TORC2 controls dendritic tiling of Drosophila sensory neurons via the Tricornered kinase signaling

Makiko Koike-Kumagai, Kei-ichiro Yasunaga, Rei Morikawa, Takahiro Kanamori

Corresponding author: Kazuo EMOTO, National Institute of Genetics, submitted by Christopher

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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 03 August 2009

Thank you for submitting your manuscript to the EMBO Journal. Please find enclosed the comments of two of the three reviewers whom we had asked to evaluate your manuscript for EMBO Journal. We are still waiting for a third report, but given the present majority recommendation I can make a preliminary decision now to save time. This decision is still subject to change should the third referee offer strong and convincing reasons for doing so. As you can see, both referees find the analysis very interesting and insightful. They raise a number of different issues with the manuscript that I would expect you to be able to resolve. Given these positive evaluations, I would therefore like to ask you to start thinking about making the requested changes and additions to the manuscript that would render the paper suitable for publication in the view of these two reviewers. 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 included in the next, final version of the manuscript. We will forward the comments of the third referee to you as soon as we receive them, together with our final editorial decision.

Thank you for the opportunity to consider your work for publication.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):
This is an interesting manuscript describing the function of the target of rapamycin complex (TORC2) in the self-avoidance and dendritic tiling of Drosophila sensory neurons. Self-avoidance and tiling among dendrites are essential for complete and non-redundant representation of sensory information. Several genes including NDR kinase Tricornered and Furry have been previously shown to be required for establishing dendritic tiling in Drosophila dendritic arborization (da) neurons. In this manuscript, the authors showed that components of the TORC2 are required for self-avoidance and tiling of class IV da neuron dendrites, whereas TORC1 is important for dendritic growth and branching. The authors further nicely showed that TORC2 components physically and genetically interact with Trc and phosphorylate Trc on a residue that is critical for Trc activity in vivo and in vitro. A membrane-tethered (dominant active) form of Trc can suppress phenotypes caused by mutations in TORC2 components. These data suggest that TORC2 activates Trc signaling pathway to regulate the dendritic tiling of class IV da neurons.

Overall, the data in this manuscript are well presented and of high quality. The advance is highly significant for both dendrite tiling and for linking TORC2 and Trc/NDR kinase pathways. I recommend publication after the authors address the following points.

What are the endogenous expression pattern of TORC2 and TORC1 components? Are they expressed in all da neurons or only in class IV da neurons? What is the sub-cellular localization of these proteins?

Are TORC2 components involved in self-avoidance or tiling in other classes of da neurons? Trc and Fry are required for tiling in all classes of da neurons and self-avoidance only in class IV da neurons. Does TORC2 also phenocopy trc and fry in other classes of da neurons?

3. The relationship between TORC2 and Hpo in regulating Trc is unclear. Both lead to phosphorylation on Thr449 and activation of Trc. So I do not understand how "TORC2 and Hpo appear to control Trc kinase activity through independent pathways" (p.17).

Referee #3 (Remarks to the Author):

In this manuscript Emoto and colleagues aimed at identifying molecular pathways involved in dendrite self-avoidance and tiling, following on their previous work. While self-avoidance is likely a general mechanism, tiling is a property of subsets of neurons that non-redundantly cover a sensory field. In a genetic screen they have noticed that dendritic self-avoidance was impaired in sin1 mutants. They set out to verify whether sin1 binding partners are also involved and found that another member of the TORC (target of rapamycin complex)2, rictor, shares a similar phenotype. They provide evidence that -while TORC1 regulates extension and branching, components of TORC2 are involved in self-avoidance and tiling. Finally, they investigate whether a previously identified regulator of self-avoidance and tiling, the NDR family kinase Trc, is part of the TORC2 pathway. The authors present biochemical and genetic data supporting the idea that Trc phosphorylation and activity require both the previously known Hippo and the TORC2. This manuscript is interesting and the biochemistry solid. The authors' conclusions are mostly convincing, but some of the genetics needs stronger support.

