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Endocytosis and intracellular trafficking contribute to necrotic neurodegeneration in C. elegans

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(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 20 June 2011

Thank you for submitting your manuscript to the EMBO journal. Your study has now been seen by three experts in the field and their comments are provided below.

As you can see, while referees #1 and 3 are more supportive of the analysis, referee #2 finds the data supporting a role for endocytosis in necrosis too circumstantial and indirect. Referee #2, and also referee #3, indicate that there are other explanations for why synaptotagmin and endocytosis mutants compensate the gain of function phenotypes induced by mec-4 or the acetylcholine receptor such as incorrect trafficking and targeting of the proteins and also by affect synaptic transmission. In other words there is not enough data to support that endocytosis directly affects necrosis - the compensatory effects observed might be due to other reasons. Further data is needed to resolve this issue for consideration here. I recognize that you also use an environmental stimulus, hypoxia, to trigger necrosis, but referee #3 also raises concerns with using sodium azide to induce hypoxia. Other issues also concern the need to monitor the endocytic process more carefully. I recognize that a lot of work is needed for consideration here, but should you be able to resolve the concerns raised in full, then we would be willing to consider a revised version. I should remind you that it is EMBO Journal 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 in this revised version. I do realize that addressing all the referees' criticisms will require a lot of additional time and effort and might be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

If you decide to thoroughly revise the manuscript for the EMBO Journal, please include a detailed

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We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

Review on the manuscript by Troulinaki and Tavernarakis

The manuscript entitled „Requirement for clathrin-mediated endocytosis and intracellular trafficking in C. elegans necrotic neurodegeneration" by Troulinaki and Tavernarakis reports that clathrin-mediated endocytosis and kinesin-mediated intracellular trafficking contribute to toxic ion channel-induced necrotic cell death in C. elegans. Undoubtedly, these findings have significant medical implications. The authors depleted/mutationally inhibited several key components of the endocytic machinery [including dynamin (DYN-1), endophilin (UNC-57) and synaptotagnin (SNT-1)], and found that these genetic interventions significantly suppress, but not completely eliminate, necrotic death of touch receptor neurons. Consistently with these data, endosome formation was found to increase during necrosis. They also report that endocytosis synergizes with autophagy, but not with lysosomal proteolysis, to facilitate necrotic demise. Thus, the endosytic system acts in parallel with autophagy, and in the same pathway with lysosomal proteolysis, to mediate this process. The manuscript is nicely written and contains several interesting data. So, it has the potential to be considered for publication in EMBO J. However, the authors should modify the manuscript along the comments I summarize below, before making the decision.

Major comments:

1, several abbreviations (e.g., ALS, AP2, ...) are not explained in the text.

2, On my screen the coloring of the last two columns of Figs. 1A, D is dark grey, while the other ones are in light grey. Why?

3, As the authors say, initiation of necrotic cell death is accompanied by enhanced formation of early and recycling endosomes. However, on Fig. 2 there is only a slight increase in the number of GFP-positive dots at the early stage of endosome formation and a sharp decrease in the number of dots at the late stage of the process. This discrepancy should be clarified.

4, The authors should examine the potential involvement of the ESCRT (endosomal sorting complexes required for transport) system too, by using vps-32 and vps-34 mutants.

5, Endocytosis and lysosomal proteolysis, but not autophagy, act in the same pathway to facilitate necrosis. However, in Fig. 3A and C, there is a clear synergestic effect between endocytosis and lysosomal proteolysis. Moreover, autophagy is known as a lysosome-mediated cellular degradation

process; thus, it is not clear why autophagy operates parallel with endocytosis in this process. In many cases during autophagic breakdown, late endosomes are fused with lysosomes, and the resulting compartment (amphisome) is further processed into the classical macroautophagic pathway.

6, The authors targeted lgg-1 (the worm Atg8 factor) by RNAi to disrupt autophagic activity. Another autophagy gene (or more) should also be tested. I also strongly suggest mutant analysis of at least one autophagy gene (e.g., atg-18, unc-51 or atg13).

7, <B>The authors should examine whether there is a synergistic effect between endocytosis and vesicle trafficking in contributing to necrotic cell death.</B>

8, The authors should check whether neurotoxin- (e.g., 6-OHDA) induced death of specific (dopaminergic) neurons can also be suppressed by downregulating the endocytic system.

9, The authors should monitor the phenotypic consequence of necrotic suppression in this model. For example, touch response in mec-4(d) vs. mec-4(d)snt-1(-) mutants should be compared.

Minor comments:

10, „..., in stroke, in trauma and other pathological conditions." (Introduction) should be „..., in stroke, trauma and other pathological conditions."

11, unc-104(e1265 mutants should be unc-104(e1265) mutants

12, At several places the authors argue for "a general requirement for clathrin-mediated endocytosis in necrotic cell death". Since necrotic cell death occurs, although at decreased levels, in mutants defective for endocytosis, the word "contribute" (instead of requirement) would be more correct.

