Rescue of Tropomyosin deficiency in Drosophila and human cancer cells by Synaptopodin reveals a role of tropomyosin α in RhoA stabilization

Jenny S Wong, Elizabeth Iorns, Michelle N Rheault, Toby M Ward, Priyanka Rashmi, Ursula Weber, Marc E Lippman, Christian Faul, Marek Mlodzik and Peter Mundel

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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 February 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the delay in getting back to you with a decision: this was primarily due to the holiday season and the consequent delay in sending the manuscript out for review and hence receiving the referees' comments. We have now received the comments from all three referees, which are enclosed below.

As you will see, all three referees express interest in the study, but all also raise significant concerns that would need to be addressed by a major revision before we could consider publication. In particular, both referees 1 and 3 comment on the confusion as to whether the effects you observe are specific to Tropomyosin 2, or also hold with other Tm isoforms. Referee 2 also highlights a number of areas where additional biochemical analysis is required: his/her points 1, 4 and 6 are particularly important. I would also highlight the concerns raised by the reviewers as to the presentation of the data (clear statements in the figure legends about statistical significance, n values etc are essential), and the need for a major re-write of the text - especially in terms of not overstating the conclusions.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the
community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on 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. Also, if you have any questions or comments about this revision, please don't hesitate to get in touch.

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

Previous work from other labs has shown that Oscar mRNA is mislocalized in Drosophila oocytes in lacking Tm2, and that in cancer cells the expression of Tm2 protein is downregulated. The report here shows that synaptopodin, a positive regulator of RhoA that induces stress fibers, and a smurf1-resistant mutant of RhoA that cannot be ubiquitinated can rescue the Tm-deficient phenotypes in both systems. The authors conclude that Tm2 induces stress fibers via its up-stream regulation of RhoA. The idea that Tm2 has a role in RhoA stabilization in cancer cells and that deficiencies in Tm2 can be rescued by synaptopodin is intriguing, novel, of general interest and potentially important. I was looking forward to reading this paper. However, there are numerous issues in the work as presented that make it difficult to know what was done, what was actually observed, how the data were analyzed and how reproducible the results are. While it may be that Tm2 has some role in the upstream regulation of RhoA, RhoA controls other proteins downstream that are involved in stress fiber assembly and function. The concluding statement in the abstract, "Our findings elucidate RhoA stabilization as the long sought mechanism by which Tm2 regulates actin dynamics." appears to be an overstatement, and is unrelated to what is well-known about direct stabilization of actin filaments by Tm. The paper is not about actin dynamics, at least in the usual parlance of the cytoskeleton field.

Major criticisms:

1. While expression of Tm2-shRNA does affect stress fiber assembly, as described, the experiments raise more questions than they answer. The authors state that the target of the Tm-specific shRNA is exon 4 of Tm2. Exon 4 is a ubiquitously-expressed exon encoded by the TPM1 gene (alpha-Tm), in the exon terminology used in Gunning's review. Therefore, it is likely that several transcripts in addition to Tm2 could be affected, including Tm3, a long form, as well as short forms. The antibody used (Sigma 311) recognizes an epitope expressed in long forms (correct reference for this is Nicholson-Flynn et al., 1996), so silencing of other forms would not be detected, but they could affect the cellular morphology as observed. The methods also refer to GIPZ Tm2 shRNA purchased from Open Biosystems; what is the sequence targeted to? It needs to be clear which experiment used which shRNA.

A further issue is that the authors do see down-regulation of what they call Tm1 (the upper band in Fig. 3B. If it is Tm1 (the form expressed in the clone provided by Gunning and used for the experiments with purified proteins in Figure 6A), it would be detected by the antibody, but it should not be downregulated by the shRNA because it is encoded by a different gene (TPM2, beta Tm). The band in the gels is probably Tm3 that differs from Tm2 in the alternatively spliced exon 6; perhaps Tm1 is not expressed in these cells. I realize the nomenclature is confusing and the literature
is vast, but if the authors are prepared to make the statements put forth in the title and abstract, they will have to sort out what they are looking at. Assuming the band labeled Tm1 is actually Tm3 (pretty likely) then this form, or any of the others encoded by TPM1 could be involved; it is difficult to argue specificity; it could just be the overall level of Tm.

2. It is frequently difficult to evaluate the results, and therefore the major claims, for the following reasons. The figures are hard to follow. Figure 6 is particularly difficult. One suggestion would be to have more experimental details in the figures, and have the title of the figure relate to the experiments, rather than stating the authors' conclusions. In some cases the experimental details are insufficient. For example, none of the images have scale bars; the bar graphs give percentages, but no values of n. Standard (deviations or errors) were given on the basis of two experiments..... In other cases, maybe the experiments were done only once. In other instances, it is difficult to evaluate the claims based on the data shown. How were "cells with stress fibers" quantified? In Fig. 2B, the Synpo panel seems to have one cell that has more stress fibers than the cells in the other panels, yet in Fig. 2C, there is a 3-fold increase in the percent of cells with stress fibers. Similarly, the data shown in Figure 4G closure was 65% for wildtype and 48% for FLAG-RhoA(K6,7R) (measured directly from the images), perhaps within the errors shown in Fig. 4H, but not to inspire confidence.

Minor points:

1. Some other less important points: The use of FLAG and GFP constructs for Tm is convenient, but there is concern that the tags are on functionally important regions of the gene. This should be acknowledged.

2. The result on the half-life is convincing, but the reference may not be relevant as it is to a study of a sarcomeric Tm in cardiac cells.

3. The results with Drosophila are interesting, but do not relate to the rest of the paper, other than that may be what led them in the direction. The authors make the link between Tm2 in Drosophila and mammalian Tm2, but there is no relationship between them, other than their being long Tms. They should make this clear.