Major comments:
1. Page 7. The conclusion that the tiling defects observed are due to loss of sin1 function is out of place at this position, since the only line of evidence shown at this point is the phenotype in a single homozygous and hemizygous mutant. This is no solid evidence that the observed defect is really due to loss of sin1. The authors present two pages later the cell-autonomous rescue of MARCM clones, which is good. Did they attempt the rescue in the mutants? They should present those data at Page 7. Is the level of sin1 protein or transcript reduced in the sin1PiggyBac line (or the appropriate reference cited or could they include the description of the insertion position ...)? Did the authors test alternative alleles (if existing) for complementation? Did the authors attempt to precisely excise the transposon? More information will be needed.
2. Page 7. While the text and the quantifications state that the number of branch points in class IV neurons is reduced in sin1 and rictor mutants, the provided images (Fig 1B and C) seem to suggest the opposite (especially for rictor). Can the authors explain this?
3. Page 10. Quantifications of the occurrence of tiling defects in Figure 2 should be presented. What
are penetrance and expressivity?

4. Page 10. In Figure 2C, D the presented example is not very well chosen since the only red branch that is obviously invading the blue field is not really easy to follow. Furthermore, all arrows in this figure point to self-avoidance defects, instead of defects in hetero-neuronal tiling, as mentioned in the text. This should be corrected and the hetero-neuronal overlaps indicated.

5. Page 12. The authors have previously shown that Trc mutants not only have tiling defects but also a significant increase of branch number. It would be important in the current manuscript to verify whether only tiling or also this other aspect of trc function is related to the TORC2 complex. What happens to branching in trans-heterozygous combinations? If mutating TORC2 or Trc results in opposite effects on branching (see also point 2), this should be also commented in the discussion in terms of Trc regulation.

6. Page 12. The control for the Sin1-Flag IP provided by Warts should be included in the presented data.

7. The biochemical evidence showing that NDR1 complexes with Rictor and Sin1 in HeLa cells is convincing. It is an interesting observation that NDR1 binds to the TORC2 complex in HeLa cells. It is a pity, though, that it is not done in flies. The authors state that appropriate antibodies are not available. But could they repeat the same experiment shown in Fig4G also for rictor and for Tor? 

8. Page 18. The phenotype of expression of Trc-myr and of the rescue with this construct of the trc mutant phenotype should be shown.

9. Fig. 4F. The graph should present the number of crossing points per branch length as in the other cases, so that the numbers can be compared.

10. The definition of tiling is quite confusing throughout the manuscript. In the introduction hetero-neuronal tiling is introduced as tiling, but then a good part of the data deals with iso-neuronal tiling. I suggest switching from iso-neuronal tiling to self-avoidance, which is more used in the recent literature. A definition of both tiling (hetero-neuronal) and self-avoidance should be included in the intro (rather than at page 9) and the distinction maintained throughout the text. It is also interesting that while hetero-neuronal tiling is a property of a subset of neurons, self-avoidance could be a more general phenomenon.

Minor comments

1. The authors only describe the class IV neuron phenotype. Did they not look at other da classes?

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6. The materials and methods are quite short: additional information about several techniques or reference to previous papers will be required (e.g. how was the phalloidin staining done? is the protocol for S2 cell spreading on concavalin described anywhere in the methods? I did not find it. At the least the appropriate citation should be referred to).

Additional Correspondence

25 August 2009

I just want to let you know that I never did receive the third report on your study and at this stage we will just go ahead with the two reports that we have.

Looking forward to seeing the revised version.

Best wishes

Editor
We would like to thank the Reviewers and the Editor for their positive and valuable comments on our paper. Following Reviewers’ suggestions, we have examined the tissue and cellular localization of TORC1 and TORC2 components. We have also added several data as we described in the point-to-point responses in detail. We have made the corresponding changes in the Results and Discussion.