Referee #2:

The study from Troulinaki and Tavernarakis aims at investigating the role of clathrin-mediated endocytosis and intracellular trafficking during necrotic neurodegeneration using genetic studies in C. elegans. The authors used various classical endocytic worm mutants and several fluorescent markers to claim that endocytosis and endosomal trafficking are required for necrosis to happen in vivo.

To induce necrosis the authors overexpress an ion channel subunit (mec-4) or use a gain of function mutation in the acetylcholine receptor (deg-3). The referee believes that inhibition of synaptic transmission being compensated by endocytic mutants or a synaptotagmin mutant is not surprising given that these will counteract each other. In other words, a synaptotagmin mutant will have a defect in exocytosis and thus for a number of reason the activity of synapses will be decreased, and there may not even be correct targeting to the membrane of the toxic ion channels etc. The same is true for the endocytic mutants where there may be decreased activity or incorrect targeting. Thus the authors should not conclude that these proteins are "critically required for the execution of necrosis" without direct evidence. The paper relies on circumstantial evidence.

Other comments:
The finding that any endocytosis mutants tested decrease necrosis is both intriguing and worrying. Many of these mutants have compromised fitness - the UNC57 mutant is paralysed (Bai et al. 2010), the UNC11 mutant has defect in several neurotransmitters transmission (Nonet et al. 1999). Many of the mutants used here are partial mutants and do not constitute 'elimination' or 'deletion' of the alleles as the authors claim. UNC11 and UNC104 deletions (that is, null alleles) are lethal (Nonet et al. 1999 and Hall and Heggecock 1991) and only partial mutants are viable. The authors should test mutants of energy metabolism etc as these will also likely rescue. Do the authors have mutants that do not rescue necrosis?

The images on Figure 2 are of insufficient quality to support the authors' claim. Most Rab5 and Rab11 signals are blurry and diffuse (hazy) and do not display the expected puncta.

In any case, this figure is of very limited value as there is no evidence that the number of early endosome relates to an enhancement of endocytosis.
There are no scale bars.

Technical comments:
1) Much of the introduction appears tangential and often inaccurate.
2) page 6, line 8-10: The acyl transferase activity of Endophilin reported in Schmidt et al Nature 1999 has been shown to be a purification contaminant in Gallop and McMahon Nature 2005 and the N-terminal region of Endophilin supposed to have such lipid modifying activity was shown to be a BAR domain in Masuda et al EMBO 2006 and Gallop el al. EMBO 2006. Please correct the sentence introducing endophilin accordingly.
3) page 7, last line: the functionality of σ2-EGFP construct was shown in Ehrlich et al Cell 2004, not Kirchhausen 1999

Referee #3:

The authors present genetic and cell biology data suggesting that endocytosis, in addition to autophagy and lysosomal proteolytic mechanisms both of which were characterized previously is required for necrosis in C. elegans. Genetic analysis indicates that endocytosis acts in the same pathway as lysosomal protein degradation to alleviate necrosis. Authophay is a separate pathway. There are several model systems to study necrotic cell death in C. elegans. The two best-studied systems use neuronal necrosis triggered by a dominat mutations of the EC-4 ion channel subunit, and the DEG-3 calcium channel subunit. This system us used, and the senior authors lab is a major player in this area of research, to find mutations that alleviate the extend of such neurodegeneration. Obviously C. elegans is an ideal system to conduct those studies in an organismal context and if would be hard, if not impossible to conduct equally comprehensive investigations in higher animal systems. We think that the current study is interesting, and eventually warrants publication, but major revisions are needed.

The major assays provided score for the extend of "suppression" of neuronal loss. What is missing entirely is the complete lack of any kinetic data. It appears imperative, to provide a time-course comparing neurodegeneration in wild type and mutant backgrounds. Such an experiment, would also help to aid the non expert reader in judging if the phenotype is indeed a genetic suppression of neurodegeration, or merely a delay of neurodegeration. Equally we would learn if the authors study the cessation of necrosis, or a delay in its onset. Once these studies are established the authors should than be more precise and consistent throughout the manuscript, to describe the phenotypes they observe. Indeed the beauty of the system is the use of defined dominant mutations causing neurodegeneration. It appears that current technology would allow for the inducible expression of these dominant alleles in adult tissues thus providing a more precise and relevant model system for careful genetic studies, as well as for cell biology based follow up examinations.

We are very much concerned as to the use of Sodium Azide to mimic hypoxia, and as to the correlation of the extend of necrosis induction, with the level of lethality. It appears that the authors aught to use hypoxia, and aught to show that this causes necrosis, suppressible by mutations in endocytosis pathways, analogous to the study by Scott et al., 2002 they cite. Effects observed upon using Sodium Azide might be an indirect consequence of altered uptake, and or efflux, or removal likely affected in membrane traffic and neuronal mutants. Indeed many of the endocytosis mutants used have pharyngeal pumping defects that may affect the NaN3 internalization.