4. There are places where the citations are too general, where the original literature should be cited.

Referee #2

In this manuscript, Wong and colleagues describe that Synaptopodin, an actin binding protein, could rescue the phenotype of Tropomyosin 2 deficiency in Drosophila and MDA-MB 231, a human breast cancer cell line. It has been published that Synaptopodin stabilizes the protein level of RhoA and regulates actin network in podocytes by blocking Smurf1-mediated RhoA ubiquitination. The authors continued to investigate and propose a similar role of Tropomyosin 2 in preventing Smurf1-mediated RhoA degradation and thereby regulating actin dynamics. Overall, this is a very interesting paper that reports a novel observation and mechanism for Tm regulation of actin cytoskeletal dynamics. The experimental designs are well described and the acquired data are of good quality. However, the manuscript could be improved substantially, as detailed below, if the authors could provide more substantive molecular and biochemical experiments to support their interpretations and the proposed model.

Detailed comments:

1. Figure 2 and 3, Synaptopodin expression induces formation of stress fibers in MDA-MB 231 cells and Tropomyosin 2 knockout cells, respectively. Formally, the authors need to demonstrate that this effect is dependent on an increase of RhoA as the proposed model suggested. For example, knockdown of RhoA or the RhoA T19N mutant or a ROCK inhibitor should reverse the effect.

2. Figure 4 and 5, the authors also need to show that the effects of lactacystin on stress fiber induction are directly through RhoA, as in comment 1 above.

3. Figure 6A, the authors show that both Tm1 and Tm2 can bind RhoA. However, Figure 6C
suggests that Tm1 does not protect RhoA. This is surprising as Tm1 and Tm2 share similar primary sequence. In this case, Figure 6B should also test the effect of Tm1 on RhoA-Smurfl binding. It would also be very interesting and critical to identify the unique domain/motif in Tm2 that mediate its function in protecting RhoA.

4. The experiment in Figure 6C has a critical problem in the current design and needs to be improved – the HA-ubiquitin signal could be from a RhoA-associated protein or even Smurfl itself. A proper assay should include a two-step IP, first with anti-FLAG IP followed by a denaturing elution (to remove associated ubiquitinated proteins) and then a second anti-FLAG IP of RhoA. This will convincingly show that the HA-Ubiquitin conjugates they are observing are covalently linked to FLAG-RhoA as opposed to a RhoA-associated protein.

5. The level of RhoA protein can be regulated by the Ubiquitin-Proteasome system through multiple mechanisms. Besides Smurfs, the Cullin3-BACURD complex has also been shown to regulate RhoA degradation. Does Tm2 also affect BACURD/Cullin3 interaction with RhoA?

6. Figure 6D, a cycloheximide-based assay using ectopically overexpressed proteins may not faithfully reflect the turnover of the endogenous protein. A radioisotope-based pulse-chase experiment and/or examining endogenous protein would be more proper. Therefore there is not enough evidence to support the authors' speculations about the distinct mechanism of Synaptopodin and Tm2 in regulating RhoA and actin.

7. Figure S2A, the authors observed a Western blot signal of Tm in MDA-MB 231 cells that they think is neither Tm1 or Tm2. However, it may be a different transcriptional variant or a differentially modified form. Therefore this Tm isoform in MDA needs to be characterized, as it is critical to confirm that it is functionally deficient. In addition, overexpression of different Tm isoforms can be carried out to see which one(s) can rescue the actin phenotype.

Referee #3

The ms reports the rescue of the TM2 phenotype by expression of Synaptopodin (Synpo) both in Drosophila oocytes scoring for oskar mRNA localisation and in cells scoring at the formation of stress fibers. Synpo and TM2 both prevent ubiquitination of RhoA and leads to its stabilisation, thus allowing the formation of actin stress fibers used for migration and possibly osk mRNA localisation. This would need to be discussed as the long actin projections that have been described for osk loc were not reported to be stress fibers.

1) The title is very specific of TM2 and seems to discard TM1 but the situation of TM1 throughout the ms is vague. What's about TM1 phenotype in flies? Can TM1 rescue TM2? Is TM1 still present when TM2 is absent in both? It seems that it is the case in cancer cells (mentioned page 7) but should be shown as a control along with the expression of synaptopodin.
Does overexpression of Tm1 induce stress fibers formation in MDA-MB 231 cells? And overexpression of Tm2 induce stress fibers formation?

2) The situation is made more complicated by the experiment in Figure 3. Here, depleting TM2 from NIH3t3 cells also deplete TM1 to the same extent, so to me, whatever the effect of Synpo, it rescues both! Not only TM2 as the title states. Try to make another shRNA specific for TM2.

3) The manuscript is not very well written. There are too many technical details in the text as well as numbers (that could be directly incorporated to the quantitation in the figures themselves) and the biology is diluted and somehow hidden. The message is actually very hard to come across and when it appears, it is very strong (sometimes too much) and seem to come out of nowhere. For instance, beginning of page 10 "Similar to MDA-MB 231 cancer cells (Figure 4B, C), the lactacystin-induced upregulation of RhoA protein abundance (Figure 5A) was associated with the reappearance of stress fibers in Tm2 knockdown cells (Figure 5B). We next quantified the percentage of stress fiber containing Tm2 knockdown cells before and after lactacystin treatment (Figure 5C). We found nosignificant difference in the percentage of stress fiber containing cells between the wild type and control knockdown fibroblasts at 0 h and 16 h. In contrast, the percentage
of stress fiber containing Tm2 knockdown fibroblasts was significantly increased after lactacystin treatment (0h: 30.96 ± 4.33 % stress fiber containing cells versus 16 h: 63.86 ± 5.15 %; P < 0.05; Student's t-test; Figure 5C). The upregulation of stress fibers by lactacystin was independent of Tm2, because it did not change Tm2 protein abundance (Figure 5A)."

This could be summarized in one sentence and put the number on top of bars in appropriate figure, so that the conclusion is more borne but a proper biological plain english description of the results.

Same for the paragraph that follows: To confirm „First, this should start with a new line and again this is too lengthy and distracts the message. As a result, I find it difficult to review this article.