Point-to-point responses to Reviewers’ comments

Referee #1:
"This is an interesting manuscript describing the function of the target of rapamycin complex (TORC2) in the self-avoidance and dendritic tiling of Drosophila sensory neurons. Self-avoidance and tiling among dendrites are essential for complete and non-redundant representation of sensory information. Several genes including NDR kinase Tricornered and Furry have been previously shown to be required for establishing dendritic tiling in Drosophila dendritic arborization (da) neurons. In this manuscript, the authors showed that components of the TORC2 are required for self-avoidance and tiling of class IV da neuron dendrites, whereas TORC1 is important for dendritic growth and branching. The authors further nicely showed that TORC2 components physically and genetically interact with Trc and phosphorylate Trc on a residue that is critical for Trc activity in vivo and in vitro. A membrane-tethered (dominant active) form of Trc can suppress phenotypes caused by mutations in TORC2 components. These data suggest that TORC2 activates Trc signaling pathway to regulate the dendritic tiling of class IV da neurons."
Overall, the data in this manuscript are well presented and of high quality. The advance is highly significant for both dendrite tiling and for linking TORC2 and Trc/NDR kinase pathways. I recommend publication after the authors address the following points."

1. What are the endogenous expression pattern of TORC2 and TORC1 components? Are they expressed in all da neurons or only in class IV da neurons? What is the sub-cellular localization of these proteins?

We attempted to made polyclonal antibodies against Drosophila Sin1 and Raptor, but unfortunately we did not obtain good antibodies working for tissue staining. Therefore, we examined the tissue localization of sin1 (TORC2) and raptor (TORC1) mRNA in the late embryonic stage by using in situ hybridization. The results indicate that both sin1 and raptor are expressed ubiquitously in all tissues with little difference in the expression patterns. To examine the cellular localization of Sin1, we expressed Sin-Flag in class IV neurons by using ppkGal4 and detected the Sin1-Flag protein by anti-Flag antibodies. Sin1-Flag is localized in the soma as well as the processes including axon and dendrites. These data are shown in Supplementary Figure S1.

2. Are TORC2 components involved in self-avoidance or tiling in other classes of da neurons? Trc and Fry are required for tiling in all classes of da neurons and self-avoidance only in class IV da neurons. Does TORC2 also phenocopy trc and fry in other classes of da neurons?

This is a very interesting point. We have to fist mention that our previous studies indicate that Trc and Fry are essential for both iso-neuronal and hetero-neuronal tiling of class IV dendrites but are dispensable for self-avoidance of other da neurons (Emoto et al, 2004). Similar to Trc and Fry, TORC2 is essential for both iso-neuronal and hetero-neuronal tiling of class IV dendrites but not for self-avoidance of other da neurons. For example, dendritic branches of sin1 and rictor class I MARCM clones do not cross each other (Supplementary Figure S2). Thus, in terms of the tiling phenotypes, TORC2 mutants phenocopy trc/fry mutants.

One phenotypic difference between trc/fry and TORC2 is that TORC2 mutants show a reduced branching phenotype (~80 % of wild-type control) whereas trc and fry mutants show an increased branching phenotype. Although it remains unclear why TORC2 and trc/fry mutants show the opposite branching phenotype, this might be due to reduction of Akt activity in TORC2 mutants. Previous studies have demonstrated that Akt is required for dendrite growth/branching in cultured neurons (Jaworski et al, 2005). Our RNAi experiments in S2 cells indicate that TORC2 is likely
essential for both Trc and Akt activities (Supplementary Figure S6). Therefore, TORC2 mutations might cause the combined defects of trc and Akt mutations, resulting in a slight reduction of the branch points. We have added this discussion in the Discussion (page 24, line 13).

3. The relationship between TORC2 and Hpo in regulating Trc is unclear. Both lead to phosphorylation on Thr449 and activation of Trc. So I do not understand how "TORC2 and Hpo appear to control Trc kinase activity through independent pathways" (p.17).