The suppression of MEC-4 (d) induced neurodegeration, could be caused be reduced MEC-4 (d) expression, altered localization and many other mechanisms. The authors provide low-resolution images and suggest the level of MEC-4 expression is not alerted in endocytosis mutants. It would help to have higher resolution pictures and the quantification of relative expression levels, and to nevertheless more carefully discuss the caveats of their approach. For instance, it is possible that perturbing membrane traffic via deletion of snt-1 reduces MEC-4(d) levels at the plasma membrane and it is important that the authors show that this is not the case.

To further investigate endocytic processes in necrosis the authors

visualize, via GFP fusion, the following proteins APS-2, the sigma subunit of the AP-2 complex, RAB-5, a early endosome marker, and RAB-11, a recycling endosome marker.
APS-2::GFP is used as a marker of clathrin coated pits and vesicles. However, as AP-2 is not essential for Clathrin mediated endocytosis it is recommended that the authors repeat their experiments using a fluorescent clathrin and show that AP-2 punctae indeed represent CME.

The authors need to establish higher resolution imaging to ensure that they are adequately measuring the number of AP-2 punctae (scale bars). The provided images (Figure 2A, B, C) suggest that AP-2 punctae are of the same size as endosomes, which appears unusual. The authors would learn much more about the involvement of endocytosis in neurodegeration with real time imaging studies, as this is a very dynamic process and the counting of ill defined punctae might be more mis-leading than leading.

In addition the authors need to be more precise in their description of membrane traffic in the introduction, for example clathrin coated vesicles, upon dissociation of clathrin, are described as 'become[ing] an early endosome', rather than fusing with early endosomes, as is the case.

1st Revision - authors' response 28 September 2011

Referee #1:

...The manuscript is nicely written and contains several interesting data. So, it has the potential to be considered for publication in EMBO J. However, the authors should modify the manuscript along the comments I summarize below, before making the decision.
We thank the Referee for the positive and encouraging comments. Our study reveals, for the first time in any metazoan, a requirement for clathrin mediated endocytosis and intracellular trafficking in necrotic neurodegeneration. Below, we detail our revisions related to each of the comments.

Major comments:

1, several abbreviations (e.g., ALS, AP2, ...) are not explained in the text.
We now define all abbreviations included in the text.

2, On my screen the coloring of the last two columns of Figs. 1A, D is dark grey, while the other ones are in light grey. Why?
The two greyer columns in Figure 1A & D depict the effects of additional *snt-1* and *unc-57* mutant alleles respectively. A different shade is also used in Figure 5D to signify the additional kinesin allele examined. We chose to use different shades in order to make it easier for the reader to discriminate between different alleles. We can use similar shades if deemed necessary.

3, As the authors say, initiation of necrotic cell death is accompanied by enhanced formation of early and recycling endosomes. However, on Fig. 2 there is only a slight increase in the number of GFP-positive dots at the early stage of endosome formation and a sharp decrease in the number of dots at the late stage of the process. This discrepancy should be clarified.
We now provide the requested clarifications. As noted in the text, the results obtained by monitoring early endosomes (RAB-5 reporter) are shown in panel B of Figure 2. The results obtained by monitoring recycling endosomes (RAB-11 reporter) are shown in panel C. In both cases, we observed considerable and significant increase of GFP-positive dots. The Referee probably refers to the slight (non statistically significant) increase and sharp decrease during early and late stages of cell death respectively, shown in panel A of Figure 2. This panel corresponds to clathrin-coated pits and clathrin-coated vesicles (APS-2 reporter), not early or recycling endosomes. We note that clathrin mediated endocytosis and trafficking are both energy-demanding processes. Early during neurodegeneration cells still maintain the capacity to carry out these processes. However, as necrosis progresses, ensuing cell damage and disorganization impairs endocytosis and intracellular trafficking, leading to pronounced decrease in the number of fluorescent dots corresponding to endosomes.

4, The authors should examine the potential involvement of the ESCRT (endosomal sorting complexes required for transport) system too, by using vps-32 and vps-34 mutants.

Per the suggestion of the Referee, we examined the potential involvement of the ESCRT system. To this end, we knocked down the *vps-32.2* and *vps-34* genes (related to the yeast Vacuolar Protein Sorting factors), in *mec-4(d)* mutants. We found that VPS-32.2 or VPS-34 deficiency does not suppress necrotic cell death (data are shown in Supplementary Figure S5). We also considered the involvement of *vps-32.1*, an additional Vacuolar Protein Sorting factor. However, knock down of this gene causes embryonic and larval lethality, as reported previously (Michelet et al. 2010 *Biol Cell* 102: 191-202; Roudier et al. 2005 *Traffic* 6: 695-705).