4) However, other statements are not enough documented. The overexpression of WT Rhoa versus Smurf resistant Rhoa is indeed shown in Figure 4D and 5E. Their level of expression is indeed higher than control cells (where it is surprisingly undetectable) but also different from one another showing that the resistant is indeed resistant. However, in both experiments, lactamycin should have been added to shown that the lower level of WT Rhoa could be rescued, suggesting that even the overexpressed version is degraded. Synpo should also have been transfected ands show that it also protects. This is important.

5) The text is not always very logical. For instance page 8, the authors state "The downregulation of Rhoa steady-state protein levels by Smurf1 mediated ubiquitination and proteasomal degradation controls protrusion formation at the leading edge of migrating cells (Wang et al, 2003) and prevents stress fiber formation in kidney podocytes (Asanuma et al, 2006). Together with the above-described findings, this raises the possibility that in the absence of Tm2, the loss of stress fibers in MDA-MB 231 results from the unopposed effects of Smurf1 on Rhoa degradation."

This calls immediately for the following questions: Is Rhoa more degraded in the absence of TM2, is it rescue by lactacystin, does Synpo does the same? This is shown in Figure 5 but instead the authors embark upon the description of the lactamycin effects in cancer cells (figure 4) and make a huge jump (and strong statement, page 9) linking the rescue of stress fibers by lactacystin and this observed by Synpo, through the stabilisation of Rhoa. However, the rescue of Rhoa stability by synpo is NOT shown and that it is not clear that it is the same mechanism. This needs to be done (crucial) and the order of figures changed. Fig5 comes before 4.

7) It seems to me that the blot in Figure 3B is flipped vertically when compared to blots in Figure 5A and E. In this 3B, the upper band for TM1 band is very strong and the lower band TM2 less strong whereas on 5A and C, the upper band is weak and the lower is strong. Although it is not super clear from the legends, the blots were generated from the same cells using the same antibody to blot.

8) Figure 6C: The text says that when synpo, TM1 and TM2 are expressed, the ubiquitination of WT Rhoa is reduced. Indeed the HA bands is less intense when compared to the control (lane 1). However the eluate is also less abundant (blot FLAG). My feeling is that the ratio HA versus FLAG is the same for all conditions. Therefore, this does NOT show that TM2 and Synpo lead to reduced Rhoa ubiquitination. This needs to be done again and quantitation of bands included. Plus it seems that TM1 has the same effect (if any) than TM2 (see my point 1)

9) It is not clear whether Synpo and TM2 act in two biochemical pathways or by forming a complex. Do they? How is the Smurf activity suppressed? Does it bind to Smurf? What is the mechanism to explain the rescue of TM2 (TM1?) by Synpo? It is the same in Drosophila? Is Rhoa the target?

Minor comments
1) Synpo seems to indeed rescue the stress fibers and reduce (not suppress) the migration as the effects are clear but small.

2) Figure 3: What is the nuclear staining observed for Synpo??

3) It is also obvious that it has been written by multiple groups as the way of referring to the materials in the figures changes. On various blots, TM2 and TM1 become tropo and tropomyosin.
4) There is also a large number of mistakes in referring to the figure number. For instance page 9, Fig3D should be 4A

5) Page 6 and Supplementary figure 1: It would be nice to see numbers of nos>synpo; Tm2eg9 females analysed. Also the numbers of egg chambers analysed per each genotype and in how many nos>synpo; Tm2eg9 egg chambers osk mRNA localization was rescued.

6) Page 6: Do eggs laid by nos>synpo; Tm2eg9 develop and hatch normally?

7) Page 7 and figure 2D: "Functionally, the restoration of stress fibers was associated with the suppression of cell migration..." data in the manuscript show there is a reduction of cell migration, but not a suppression.

8) Page 7: "...it may also rescue Tm2 deficiency in fibroblasts where...": the term "deficiency" not appropriate, depletion is better.

9) MG132 should also possibly be used in addition to Lactamycin

1st Revision - authors' response 05 June 2011

Point-to-point response to the Referees' critiques

Referee #1

Previous work from other labs has shown that Oscar mRNA is mislocalized in Drosophila oocytes in lacking Tm2, and that in cancer cells the expression of Tm2 protein is downregulated. The report here shows that synaptopodin, a positive regulator of RhoA that induces stress fibers, and a smurf1-resistant mutant of RhoA that cannot be ubiquitinated can rescue the Tm-deficient phenotypes in both systems. The authors conclude that Tm2 induces stress fibers via its up-stream regulation of RhoA. The idea that Tm2 has a role in RhoA stabilization in cancer cells and that deficiencies in Tm2 can be rescued by synaptopodin is intriguing, novel, of general interest and potentially important. I was looking forward to reading this paper. However, there are numerous issues in the work as presented that make it difficult to know what was done, what was actually observed, how the data were analyzed and how reproducible the results are. While it may be that Tm2 has some role in the upstream regulation of RhoA, RhoA controls other proteins downstream that are involved in stress fiber assembly and function. The concluding statement in the abstract, "Our findings elucidate RhoA stabilization as the long sought mechanism by which Tm2 regulates actin dynamics." appears to be an overstatement, and is unrelated to what is well-known about direct stabilization of actin filaments by Tm. The paper is not about actin dynamics, at least in the usual parlance of the cytoskeleton field.

Answer: We revised our concluding statement to down tone our overall conclusion. It now reads: “Our findings elucidate RhoA stabilization by structurally unrelated actin binding proteins as a conserved mechanism for the regulation of stress fiber dynamics and cell motility in a cell type specific fashion.”