We agree that "through independent pathways" is confusing presentation. Our data indicate that TORC2 and Hpo are both required for Trc phosphorylation on Thr449 (Figure 3). In the previous paper, we have shown that Hpo directly phosphorylates Trc on Thr449 in vivo and in vitro (Emoto et al, 2006). In addition, our RNAi experiments in S2 cells suggest that TORC2 does not directly regulate Hpo kinase activity and vice versa (Figure 5). Based on these results, we speculate that TORC2 regulates the Hpo-dependent Trc phosphorylation in an indirect manner. For example, TORC2 may function in recruitment of Trc to the plasma membrane because a recent paper indicates that the membrane translocation is crucial for Trc phosphorylation by Hpo (Hergovich et al, 2005). Alternatively, TORC2 may promote Trc assembly with Mobs by phosphorylating Mobs. Therefore, we have changed "TORC2 and Hpo appear to control Trc kinase activity through independent pathway" to "Trc may regulate the Hpo-dependent Trc phosphorylation in an indirect manner. (page 25, lane 10).

Referee #3:

"In this manuscript Emoto and colleagues aimed at identifying molecular pathways involved in dendrite self-avoidance and tiling, following on their previous work. While self-avoidance is likely a general mechanism, tiling is a property of subsets of neurons that non-redundantly cover a sensory field. In a genetic screen they have noticed that dendritic self-avoidance was impaired in sin1 mutants. They set out to verify whether sin1 binding partners are also involved and found that another member of the TORC (target of rapamycin complex)2, rictor, shares a similar phenotype. They provide evidence that -while TORC1 regulates extension and branching, components of TORC2 are involved in self-avoidance and tiling. Finally, they investigate whether a previously identified regulator of self-avoidance and tiling, the NDR family kinase Trc, is part of the TORC2 pathway. The authors present biochemical and genetic data supporting the idea that Trc phosphorylation and activity require both the previously known Hippo and the TORC2. This manuscript is interesting and the biochemistry solid. The authors' conclusions are mostly convincing, but some of the genetics needs stronger support."

Major comments:

1. Page 7. The conclusion that the tiling defects observed are due to loss of sin1 function is out of place at this position, since the only line of evidence shown at this point is the phenotype in a single homozygous and hemizygous mutant. This is no solid evidence that the observed defect is really due to loss of sin1. The authors present two pages later the cell-autonomous rescue of MARCM clones, which is good. Did they attempt the rescue in the mutants? They should present those data at Page 7. Is the level of sin1 protein or transcript reduced in the sin1PiggyBac line (or the appropriate reference cited or could they include the description of the insertion position ...)? Did the authors test alternative alleles (if existing) for complementation? Did the authors attempt to precisely excise the transposon? More information will be needed.

Following the reviewer’s suggestion, we attempted the genetic rescue in sin1 and rictor mutants by neuronal expression of sin1 and rictor, respectively, and found significant rescue of the tiling defects in both mutants. We have added these data in Figure 1D-F and Figure 2G. We have also cited the appropriate reference about the sin1 PiggyBac line (Hietakangas and Cohen, 2007). As far as we searched extensively, there is no other available sin1 allele. To strengthen the genetic evidence that the PBc insertion in the sin1 locus indeed causes the tiling defects, we examined the trans-heterozygous combinations of sin1PiggyBac and a small chromosomal deficiency (Df) that uncovers sin1 and gene observed an identical dendritic tiling defects (Figure 1F). Taken together with the genetic rescue data in mutants and MARCM clones, we believe that we have presented enough genetic evidence to argue that the PBc insertion in sin1 locus causes the tiling phenotype in class IV neurons.
2. Page 7. While the text and the quantifications state that the number of branch points in class IV neurons is reduced in sin1 and rictor mutants, the provided images (Fig 1B and C) seem to suggest the opposite (especially for rictor). Can the authors explain this?

We had the same impression when we first looked at these images. However, in our non-biased quantifications (we carried out them in blind), the branch point number in sin1 and rictor mutants is reduced to ~80 % of that in wild-type control, whereas the total branch length is not significantly changes. Probably, because sin1 and rictor mutants have many crossing points between the terminal branches, we may have the impression that TORC2 mutants would have more branch points than wild-type control. Actually, the branch points in the wild-type image (Figure 1A) is "81" whereas those in sin1 (Figure 1B) and rictor (Figure 1C) are "63" and "71", respectively, which are almost corresponding to the average (~80 %).