5, Endocytosis and lysosomal proteolysis, but not autophagy, act in the same pathway to facilitate necrosis. However, in Fig. 3A and C, there is a clear synergestic effect between endocytosis and lysosomal proteolysis. Moreover, autophagy is known as a lysosome-mediated cellular degradation process; thus, it is not clear why autophagy operates parallel with endocytosis in this process. In many cases during autophagic breakdown, late endosomes are fused with lysosomes, and the resulting compartment (amphisome) is further processed into the classical macroautophagic pathway.
We agree with the Referee about the slight additional suppression of necrosis by combining endocytosis and lysosomal proteolysis mutants shown in Figure 3A & C. Because of the ambivalence of this observation, we have repeated these experiments multiple times and assayed several hundreds of animals in an effort to obtain the most accurate quantification of cell death for each mutant. Our cumulative statistical analysis shows that there is no significant difference between the levels of necrotic cell death in the double *cad-1(j1);mec-4(d)* and the triple *cad-1(j1);snt-1(md290);mec-4(d)* mutants (P value=0.1059) (Figure 3A). Similarly, there is no statistically significant difference between *vha-2(RNAi);mec-4(d)* and *vha-2(RNAi);unc-57(e1190);mec-4(d)* animal populations (P value=0.0876) (Figure 3C). The combined suppression is close to that of the mutant with the strongest effect. As the Referee correctly points out, in many cases late endosomes are fused with lysosomes and the resulting compartment is then following the lysosomal degradation pathway. However, in addition to this route early endosomes can also follow the recycling route. This notion is supported by our *in vivo* monitoring analysis, which reveals that the number of recycling endosomes (represented by GFP::RAB-11 fluorescent puncta) increases during early stages of necrosis. To obtain further support about the involvement of endosome recycling, we knocked down RME-1, a protein with a key role in receptor recycling from the endosome to the plasma membrane (Shi et al. 2007, *Curr Biol* 17: 1913-24). We found that RME-1 deficiency significantly suppresses *mec-4(d)*-induced neurodegeneration (data are shown in Supplementary Figure S8). Based on the totality of our findings, we suggest that both autophagy and endocytosis converge on the lysosomal degradation pathway to mediate necrotic cell death. We now discuss these points in the main text.

6, The authors targeted lgg-1 (the worm Atg8 factor) by RNAi to disrupt autophagic activity. Another autophagy gene (or more) should also be tested. I also strongly suggest mutant analysis of at least one autophagy gene (e.g., atg-18, unc-51 or atg13).
Per the suggestion of the Referee, we have tested two more key autophagy regulators, *bec-1* and *unc-51*, in combination with both endocytosis and trafficking mutants, for their effect on neurodegeneration. The results with these two additional genes corroborate our previous findings with LGG-1/LC3. We observed further suppression of cell death upon knock down of either *bec-1* or *unc-51* in endocytosis mutants, while there was no significant synthetic effect with trafficking deficient animals (the data are shown in Supplementary Figure S4).

7, The authors should examine whether there is a synergistic effect between endocytosis and vesicle trafficking in contributing to necrotic cell death.
Per the suggestion of the Referee, we knocked down both *unc-116* and *unc-104* genes in the *snt-1(md290);mec-4(d)* and *unc-57(e1190);mec-4(d)* double mutants. We found that the combined inactivation of these two cellular processes does not further suppress necrotic cell death. The relevant data are now included in Figure 5F and discussed in the main text.

8, The authors should check whether neurotoxin- (e.g., 6-OHDA) induced death of specific (dopaminergic) neurons can also be suppressed by downregulating the endocytic system.
To address this point, we used 6-OHDA to induce neurotoxin death of dopaminergic neurons (per the report of Nass et al. *PNAS* 2002, 99: 3264-9), in the endocytotic mutants *snt-1(md290)*, *unc-57(e1190)* and *unc-11(e47)*. We found that downregulation of the endocytotic system protects the dopaminergic neurons from neurotoxin-induced cell death (the results are shown in Supplementary

Figure S3B).

9, The authors should monitor the phenotypic consequence of necrotic suppression in this model. For example, touch response in mec-4(d) vs. mec-4(d)snt-1(-) mutants should be **compared.**
We have now performed the phenotypic characterization requested by the Referee. Similarly to all previously characterized suppressors of *mec-4(d)*-induced neurodegeneration, we found that the mutations in both the endocytotic genes *snt-1* and *unc-57*, and in the kinesin genes, *unc-116* and *unc-104*, do not revert the touch response defect of the *mec-4(d)* mutants (the data are shown in Supplementary Figure S2). The reason is that, although touch receptor neurons survive in the double mutants, they still lack a functional touch transduction ion channel, since they express the mutant MEC-4(d) subunit. This is the case for all genetic suppressors of necrosis induced by *mec-4(d)* reported in the literature (see for example Xu et al. 2001, *Neuron* 31: 957-971; Syntichaki et al. 2002, *Nature* 419: 939-944; Syntichaki et al. 2005, *Curr Biol* 15 (13), 1249-1254; Artal-Sanz et al. 2006, *J Cell Biol* 173, 231-239; Samara et al. 2007, *Cell Death Differ* 15: 105-112).

Minor comments:

10, ...,in stroke, in trauma and other pathological conditions." (Introduction) should be ..., in stroke, trauma and other pathological conditions."
We have now rephrased the text accordingly.

11, unc-104(e1265 mutants should be unc-104(e1265) mutants
We have corrected the allele designation.

