain of GW182. In mammalian cells, DICER and AGO2, but not GW182 (also known as TNRC6), are targeted for selective autophagic degradation. The degradation of DICER and AGO2 is mediated by the selective autophagy receptor NDP52, which associates with the cargoes and also with Atg8/LC3. No identifiable NDP52 homologs are present in *C. elegans*. In addition to the Atg8/LC3 interacting receptor, a family of scaffold proteins is required for conferring cargo selectivity and efficient autophagic degradation. The presence of distinct autophagy receptor and scaffold proteins may determine different components of the RISC complex are degraded by autophagy in *C. elegans* and mammals. This has been clearly discussed in the revised manuscript (Page 12, lines 9-15).

8. Accumulation of AIN-1 is present in aggregates that are exclusively formed in absence of autophagy, and not under physiological conditions, in wild-type embryos. How is the AIN-1 degradation regulated in wild-type embryos? Is there a steady turnover of AIN-1? Does it differ in different stages of development, etc? it is recommended that a paragraph is added where this topic is discussed.

During embryogenesis, AIN-1::GFP is diffusely localized in the cytoplasm and exhibits no difference in different embryonic stages. Expression levels of AIN-1::GFP are elevated and accumulate into a large number of aggregates in autophagy mutants. EPG-7, the scaffold protein required for AIN-1 degradation, is also diffusely localized in the cytoplasm in wild type embryos and accumulates into numerous aggregates in autophagy mutants, suggesting that AIN-1 is steadily removed by EPG-7-mediated autophagic degradation during embryogenesis. This has been discussed in the revised manuscript (Page 11, lines 6-10).

9. The last part of the Results section, regarding P-bodies, is out of the scope of the manuscript and we would recommend its removal.

As suggested by the reviewer, the Results section on P bodies has been removed in the revised manuscript.

2nd Editorial Decision 04 April 2013

We have now received the reports from all referees, which are copied below. Referees 2 and 3 have some minor suggestions that I would like you to address before we proceed with the official acceptance of your manuscript.

If feasible, I think that a model of how autophagy modulates the miRNA pathway would be a nice addition. It would also be great if you could address referee 3's concern. However, if this would be a major experiment to perform, then we can discuss this issue further.

I also noticed that the test used to calculate the p-values in figure 1 still needs to be specified in the figure legend, that the number of animals used need to be mentioned in the legend for SF2, and that the error bars need to be defined in the legend for SF3Z.

Finally, I have a few suggestions for minor changes to the abstract (I also agree with referee 2 that GW182 should be included in the title):

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MicroRNAs (miRNAs) post-transcriptionally repress gene expression via the miRNA-induced silencing complex (miRISC), which includes miRNA, Argonaute (Ago) and a GW182 family member. Here we show that in C. elegans, miRNA-mediated gene silencing is modulated by macroautophagy, a lysosome-mediated degradation process. Loss of autophagy activity suppresses developmental defects caused by partially impaired silencing of miRNAs targets including the let-7 family and lsy-6. The C. elegans GW182 homolog AIN-1 is itself selectively degraded by autophagy and colocalizes with the p62 homolog SQST-1 in autophagy mutants. Thus, autophagy activity modulates miRNA-mediated gene silencing and degrades a core miRISC component.

Please let me know if you agree with these changes. Please also feel free to contact me if you have any further comments or questions.

I look forward to seeing a new revised version of your manuscript as soon as possible.

REFEREE REPORTS:

Referee #1:

This revised manuscript is suitable for publication.

Referee #2:

The authors have adequately addressed the comments I suggested in the first round of the evaluation process. Thus, the manuscript is now suitable for publication in EMBO Reports. I suggest two additional, minor (not essential) points at this stage that may further improve the general quality:
1, change the title as "... AIN-1/GW182..."
2, draw a model showing how autophagy modulates the miRNA pathway (as Fig. 5)
There is no need to turn back the manuscript to this referee.

Referee #3:

In the revised version of the manuscript, the authors addressed most of the questions raised. Point 4, however, was not fully addressed. As the authors clearly state the nature of the stress behind water absence is not known. It would be highly appreciated if stress was induced by reported autophagy inducers and inhibitors, instead of water absence.

2nd Revision - authors' response 08 April 2013

Responses to the reviewer's comments:

1. If feasible, I think that a model of how autophagy modulates the miRNA pathway would be a nice addition.

A model showing the role of autophagy in the miRNA pathway has been included in Fig 4U in the revised manuscript.

2. I also noticed that the test used to calculate the p-values in figure 1 still needs to be specified in the figure legend, that the number of animals used need to be mentioned in the legend for SF2, and that the error bars need to be defined in the legend for SF3Z.

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In the revised manuscript, we have specified the test used to calculate the p-values in Figure 1, the number of animals used for SF2 and the error bars in SF3Z.

3. Finally, I have a few suggestions for minor changes to the abstract (I also agree with referee 2 that GW182 should be included in the title):

These changes have been incorporated in the revised manuscript. GW182 has also been included in the title.

Referee #2:
1, change the title as "... AIN-1/GW182..."

This has been changed.

2, draw a model showing how autophagy modulates the miRNA pathway (as Fig. 5)

A model showing the role of autophagy in the miRNA pathway has been included in Fig 4U in the revised manuscript.

Referee #3:

1. In the revised version of the manuscript, the authors addressed most of the questions raised. Point 4, however, was not fully addressed. As the authors clearly state the nature of the stress behind water absence is not known. It would be highly appreciated if stress was induced by reported autophagy inducers and inhibitors, instead of water absence.

GFP::ALG-1 and ALG-2::GFP accumulate into aggregates upon stress conditions in mutants of genes acting at different steps of the autophagy pathway, indicating that formation of aggregates is unlikely due to elevated autophagy activity. *C. elegans* embryogenesis is independent of external nutrients and thus, the autophagy activity in *C. elegans* embryos occurs at a basal level and autophagic degradation of protein aggregates is not induced by inactivation of Tor signaling, at which the autophagy inducer rapamycin acts.

Treatment with autophagy inhibitors or inducers will not help us to determine the nature of the stress. First, the *C. elegans* embryo is enclosed in a tough eggshell that is impermeable to most solutes. Second, reported autophagy inducers such as rapamycin and inhibitors such as Wortmannin exhibit other effects in addition to autophagy. We tested whether it is caused by the osmotic stress. However, RNAi inactivation of *cyk-3*, which is required for cellular osmotic regulation, didn't affect the expression pattern of ALG-2::GFP in *epg-8* mutants. We are performing genetic screens to identify mutants that result in accumulation of GFP::ALG-1 and ALG-2::GFP aggregates in autophagy mutants, which will provide insights into how the formation of these aggregates is regulated.

3rd Editorial Decision 09 April 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

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