Major criticisms:

1. While expression of Tm2- shRNA does affect stress fiber assembly, as described, the experiments raise more questions than they answer. The authors state that the target of the Tm-specific shRNA is exon 4 of Tm2. Exon 4 is a ubiquitously-expressed exon encoded by the TPM1 gene (alpha-Tm), in the exon terminology used in Gunning's review. Therefore, it is likely that several transcripts in addition to Tm2 could be affected, including Tm3, a long form, as well as short forms. The antibody used (Sigma 311) recognizes an epitope expressed in long forms (correct reference for this is Nicholson-Flynn et al., 1996), so silencing of other forms would not be detected, but they could affect the cellular morphology as observed. The methods also refer to GIPZ Tm2 shRNA purchased from Open Biosystems; what is the sequence targeted to? It needs to be clear which experiment used which shRNA.

A further issue is that the authors do see down-regulation of what they call Tm1 (the upper band in
If it is Tm1 (the form expressed in the clone provided by Gunning and used for the experiments with purified proteins in Figure 6A), it would be detected by the antibody, but it should not be downregulated by the shRNA because it is encoded by a different gene (TPM2, beta Tm). The band in the gels is probably Tm3 that differs from Tm2 in the alternatively spliced exon 6; perhaps Tm1 is not expressed in these cells. I realize the nomenclature is confusing and the literature is vast, but if the authors are prepared to make the statements put forth in the title and abstract, they will have to sort out what they are looking at. Assuming the band labeled Tm1 is actually Tm3 (pretty likely) then this form, or any of the others encoded by TPM1 could be involved; it is difficult to argue specificity; it could just be the overall level of Tm.

**Answer:** We greatly appreciate these insightful comments by the Referee. The GIPZ Tm2 shRNA (Open Biosystems, Catalog Number RMM4431-98754654, Clone ID V2LMM_25293 shRNA) is targeted to the following sequence: CACTACATATGTAATTGGT. When blasted, the shRNA matches isoforms TPM1, transcript variant 2, 3, 4, 6, and 7. This information is now included in the revised paper. We also added the correct reference, Nicholson-Flynn et al., 1996

We agree with the Referee that it is difficult to argue specificity and that it could just be the overall level of Tm. We now discuss this issue in the revised manuscript. Moreover, following the Referee’s suggestion, we removed the isoform labels from Figure 3B and supplementary Figure S2A.

2. It is frequently difficult to evaluate the results, and therefore the major claims, for the following reasons. The figures are hard to follow. Figure 6 is particularly difficult. One suggestion would be to have more experimental details in the figures, and have the title of the figure relate to the experiments, rather than stating the authors’ conclusions. In some cases the experimental details are insufficient. For example, none of the images have scale bars; the bar graphs give percentages, but no values of n. Standard (deviations or errors) were given on the basis of two experiments..... In other cases, maybe the experiments were done only once. In other instances, it is difficult to evaluate the claims based on the data shown. How were "cells with stress fibers" quantified? In Fig. 2B, the Synpo panel seems to have one cell that has more stress fibers than the cells in the other panels, yet in Fig. 2C, there is a 3-fold increase in the percent of cells with stress fibers. Similarly, the data shown in Figure 4G closure was 65% for wildtype and 48% for FLAG-RhoA(K6,7R) (measured directly from the images), perhaps within the errors shown in Fig. 4H, but not to inspire confidence.

**Answer:** As suggested by the Referee, we improved the flow of the figures by adding more experimental details in the figure, and modifying the title of the figure to better relate to the experiments. The “n” for all studies is given in the methods section.

Please note that while the images in the various figures are representative, all experiments were done at least 3 times and analyzed quantitatively to ensure statistical significance. This is indicated in the Results or Methods sections.

Stress fibers were counted manually in each individual cell in independent images. A stress fiber was defined as a phallolidin-positive structure that was represented by a line spanning across the length of the cell. This is now clearly stated in the revised Methods section.

**Minor points:**

1. Some other less important points: The use of FLAG and GFP constructs for Tm is convenient, but there is concern that the tags are on functionally important regions of the gene. This should be acknowledged.

**Answer:** The referee’s point is well taken. The full-length cDNAs for Tm1 and Tm2 were cloned into FLAG and GFP expression vectors as documented within the manuscript. By sequence verification we ensured that no functionally important regions were affected. We acknowledge and comment on the presence of the tags in the revised manuscript.

2. The result on the half-life is convincing, but the reference may not be relevant as it is to a study of a sarcomeric Tm in cardiac cells.

**Answer:** We agree with the Referee. We now mention more clearly in the revised manuscript that the obtained value was derived from the study of Tm in cardiac cells.

In addition, in response to a suggestion by Referee 2, we examined the effect of cycloheximide on the turnover of endogenous synaptopodin and tropomyosin. We found that the half-life of endogenous synaptopodin (t = 17.1 ± 2.4 h, **new Figure 7D**) in podocytes is very similar to that of exogenous synaptopodin (t = 14.6 ± 4.5 h) in HEK293 cells. The results of the studies with...
ectopically expressed proteins are now displayed in Supplementary Figure S3D.

3. The results with Drosophila are interesting, but do not relate to the rest of the paper, other than that may be what led them in the direction. The authors make the link between Tm2 in Drosophila and mammalian Tm2, but there is no relationship between them, other than their being long Tms. They should make this clear.

**Answer:** Clearly future studies will be required to establish more precisely the molecular link between Tm2 in Drosophila and mammalian Tm2. We added a statement in the revised paper to make this clear.

4. There are places where the citations are too general, where the original literature should be cited

**Answer:** We tried to replace more general citations with original literature and would be happy to receive additional suggestions for specific changes from the referee.

**Referee #2**

1. Figure 2 and 3, Synaptopodin expression induces formation of stress fibers in MDA-MB 231 cells and Tropomyosin 2 knockdown cells, respectively. Formally, the authors need to demonstrate that this effect is dependent on an increase of RhoA as the proposed model suggested. For example, knockdown of RhoA or the RhoA T19N mutant or a ROCK inhibitor should reverse the effect.

**Answer:** As suggested by the Referee, we now show that the ROCK inhibitor Y-27632 blocks synaptopodin induced stress fibers in tropomyosin knockdown fibroblasts (new Figure 5A, B) and MDA-MB 231 cells (new Supplementary Figure S3A, B).