12, At several places the authors argue for "a general requirement for clathrin-mediated endocytosis in necrotic cell death". Since necrotic cell death occurs, although at decreased levels, in mutants defective for endocytosis, the word "contribute" (instead of requirement) would be more correct.
Following the suggestion of the Referee, we have now rephrased the text and also provide the relevant clarifications throughout.

Referee #2:

To induce necrosis the authors overexpress an ion channel subunit (mec-4) or use a gain of function mutation in the acetylcholine receptor (deg-3). The referee believes that inhibition of synaptic transmission being compensated by endocytic mutants or a synaptotagmin mutant is not surprising given that these will counteract each other. In other words, a synaptotagmin mutant will have a defect in exocytosis and thus for a number of reason the activity of synapses will be decreased, and there may not even be correct targeting to the membrane of the toxic ion channels etc. The same is true for the endocytic mutants where there may be decreased activity or incorrect targeting. Thus the authors should not conclude that these proteins are "critically required for the execution of necrosis" without direct evidence. The paper relies on circumstantial evidence.
To address this issue, we performed a number of experiments. First, we monitored the expression, stability and correct localization of the toxic MEC-4 ion channel in live animals by using a full length GFP reporter. We found that neither is affected in the endocytosis mutants we examined (the data is included in Figures 1 & 5). Second, to further establish that neuronal signalling and synaptic transmission defects are not the underlying reason for suppression of necrosis, we examined the behavioural output of touch receptor neurons (sensitivity to light touch) in single synaptotagmin and other endocytosis mutants (*snt-1, unc-57, unc-116 and unc-104*). We find that these mutants show normal response to touch, indicating that signalling by these neurons is preserved (shown in Supplementary Figure S2). Finally, to investigate whether suppression of neurodegeneration is merely a consequence of interfering with synaptic activity, we examined two other key proteins involved in synaptic function and exocytosis or neurotransmitters, synaptobrevin (SNB-1) and syntaxin (UNC-64). None of these proteins is required for necrosis, since their knock down did not suppress cell death (data shown in Supplementary Figure S6). Thus, suppression of neurodegeneration is specific to impairment of endocytosis and not a collateral effect of defective synaptic function.
In closing our response to this comment, we respectfully note that our conclusions are not merely based on circumstantial evidence. Instead, we demonstrate a clear contribution of endocytic processes in necrotic neurodegeneration by establishing, for the first time, a genetic requirement for

endocytosis and intracellular trafficking in necrosis, by monitoring endocytosis *in vivo* during the course of cell death, and by examining the link between endocytosis, trafficking and previously characterised necrotic pathways.

Other comments:

The finding that any endocytosis mutants tested decrease necrosis is both intriguing and worrying. Many of these mutants have compromised fitness - the UNC57 mutant is paralysed (Bai et al. 2010), the UNC11 mutant has defect in several neurotransmitters transmission (Nonet et al. 1999). Many of the mutants used here are partial mutants and do not constitute 'elimination' or 'deletion' of the alleles as the authors claim. UNC11 and UNC104 deletions (that is, null alleles) are lethal (Nonet et al. 1999 and Hall and Heggecock 1991) and only partial mutants are viable. The authors should test mutants of energy metabolism etc as these will also likely rescue. Do the authors have mutants that do not rescue necrosis?
Following the suggestion of the Referee, we tested a number of mutants that either directly or indirectly affect energy metabolism. Specifically, we tested the *clk-1(qm30)*, *isp-1(qm150)* and *mev-1(kn1)* mitochondrial metabolism mutants (encoding the highly conserved demethoxyubiquinone hydroxylase necessary for the biosynthesis of ubiquinone coenzyme Q, the Rieske iron sulphur protein which is a subunit of the mitochondrial complex III, and the succinate dehydrogenase, cytochrome b subunit, respectively). None of these energy metabolism-deficient mutants suppressed necrotic neurodegeneration (data shown in Supplementary Figure S7). In addition, we tested the *daf-2(e1370)*, *age-1(hx546)* and *daf-16(m26)* mutants which interfere with the insulin/IGF-1 signalling pathway that indirectly influences metabolic processes in the cell. Similarly to the mitochondrial mutants, we observed no effect on necrosis in these genetic backgrounds (data shown in Supplementary Figure S7). Thus, suppression of necrosis is not merely a result of defective energy metabolism. We also note that compromised fitness does not correlate with suppression of necrotic cell death since many of the mutants we tested, which show compromised fitness (for example *mev-1*, *isp-1*, *snb-1*, *unc-64*, etc.), have no observable effect on neurodegeneration, whatsoever (Supplementary Figures S6 & S7; see also the previous comment above).