3. Page 10. Quantifications of the occurrence of tiling defects in Figure 2 should be presented. What are penetrance and expressivity?

We have shown the quantification data in Figure 2G. The penetrance of the hetero-tiling phenotype is almost same as the iso-neuronal tiling phenotype.

4. Page 10. In Figure 2C, D the presented example is not very well chosen since the only red branch that is obviously invading the blue field is not really easy to follow. Furthermore, all arrows in this figure point to self-avoidance defects, instead of defects in hetero-neuronal tiling, as mentioned in the text. This should be corrected and the hetero-neuronal overlaps indicated.

Following the comments, we have replaced the Figure 2C with a better representative image. Also, we have changed the arrow positions (Figure 2A-F).

5. Page 12. The authors have previously shown that Trc mutants not only have tiling defects but also a significant increase of branch number. It would be important in the current manuscript to verify whether only tiling or also this other aspect of trc function is related to the TORC2 complex. What happens to branching in trans-heterozygous combinations?. If mutating TORC2 or Trc results in opposite effects on branching (see also point 2), this should be also commented in the discussion in terms of Trc regulation.

We agree that this is a very important point. As the reviewer pointed out, TORC2 mutants show a reduced branching phenotype (~80 % of wild-type) whereas trc/fry mutants show an increased branching phenotype. Although it remains unclear why TORC2 and trc mutants show the opposite branching phenotype, this might be due to reduction of Akt activity in TORC2 mutants. Previous studies have demonstrated that Akt activity is required for dendrite growth/branching in cultured neurons (Jaworski et al, 2005). Our RNAi experiments in S2 cells indicate that TORC2 is likely essential for both Trc and Akt activities (Supplementary Figure S4). Therefore, TORC2 mutations might cause the combined defects of trc and Akt mutations, resulting in a slight reduction of the branch points. Indeed, no branching phenotype was observed in trans-heterozygous combinations. We have added this point in the Discussion (page 24 line 13).

6. Page 12. The control for the Sin1-Flag IP provided by Warts should be included in the presented data.

We have added this data in Figure 4G.

7. The biochemical evidence showing that NDR1 complexes with Rictor and Sin1 in HeLa cells is convincing. It is an interesting observation that NDR1 binds to the TORC2 complex in HeLa cells. It is a pity, though that it is not done in flies. The authors state that appropriate antibodies are not available. But could they repeat the same experiment shown in Fig4G also for rictor and for Tor?

To answer this question, we obtained two different antibodies against Tor from other labs. Unfortunately, however, we have eventually realized that these antibodies did not work at least by our hands. Therefore, at this moment, we could not show more data about the physical interactions between Trc and TORC2 components in Drosophila sensory neurons.
8. Page 18. The phenotype of expression of Trc-myr and of the rescue with this construct of the trc mutant phenotype should be shown.

*We have added the data in Supplementary Figure S4.*

9. Fig. 4F. The graph should present the number of crossing points per branch length as in the other cases, so that the numbers can be compared.

*We have corrected this point (Figure 4F).*

10. The definition of tiling is quite confusing throughout the manuscript. In the introduction hetero-neuronal tiling is introduced as tiling, but then a good part of the data deals with iso-neuronal tiling. I suggest switching from iso-neuronal tiling to self-avoidance, which is more used in the recent literature. A definition of both tiling (hetero-neuronal) and self-avoidance should be included in the intro (rather than at page 9) and the distinction maintained throughout the text. It is also interesting that while hetero-neuronal tiling is a property of a subset of neurons, self-avoidance could be a more general phenomenon.