2. Figure 4 and 5, the authors also need to show that the effects of lactacystin on stress fiber induction are directly through RhoA, as in comment 1 above.

**Answer:** We now show that the ROCK inhibitor Y-27632 blocks lactacystin induced stress fibers in tropomyosin knockdown fibroblasts (new Figure 5A, C) and MDA-MB 231 cells (new Supplementary Figure S3A, C).

3. Figure 6A, the authors show that both Tm1 and Tm2 can bind RhoA. However, Figure 6C suggests that Tm1 does not protect RhoA. This is surprising as Tm1 and Tm2 share similar primary sequence. In this case, Figure 6B should also test the effect of Tm1 on RhoA-Smurf1 binding. It would also be very interesting and critical to identify the unique domain/motif in Tm2 that mediate its function in protecting RhoA.

**Answer:** As suggested by Referee 1 and now discussed in the amended Discussion section, it is likely that the overall level of Tm rather than specific isoforms is critical for the induction of stress fibers by Tm. In keeping with this idea, a novel study by Tojkander and colleagues (Current Biology, 2011) shows that depletion of either Tm1, Tm2/3, Tm4 or Tm5NM1/2 compromises stress fiber formation. Thus, in the context of not focusing on a particular isoform in this revised manuscript, but rather on Tm levels as a whole, we believe it is most appropriate to investigate isoform specific effects (such as why overexpression of Tm1 does not prevent the ubiquitination of RhoA) in future studies, formally focused on the many Tm isoforms (Tm1, 2, 3, etc.).

4. The experiment in Figure 6C has a critical problem in the current design and needs to be improved - the HA-ubiquitin signal could be from a RhoA-associated protein or even Smurf1 itself. A proper assay should include a two-step IP, first with anti-FLAG IP followed by a denaturing elution (to remove associated ubiquitinated proteins) and then a second anti-FLAG IP of RhoA. This will convincingly show that the HA-Ubiquitin conjugates they are observing are covalently linked to FLAG-RhoA as opposed to a RhoA-associated protein.

**Answer:** The assay employed in Figure 6C (now Figure 7C) has been validated and published before by Dr. Wrana’s group (Wang et. al, Science, 2003: 302, 1775) and our group (Asanuma et al., Nat Cell Biol. 2006, 8: 485). Together with the data obtained from the rescue studies with Smurf1-resistant RhoA (Figure 4D, 6E) and the competitive inhibition of RhoA binding to Smurf-1 (Figure 7B), these data strongly support the notion that, like synaptopodin (Asanuma et al., Nat Cell Biol. 2006, 8: 485), tropomyosin also can block the Smurf-1 mediated ubiquitination and
proteasomal degradation of RhoA. Thus, the proposed two-step Co-IP would not add further information.

5. The level of RhoA protein can be regulated by the Ubiquitin-Proteasome system through multiple mechanisms. Besides Smurfs, the Cullin3-BACURD complex has also been shown to regulate RhoA degradation. Does Tm2 also affect BACURD/Cullin3 interaction with RhoA?

Answer: As Smurf1-resistant RhoA(K6,7R) is sufficient to rescue stress fibers in Tm2 depleted cells (Fig. 4D), Tm2 probably does not affect the Cullin3-BACURD mediated degradation of RhoA. This is now discussed in the revised Discussion section.

6. Figure 6D, a cycloheximide-based assay using ectopically overexpressed proteins may not faithfully reflect the turnover of the endogenous protein. A radioisotope-based pulse-chase experiment and/or examining endogenous protein would be more proper. Therefore there is not enough evidence to support the authors' speculations about the distinct mechanism of Synaptopodin and Tm2 in regulating RhoA and actin.

Answer: In response to this important concern, we examined the effect of cycloheximide on the turnover of endogenous synaptopodin and tropomyosin. We found that the half-life of endogenous synaptopodin (t = 17.1 ± 2.4 h, new Figure 7D) in podocytes is very similar to that of exogenous synaptopodin (t = 14.6 ± 4.5 h) in HEK293 cells. The results of the studies with ectopically overexpressed proteins are now displayed in Supplementary Figure S3D.

7. Figure S2A, the authors observed a Western blot signal of Tm in MDA-MB 231 cells that they think is neither Tm1 or Tm2. However, it may be a different transcriptional variant or a differentially modified form. Therefore this Tm isoform in MDA needs to be characterized, as it is critical to confirm that it is functionally deficient. In addition, overexpression of different Tm isoforms can be carried out to see which one(s) can rescue the actin phenotype.

Answer: While the precise molecular identity of the additional variant in Figure S2A is unknown, it is not sufficient to induce stress fibers in MDA-MB 231 cells. As suggested by Referee 1 and now discussed in the amended Discussion section, it is likely that the overall level of Tm rather than specific isoforms is critical for the induction of stress fibers by Tm. In keeping with this idea, a novel study by Tojkander and colleagues (Current Biology, 2011) shows that depletion of either Tm1, Tm2/3, Tm4 or TmSNM1/2 compromises stress fiber formation. We now discuss this issue in the revised manuscript.

Referee #3

The ms reports the rescue of the TM2 phenotype by expression of Synaptopodin (Synpo) both in Drosophila oocytes scoring for oskar mRNA localisation and in cells scoring at the formation of stress fibers. Synpo and TM2 both prevent ubiquitination of RhoA and leads to its stabilization, thus allowing the formation of actin stress fibers used for migration and possibly oskar mRNA localisation. This would need to be discussed as the long actin projections that have been described for osk loc were not reported to be stress fibers.

Answer: We added the suggested discussion to the revised manuscript.

1) The title is very specific of TM2 and seems to discard TM1 but the situation of TM1 throughout the ms is vague. What's about TM1 phenotype in flies? Can TM1 rescue TM2? Is TM1 still present when TM2 is absent in both? It seems that it is the case in cancer cells (mentioned page 7) but should be shown as a control along with the expression of synaptopodin. Does overexpression of Tm1 induce stress fibers formation in MDA-MB 231 cells? And overexpression of Tm2 induce stress fibers formation?