The images on Figure 2 are of insufficient quality to support the authors' claim. Most Rab5 and Rab11 signals are blurry and diffuse (hazy) and do not display the expected puncta. In any case, this figure is of very limited value as there is no evidence that the number of early endosome relates to an enhancement of endocytosis.
To address this point, we have now repeated the imaging experiments and provide better quality images (Figure 2). We would like to clarify that we don't declare/state that there is an enhancement of endocytosis based solely on the number of clathrin-coated pits and clathrin-coated vesicles (Figure 2A; APS-2 reporter). As we point out in the text, monitoring of the APS-2::GFP fluorescent spots, suggests that there in no enhancement of clathrin-mediated endocytosis during the first stages of necrosis. By contrast, the increased number of the early and late endosomes (shown in Figure 2B & C) indicates an upregulation of the endosomal pathway. Early endosomes are not formed solely through clathrin-mediated endocytosis but also through the trans-Golgi. Finally, we note that the images in Figure 2 are offered as representative cases only. Instead the quantification shown in the graphs was performed by counting fluorescent puncta in the six touch receptor neurons of more than one hundred animals (>600 measurements) per strain or condition, as described in the Materials and Methods section. This practice meets or exceeds the standards used for similar quantifications in the literature (Ruck et al. 2011, Autophagy 7: 386-400; Sato et al. 2005, Nat Cell Biol 7: 559-69; Samara et al. 2007, *Cell Death Differ* 15: 105-112).

There are no scale bars.
We have now added appropriate scale bars to the images.

Technical comments:

1) Much of the introduction appears tangential and often inaccurate.
We have revised the introduction to accommodate this point. We would like to note that it is not our intention, nor is it possible given the space constrains, to provide a comprehensive review of endocytosis or necrotic cell death in the introduction. For this reason, as it is commonly practiced, we chose to only introduce the factors, genes and pathways pertinent to our study. With regard to the introduction, our objective is to equip the reader with the information necessary to follow our study.

We hope the Referee finds our revisions satisfactory. We could incorporate additional material if necessary.

2) page 6, line 8-10: The acyl transferase activity of Endophilin reported in Schmidt et al Nature 1999 has been shown to be a purification contaminant in Gallop and McMahon Nature 2005 and the N-terminal region of Endophilin supposed to have such lipid modifying activity was shown to be a BAR domain in Masuda et al EMBO 2006 and Gallop el al. EMBO 2006. Please correct the sentence introducing endophilin accordingly.
We thank the Referee for pointing out this controversy in the published literature. We have now revised the text to accommodate this point.

3) page 7, last line: the functionality of AP2-EGFP construct was shown in Ehrlich et al Cell 2004, not Kirchhausen 1999
Indeed, Ehrlich et al. (*Cell* 118: 591-605, 2004) have shown that both AP2 and clathrin can be used for monitoring clathrin coated pits in living cells. We have now added this reference. We provided the earlier review by Kirchhausen, which discusses adaptors that participate in clathrin-mediated endocytosis, for more general information on AP2.

Referee #3:

...Obviously C. elegans is an ideal system to conduct those studies in an organismal context and if would be hard, if not impossible to conduct equally comprehensive investigations in higher animal systems. We think that the current study is interesting, and eventually warrants publication, but major revisions are needed.
We thank the Referee for the encouraging comments. Endocytic processes were long suspected to be involved in neurodegenerative diseases in humans. Our study uses multiple genetic models of neurodegeneration in *C. elegans* to establish a cause-and-effect link between endocytosis, intracellular trafficking and necrotic cell death. We do hope the Referee finds our revisions, described below, adequate for publication of our study in *EMBO J*.

The major assays provided score for the extend of "suppression" of neuronal loss. What is missing entirely is the complete lack of any kinetic data. It appears imperative, to provide a time-course comparing neurodegeneration in wild type and mutant backgrounds. Such an experiment, would also help to aid the non expert reader in judging if the phenotype is indeed a genetic suppression of neurodegeneration, or merely a delay of neurodegeneration. Equally we would learn if the authors study the cessation of necrosis, or a delay in its onset. Once these studies are established the authors should than be more precise and consistent throughout the manuscript, to describe the phenotypes they observe.
We have now performed the time-course analysis of neurodegeneration requested by the Referee in the single *mec-4(d)* mutant and in the double mutants of endocytosis and trafficking (*snt-1(md290);mec-4(d)*, *unc-57(e1190);mec-4(d)*, *unc-116(e2310);mec-4(d)* and *unc-104(e1265);mec-4(d)*). This analysis reveals that suppression of neurodegeneration is not merely a delay of cell death (the kinetics data are shown in Supplementary Figure S1). Necrotic cell death is reduced in all the double mutants not only at the L1 stage but also at L2 and even at L3-L4 stages of development. Thus, impairment of endocytosis or trafficking does not delay neurodegeneration.