*To our knowledge, "iso-neuronal tiling" and "self-avoidance " define different mechanisms (Gao, 2007): iso-neuronal tiling functions in class IV neurons to avoid crossing of homotypic branches in the same neurons, whereas self-avoidance functions in all da neurons to ensure the proper spacing of dendritic branches. In TORC2 mutants, although both iso-neuronal and hetero-neuronal tiling in class IV neurons are defective, class I dendrites did not show obvious self-avoidance defects (Supplementary Figure S2). Therefore, we feel that it is better to use the terms "hetero-neuronal tiling" and "iso-neuronal tiling" in this paper. Following the reviewerí's suggestion, we have defined the terms in the Introduction (page 5, lane 13).*

Minor comments
1. The authors only describe the class IV neuron phenotype. Did they not look at other da classes?

*This is an interesting point. As shown in the manuscript, TORC2 mutants show both iso-neuronal and hetero-neuronal tiling defects in class IV dendrites. In contrast, other da neurons such as class I neurons do not show obvious dendrite defect, although the branch number is slightly reduced compared to that of wild-type (Supplementary Figure S2). Therefore, TORC2 is required for iso-neuronal and hetero-neuronal tiling but not for self-avoidance. These TORC2 phenotypes are consistent with what observed in trc mutants (Emoto et al, 2004; Soba et al, 2007).*

2. Page 3. In the introduction the authors should summarize what is known about the composition of the TORC1 and 2 complexes in their system of analysis, Drosophila.

*We have added a brief introduction that TORC1 and TORC 2 are both evolutionarily conserved in the functions as well as the compositions (page 3, lane 12). As far as we know, no information has been reported about the composition and the function of TORC1 and TORC2 complex in Drosophila da sensory neurons.*

3. Page 7. The argument that the observed overlap of terminal branches is unlikely to derive from abnormal stratification because of the 10µm space in which they grow is not very compelling: how thick are the fine processes that are seen to overlap? With high z resolution confocal imaging it should be possible to resolve whether two crossing branches are separate or very close to one another.

*This is our wrong presentation. We have to describe as 1µm instead of 10 µm. The diameter of dendritic branches is ~1 µm in average, thus dendrites of da neurons innervate in a two-dimensional fashion between the epidermis and the underlying musculature. We have confirmed that the crossing branches are in contact directly with each other.*

4. Page 15 and 17. I am surprised that we are presented a total loss of OA-induced T449P signal both in TORC2 and in Hpo mutants. How do the authors explain this?
We repeated the same RNAi experiments several times, and the data were completely reproducible. Thus, the RNAi data indicate that TORC2 and Hpo are both required for Trc phosphorylation on Thr449 (Figure 3). In the previous paper, we have shown that Hpo directly phosphorylates Trc on Thr449 in vivo and in vitro (Emoto et al, 2004). In addition, our RNAi experiments in S2 cells suggest that TORC2 does not directly regulate Hpo kinase activity and vice versa (Figure 5E). Based on these results, we speculate that TORC2 regulates the Hpo-dependent Trc phosphorylation in an indirect manner. For example, TORC2 may function in the recruitment of Trc to the plasma membrane because a recent paper indicates that the membrane translocation is crucial for NDRI phosphorylated by MST (Hergovich et al, 2005). Alternatively, TORC2 may promote complex formation of Trc with Mobs by phosphorylating Mobs. We have added this point in the Discussion (page 25, lane 10).

5. The manuscript is in general nicely written and clear. The discussion and the abstract, though, appear not to have gone through careful proofreading in terms of grammar. This should be improved

We have improved the grammar issue by English edition.

6. The materials and methods are quite short: additional information about several techniques or reference to previous papers will be required (e.g. how was the phalloidin staining done? is the protocol for S2 cell spreading on concavalin described anywhere in the methods? I did not find it. At the least the appropriate citation should be referred to).

We have referred the appropriate citation (Rogers et al, 2003) in the Materials and Methods.
stronger line of evidence that sin1 is responsible comes from the rescue experiments: they should be mentioned here to support the conclusion that mutation in sin1 causes the observed phenotype.

Additional Correspondence 30 September 2009

Thank you so much for the email. We are very happy to learn that our paper is almost acceptable for the EMBO journal. As for the points by the Referee #3, it seems to us unnecessary to add the sentence into the text at page7, because we mention the same points in the latter part. Thus, we would like to proceed the things in the present form.