Answer: In response to this comment and a similar critique by referee 1, we revised the title, which now reads” Rescue of Tropomyosin deficiency in Drosophila and human cancer cells by synaptopodin reveals a role of tropomyosin in RhoA stabilization”. In flies, TM1 is not sufficient to rescue the phenotype of Tm2 mutants. As described in the introduction, the loss of stress fibers NIH3T3 cells and MDA-MB-231 breast cells can be rescued by the reintroduction of Tm1 (Bharadwaj et al, 2005b; Prasad et al, 1993). Tm2 can also cooperate with TM1 to restore stress fibers in DT cells (Shah et al., Somat Cell Mol Genet. 1998, 24: 273-280).
2) The situation is made more complicated by the experiment in Figure 3. Here, depleting TM2 from NIH3T3 cells also deplete TM1 to the same extent, so to me, whatever the effect of Synpo, it rescues both! Not only TM2 as the title states. Try to make another shRNA specific for TM2.

**Answer:** We agree with Referees 1 and 3 that it is difficult to argue isoform specificity and that it could just be the overall level of Tm in the cultured cells. In keeping with this idea, a novel study by Tojkander and colleagues (Current Biology, 2011) show that depletion of either Tm1, Tm2/3, Tm4 or Tm5NM1/2 compromises stress fiber formation. We now discuss this issue in the revised manuscript.

3) The manuscript is not very well written. There are too many technical details in the text as well as numbers (that could be directly incorporated to the quantitation in the figures themselves) and the biology is diluted and somehow hidden. The message is actually very hard to come across and when it appears, it is very strong (sometimes too much) and seems to come out of nowhere.

For instance, beginning of page 10 "Similar to MDA-MB 231 cancer cells (Figure 4B, C), the lactacystin-induced upregulation of RhoA protein abundance (Figure 5A) was associated with the reappearance of stress fibers in Tm2 knockdown cells (Figure 5B). We next quantified the percentage of stress fiber containing Tm2 knockdown cells before and after lactacystin treatment (Figure 5C). We found no significant difference in the percentage of stress fiber containing cells between the wild type and control knockdown fibroblasts at 0 h and 16 h. In contrast, the percentage of stress fiber containing Tm2 knockdown fibroblasts was significantly increased after lactacystin treatment (0h: 30.96 ± 4.33 % stress fiber containing cells versus 16 h: 63.86 ± 5.15%; P < 0.05; Student’s t test; Figure 5C). The upregulation of stress fibers by lactacycin was independent of Tm2, because it did not change Tm2 protein abundance (Figure 5A)." This could be summarized in one sentence and put the number on top of bars in appropriate figure, so that the conclusion is more borne but a proper biological plain english description of the results.

**Answer:** Following this suggestions and a similar request by the Editor, the manuscript underwent a major re-write to enhance the overall clarity and avoid overstating the conclusions. The language of the manuscript has been revised to enhance clarity and readability. We also have softened and toned down conclusions that may have been too strong. Furthermore, many of the technical details have been moved to either the Methods section or the figure legends. Moreover all quantification values have been relocated to the relevant figure legends, thereby markedly increasing the flow of the manuscript.

Same for the paragraph that follows: To confirm this, this should start with a new line and again this is too lengthy and distracts the message. As a result, I find it difficult to review this article.

**Answer:** Again, as suggested by the Referee, we modified the writing to enhance clarity and readability.

4) However, other statements are not enough documented. The overexpression of WT RhoA versus Smurf resistant RhoA is indeed shown in Figure 4D and 5E. Their level of expression is indeed higher than control cells (where it is surprisingly undetectable) but also different from one another showing that the resistant is indeed resistant. However, in both experiments, lactacycin should have been added to show that the lower level of WT RhoA could be rescued, suggesting that even the overexpressed version is degraded. Synpo should also have been transfected ands show that it also protects. This is important.

**Answer:** We did not add lactacycin in the overexpression experiments because lactacycin by itself is sufficient to rescue stress fibers, thereby making it impossible to discriminate between the effects of Smurf1 resistant RhoA versus lactacycin. The fact that synaptopodin rescues stress fiber formation is evidenced throughout the manuscript (Figures 2B,C, 3C, D, 5A,B, Supplementary Figure S3A,B) and the biochemistry showing the protection of RhoA by synaptopodin was the subject of our Nature Cell Biology manuscript (Asanuma et al., 2006).

5) The text is not always very logical. For instance page 8, the authors state "The downregulation of RhoA steady-state protein levels by Smurf1 mediated ubiquitination and proteasomal degradation controls protrusion formation at the leading edge of migrating cells (Wang et al, 2003) and prevents stress fiber formation in kidney podocytes (Asanuma et al, 2006). Together with the above-described findings, this raises the possibility that in the absence of Tm2, the loss of stress fibers in MDA-MB 231 results from the unopposed effects of Smurf1 on RhoA degradation." This calls immediately for the following questions: Is RhoA more degraded in the absence of TM2, is it rescue by lactacycin,
does Synpo does the same. This is shown in Figure 5 but instead the authors embark upon the
description of the lactamycin effects in cancer cells (figure 4) and make a huge jump (and strong
statement, page 9) linking the rescue of stress fibers by lactacystin and this observed by Synpo,
through the stabilisation of RhoA. However, the rescue of RhoA stability by synpo is NOT shown
and that it is not clear that it is the same mechanism. This needs to be done (crucial) and the order
of figures changed. Fig 5 comes before 4.

Answer: As suggested by the referee, we reversed the order of the figures. As mentioned
above, he manuscript underwent a major re-write to enhance the overall clarity and to avoid
overstating the conclusions.