Indeed the beauty of the system is the use of defined dominant mutations causing neurodegeneration. It appears that current technology would allow for the inducible expression of these dominant alleles in adult tissues thus providing a more precise and relevant model system for careful genetic studies, as well as for cell biology based follow up examinations.
We appreciate the recommendation for a follow-up study using inducible expression of dominant alleles. We have previously attempted to develop such a system for the dominant *mec-4(d)* allele based on the *smg-1* induction system and the heat inducible *hsp-16* promoter. However, we observed that the leaky expression allowed under non-induced conditions by currently available methodologies, precludes their use in real practice (see also Harbinder et al. 1997, *PNAS* 94: 13128-33; and Link 2006, *Exp Gerontol* 41: 1007-13 for a discussion about the caveats of these systems). Unfortunately, although attempts have been made, a Tet-on/off system for inducible expression of transgenes has yet to be fully implemented in *C. elegans.*

We are very much concerned as to the use of Sodium Azide to mimic hypoxia, and as to the correlation of the extent of necrosis induction, with the level of lethality. It appears that the authors aught to use hypoxia, and aught to show that this causes necrosis, suppressible by mutations in endocytosis pathways, analogous to the study by Scott et al., 2002 they cite. Effects observed upon using Sodium Azide might be an indirect consequence of altered uptake, and or efflux, or removal likely affected in membrane traffic and neuronal mutants. Indeed many of the endocytosis mutants used have pharyngeal pumping defects that may affect the NaN3 internalization.
Per the suggestion of the Referee, we have now repeated the hypoxia experiments using low oxygen concentration in a hypoxic chamber as described previously in Scott et al., 2002 *Science* 296: 2388-91. We found that, similarly to our observations with sodium azide, mutations in three different endocytotic genes, *snt-1*, *unc-57* and *unc-11* significantly protect from animal death induced by hypoxia (data are shown in Supplementary Figure S3A).

The suppression of MEC-4 (d) induced neurodegeration, could be caused be reduced MEC-4 (d) expression, altered localization and many other mechanisms. The authors provide low-resolution images and suggest the level of MEC-4 expression is not alerted in endocytosis mutants. It would help to have higher resolution pictures and the quantification of relative expression levels, and to nevertheless more carefully discuss the caveats of their approach. For instance, it is possible that perturbing membrane traffic via deletion of snt-1 reduces MEC-4(d) levels at the plasma membrane and it is important that the authors show that this is not the case.
Following the suggestion of the Referee, we have repeated the imaging experiments and provide higher quality images (shown in Figures 1 & 5). We also monitored and quantified the expression, stability and correct localization of the toxic MEC-4 ion channel in live animals by using a full length GFP reporter. We found that none of the above is affected in the endocytosis mutants we examined (the quantification data are also included in Figures 1 & 5).

To further investigate endocytic processes in necrosis the authors visualize, via GFP fusion, the following proteins APS-2, the sigma subunit of the AP-2 complex, RAB-5, a early endosome marker, and RAB-11, a recycling endosome marker. APS-2::GFP is used as a marker of clathrin coated pits and vesicles. However, as AP-2 is not essential for Clathrin mediated endocytosis it is recommended that the authors repeat their experiments using a fluorescent clathrin and show that AP-2 punctae indeed represent CME.
Prior to the submission of our manuscript, we had considered and also attempted to use a fluorescent clathrin reporter, as the Referee suggests, to visualize and monitor clathrin-mediated endocytosis. However, this proved to be an ineffective and non-specific approach for the following reason: It has been shown that Ca2+ promotes the oligomerization of clathrin light chains (Nathke et al. 1990 *J Biol Chem* 265: 18621-7). Because cytoplasmic Ca2+ concentration is highly increased during necrotic cell death, fluorescent puncta do not specifically correspond to clathrin coated pits and vesicles, but also to clathrin light chain oligomers. To avoid this caveat, we used the more specific AP2 reporter. We note that AP2 (the σ subunit) has been tagged with a fluorescent protein and used equivalently with clathrin for monitoring of clathrin coated pits in living cells (Ehrlich et al. 2004, *Cell* 118: 591-605). The AP2 adaptor complex consists of two large subunits (α and β2), one medium (μ) and one small (σ) subunit, and serves to link the clathrin coat, through the hinge region of β2 with proteins to be endocytosed (Shih et al. 1995 *J Biol Chem* 270: 31083-90). Thus, an AP2 reporter is a reliable and more specific indicator of clathrin coated pits under conditions of necrosis where elevated Ca2+ concentration interferes with a clathrin-based reporter.

The authors need to establish higher resolution imaging to ensure that they are adequately measuring the number of AP-2 punctae (scale bars). The provided images (Figure 2A, B, C) suggest that AP-2 punctae are of the same size as endosomes,which appears unusual. The authors would learn much more about the involvement of endocytosis in neurodegeration with real time imaging studies, as this is a very dynamic process and the counting of ill defined punctae might be more mis-leading than leading.
We have now repeated the analysis and provide better quality images. We note that the size and morphology of the puncta we observe is typical and comparable with that of previous reports in the literature (see for example Shi et al. 2009, *EMBO J* 28: 3290-302; in our case, a higher magnification is used to look inside individual neurons). In addition, we would like to clarify that the images in Figure 2 are only provided as representative. The actual quantification shown in the graphs is performed by counting fluorescent puncta in all six touch receptor neurons of more than one hundred animals (>600 measurements in each case; described in the Materials and Methods).

Hence, our approach is more stringent that what is typically practiced in the literature (Ruck et al. 2011, Autophagy 7: 386-400; Sato et al. 2005, Nat Cell Biol 7: 559-69; Samara et al. 2007, *Cell Death Differ* 15: 105-112). We also note that to our knowledge, this is the first time endocytic processes are monitored *in vivo*, during the course of necrotic neurodegeneration. Given the stochastic incidence of necrosis in our model, we performed a horizontal analysis, averaging over a large number of animals, to obtain statistically reliable data.