The stabilization of RhoA by synaptopodin has already been described in detail before (Asanuma et
al., 2006). However, in response to this and a similar critique by Referee 2, we now show that the
ROCK inhibitor Y-27632 blocks both synaptopodin and lactacystin induced stress fibers in
tropomyosin knockdown fibroblasts (new Figure 5) and MDA-MB 231 cells (new Supplementary
Figure S3A-C). Together, with the observation that similarly to lactacystin and synaptopodin,
Smurf1-resistant RhoA(K76,7R) can also restore stress fibers in tropomyosin depleted fibroblasts or
MDA-MB 231 cancer cells, these data further support our overall conclusion that - like
synaptopodin (Asanuma et al., 2006) - tropomyosin can promote stress fibers by increasing RhoA
signaling.

7) It seems to me that the blot in Figure 3B is flipped vertically when compared to blots in Figure
5A and E. In this 3B, the upper band for TM1 band is very strong and the lower band TM2 less
strong whereas on 5A and C, the upper band is weak and the lower is strong. Although it is not
super clear from the legends, the blots were generated from the same cells using the same antibody
to blot.

Answer: We apologize and thank the Referee for pointing this out. The mistake has been corrected.

8) Figure 6C: The text says that when synpo, TM1 and TM2 are expressed, the ubiquitination of WT
Rho is reduced. Indeed the HA bands is less intense when compared to the control (lane 1).
However the eluate is also less abundant (blot FLAG). My feeling is that the ratio HA versus FLAG
is the same for all conditions. Therefore, this does NOT show that TM2 and Synpo lead to reduced
RhoA ubiquitination. This needs to be done again and quantitation of bands included. Plus it seems
that TM1 has the same effect (if any) than TM2 (see my point 1)

Answer: We quantified the bands in Figure 6C (now Figure 7C). With the exception of Lane 3,
which is a negative control, all other FLAG bands show comparable density values, in particular, the
relevant lanes 2 and 4-7 (FLAG blot density per lane: lane 1: 1313821, lane 2: 907107, lane 3:
482110, lane 4: 908718, lane 5: 765224, lane 6: 765224, lane 7: 1186643). Thus, Tm2 and synaptopodin (as
published before: Asanuma et al. 2006), but not TM1 or actinin-4 (as published before: Asanuma et
al. 2006), can block the ubiquitination of RhoA. These data are in line with the observed inhibition
of RhoA binding to Smurf1 by Tm2 (Figure 7B) and synaptopodin (Asanuma et al. 2006) and the
rescue of stress fibers by Smurf1-resistant RhoA (K6,7R).

9) It is not clear whether Synpo and TM2 act in two biochemical pathways or by forming a complex.
Do they? How is the Smurf activity suppressed? Does it bind to Smurf? What is the mechanism to
explain the rescue of TM2 (TM1?) by Synpo? Is RhoA the target?

Answer: We did not formally test whether synaptopodin and TM2 form a biochemical complex but
it is unlikely because fibroblasts or MDA-MB 231 cells do not express endogenous synaptopodin. In
podocytes, the two proteins are spatially segregated: Tm is exclusively found in the cell body and
major processes (Drenckhahn et al, 1990), whereas synaptopodin is restricted to foot processes
(Mundel et al. 1991).

Please not that synaptopodin (Asanuma et al. 2006) and Tm do not suppress the activity of Smurf,
but instead competitively block the interaction of Smurf1 with its substrate RhoA.

Regarding the rescue of Tm2 deficiency by synaptopodin in Drosophila, future studies will be
worthwhile to test in detail whether synaptopodin rescues Tm2 deficiency by virtue of restoring
RhoA signaling, through the restoration of Oskar induced long actin projections or via regulation of
oskar mRNA trafficking or anchoring. This is now mentioned in the revised discussion section.

Minor comments
1) Synpo seems to indeed rescue the stress fibers and reduce (not suppress) the migration as the effects are clear but small.

Answer: The point is well taken. Synaptopodin reduces cell migration but does not suppress it. The text has been corrected accordingly.

2) Figure 3: What is the nuclear staining observed for Synpo??

Answer: As described in the manuscript, the nuclear staining is due to a nonspecific cross-reaction of the secondary antibody and also seen in the cells that were infected with the empty vector instead of synaptopodin.

3) It is also obvious that it has been written by multiple groups as the way of referring to the materials in the figures changes. On various blots, TM2 and TM1 become tropo and tropomyosin.

Answer: We took great care to make the writing style of the paper more harmonious during the revision, including the labeling of tropomyosin in the various Western blot figures. Again, we are now overall focusing on total Tm rather than placing unwarranted focus on a particular isoform.

4) There is also a large number of mistakes in referring to the figure number. For instance page9, Fig3D should be 4A.

Answer: We thank the Referee for pointing this out. We have corrected these mistakes in the revised manuscript. We also carefully checked the other figures for correct labeling and quotation.

5) Page 6 and Supplementary figure 1: It would be nice to see numbers of nos>synpo; Tm2eg9 females analysed. Also the numbers of egg chambers analysed per each genotype and in how many nos>synpo; Tm2eg9 egg chambers osk mRNA localization was rescued.

Answer: We analyzed the ovaries of 10-15 females per genotype. The average normal osk mRNA localization was in wild type 82 % +/- 10 (n = 325), in nos-synpo 80 ± 7 % (n=478), nos-synpo, Tm2eg9 59 ± 14% (n = 393) and Tm2eg9 35% ± 6 % (n = 302) from 3 independent experiments. This information is now included in the methods section or legend to Figure 1A.

6) Page 6: Do eggs laid by nos>synpo; Tm2eg9 develop and hatch normally?

Answer: Yes, they do. This is now mentioned in the revised Results section.

7) Page 7 and figure 2D: "Functionally, the restoration of stress fibers was associated with the suppression of cell migration..." data in the manuscript show there is a reduction of cell migration, but not a suppression.

Answer: As mentioned before, we revised our statement, which now reads ” Functionally, the restoration of stress fibers was associated with the reduction of cell migration..."

8) Page 7: "...it may also rescue Tm2 deficiency in fibroblasts where...": the term "deficiency" not appropriate, depletion is better.