In addition the authors need to be more precise in their description of membrane traffic in the introduction, for example clathrin coated vesicles, upon dissociation of clathrin, are described as 'become[ing] an early endosome', rather than fusing with early endosomes, as is the case.
We have now revised the introduction and rephrased the corresponding sentences.

2nd Editorial Decision 30 October 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked referee #1 and 3 to review the revised version and I have now received their comments.

As you can see below, the referees appreciate the introduced changes and are supportive of publication here. Referee #3 has a few remaining concerns that can be addressed with appropriate changes in the discussion and by further clarifying how some of the experiments were done. Once we receive the revised version, we will proceed with the acceptance of the paper for publication here.

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

the authors have adequately addressed the comments raised by the referees on this manuscript. The material convincingly reveals an important finding, namely the contribution of the edocytotic and intracellular trafficking systems to necrotic cell death in C. elegans. This certainly has significant medical implications. I now find the manuscript suitable for publication in EMBO Journal.

There is only a single minor discrepancy that the authors should explain before the acceptance. They have silenced VPS-34 and found no effect on necrotic cell death. However, vps-34 (which encodes a type III PI3 kinase) is an essential component of the autophagic machinery, and autophagy has also been found to contribute to necrotic cell death in this organism.

My suggestion for the title:
Endocytosis and intracellular trafficking contribute to necrotic cell death in C. elegans.

Referee #3:

Overall the authors appeared to address most of the questions and concerns we had. However, there is once major point, which is still not clear to us.

In essence, taken the data provided, and the description of the experimental systems, we are still not entirely convinced that counting the number of necrotic neurons, at a give stage of development is a precise measurement of the extend of neurodegeneration. It was published that necrotic corpses are eliminated (like apoptotic corpses) by the engulfment machinery (Driscoll and Hengartner labs, 2000, Nature Cell Biology). This is clearly consistent with Supplementary Figure 1, were a decrease in the number of corpses in mec-4 mutants is shown, as worms progress into later stages of larval development. Thus, given that in most experiments the number of necrotic neurons is scored in the late L1 stage, it is not clear to us, if by that stage some necrotic neurons already disappeared, thus

confounding the interpretation of the entire dataset. In line with this argument, I think I am right to assume that some of the authophagy mutants analyzed might develop slower than wild type. Thus, if there were to be more time to necrose (before counting late stage L1 worms), merely counting the number of necrotic neurons at the late L1 stage would again confuse the interpretation of data

We are not entirely convinced that Figure 2 adds much to the paper.

In general, the data make sense if results are interpreted the way the authors interpret them. Obviously, we can not be absolutely sure, even if excessive additional experiments were to be done, that the data are indeed correctly inteprted. Thus, more care needs to needs to be taken, to at least mention this in the discussion. Other reviewers, raised and the athors responded to the issue of the dominant MEC-4 not being properly expressed in some of the endoytosis and trafficking mutants. The authors provide plausible arguments that this is not altered by we can not be absolutely sure. Similarly the reduction in 6OHDA sensitivity might be explained by those membrance trafficking mutants directly or indirectly affecting the DAT-1 dompamine trasnporter, which is required to take up 6OHDA into dopaminergic neurons, to cause their degeneration.

2nd Revision - authors' response 07 November 2011

We would like to thank you and the Referees for your comments and suggestions. We have now revised our manuscript to address these points.

Per the suggestion of the first Referee we have changed the title to: “Endocytosis and intracellular trafficking contribute to necrotic neurodegeneration in C. elegans”. We also comment on the involvement of vps-34 in autophagy.

With regard to the comments of the third Referee, we note that we have used previously published and widely used methodologies for assessing and quantifying necrotic cell death in *C. elegans*. We now provide detailed description of the implemented experimental procedures in the Materials and Methods section.

The Referee also points out a potential caveat, related to mutants showing a developmental delay. We generally observe that in such mutants, necrosis, which follows the onset of expression of the neurotoxic *mec-4(d)* allele, is also delayed accordingly. Thus, still neurons undergo necrosis during the (now slightly delayed) L1 stage. In fact, cell death commences during embryogenesis for some neurons. We also monitored necrosis in embryos and confirmed that necrosis suppression is not merely due to cells dying earlier than the assay point in developmentally delayed mutants.

Finally, regarding the 6-OHDA experiment and the effects of endocytosis and trafficking on the surface expression of the dopamine transporter DAT-1, we now include a relevant note in the text. The Referee suggests that suppression of necrosis by endocytosis or trafficking defects might be due to impaired plasma membrane surface expression of DAT-1 and consequent lower uptake of the neurotoxin. As we explain, although we cannot exclude this possibility, we consider it unlikely because these mutants do not display phenotypes associated with defective dopaminergic signalling, and the localization of other membrane proteins such as MEC-4 is not affected.