Answer: As suggested by the referee, we replaced “deficiency” with depletion

9) MG132 should also possibly be used in addition to Lactamycin.

Answer: In preliminary studies we found comparable effects for MG132 on the actin cytoskeleton. To make the data comparable with our published results in podocytes (Asanuma et al. 2006), we elected to work primarily with lactacystin.

As we just discussed, I would like to invite you to submit a revised version of your manuscript, addressing the points raised by the referees as you laid out in your point-by-point response, and also incorporating the experiment using the tropomyosin beta knockdown and lactacystin treatment - to start to address the question of isoform specificity.

I look forward to receiving your revision in due course.
REFEREE REPORTS

Referee #1

This manuscript has "novelty", "broad biological significance", is "important to the field", but it does not have "strong evidence for the conclusions drawn."

I have spent some time reviewing the resubmitted manuscript, and I am disappointed by the authors' answers and solutions to the critiques by the reviewers of the initially submitted manuscript. Either the authors did not understand my points or simply did not care about getting it right. Instead of addressing the issue of what Tm isoform(s) they are detecting/affecting in their work, they just replaced "Tm2" with "Tm" through most of the manuscript, "removing the isoform labels" as their response to my major criticism 1. In addition, there continues to be the potential misleading impression that the Tm2 in the mammalian cells is the same thing as the Tm2 in the Drosophila work. In the text they list the forms identified by a BLAST search with the sequence (presumably the all the alpha gene isoforms that referred to), but they do not relate these to the isoform names in the text, or those detected by the antibody. Their shRNA will knock down all the products of the TPM1 (alpha gene) but not the three other genes. Furthermore, the antibody they are using will detect the long products of the TPM1 and TMP2 (and probably TPM3) genes, but not the short forms encoded by the TPM1, TPM3 or TPM4 genes. Hence they have NO EVIDENCE that they knocked down Tm, as they repeatedly state, resulting in an even more misleading interpretation of the results by the authors.

In response to one criticism under my #2, they simply eliminated the figure without explanation. There are still no scale bars. They say they have an n=3, even though they stated n=2 for some experiments before; the bar graphs did not seem to change with the increased n.

In response to minor point 1, they seem to have missed the point that the presence of a GFP or FLAG tag on Tm can affect the FUNCTION of the protein, since the end of Tm is a critical functional domain. Since the affinity experiments only probe for the tag, it is impossible for the reader to know what else is in the mix. There could be numerous proteins from the cell extracts, and that there is no direct interaction. Only showing the immunoblot results do not provide evidence for direct interaction between Rho and Tm-a major conclusion of the paper. Without the missing evidence, the model is not justified. Actually, this is a major point.

Some of the figures remain hard to follow. For example, what are the lanes in Fig. 4E?

Unfortunately, my impression is that the conclusions are misleading in relation to the experimental data.

Referee #2

The authors did a nice job of revising the paper and addressing all of our criticisms except one, which is the failure to do a two step IP to confirm that the ubiquitination signal in the RhoA IP is not due to an associated protein. After checking the manuscript referred to in the rebuttal for this method (Wang et al, 2003), Wang et al in fact employed a double IP to confirm direct ubiquitination of RhoA. Ideally the authors should do the experiment, but given the overall strength of the revised version, I would be comfortable if the authors discussed this caveat in the text.

Referee #3

1) The manuscript has improved in its form and is easier to read and understand. I still don't warm too much to it because I find the conclusions quite surprising, ie that two proteins that are so biochemically different can have the same role on the stability of RhoA (preventing its ubiquitination by competing with SmurfA) and leading to the formation of stress fibers. What I find hard to understand is how this inhibition can be achieved if the two proteins are so different. This is not discussed. Is it a competitive inhibition for both proteins?
2) It is also not clear whether the endogenous proteins do compensate for each other. They do when overexpressed, but this might be due to an overflow of the system in term of localization and stability.

In this regard, it is not clear to me why in Fibroblasts, where both TM and Synpo are theoretically expressed, they do not act as a redundant manner. In theory, RhoA stability should not be affected but TmKO does affect RhoA stability and stress fiber formation. This should be discussed perhaps in the context of the localization and stability of the two proteins, which are clearly non-overlapping. And if there is no redundancy, what is the biology behind this shared protein function? How can the cell use it?

3) The use of 3 different cell systems, all with different properties is hard to follow and should be described properly at the beginning of the text.

For instance, I am not entirely sure whether fibroblasts express TM and Synpo, as I could not find this info provided in the manuscript (see my previous comment).

4) I was also surprised to find that the Synpo expressed in flies is the vertebrate version. Do flies express a Synpo orthologue? Why start with this experiment that is more a confirmation of the experiments in mammalian cells

5) page 9: The induction of stress fibers [by lactamycin in MDA-MB] was independent of Tm because it did not change Tm protein abundance.

Given that Synpo is itself rather unstable, could lactamycin acts through SYnpo and not directly on RhoA?? This should be shown.

6) The authors rely heavily on their previous publications for statement (Asanuma et al). They should not overdo it as manuscripts should stand for themselves.

7) page11: "This control also suggests that the GFP tag is biochemically inert, which is reassuring. While interference by FLAg or GFP tags can never be fully excluded, our control experiments in general throughout this study do not raise such concern".

I find the wording related to this important control a little funny (especially the reassuring!).

8) I also found that the ms has lost its edge with the issue of the Tm isoforms. But I am not a specialist in this field and it is perhaps not important.

2nd Revision - authors' response 21 November 2011

List of incorporated changes

1. We now show that gene silencing of TPMβ is not sufficient to cause a loss of stress fibers in NIH3T3 fibroblasts (new Supplementary Figure 3).

2. We added scale bars to all figures.

3. The manuscript underwent a major re-write to enhance the overall clarity and avoid overstating conclusions.

We added the following information about the shRNAs to the Methods section: "The GIPZ TPMα shRNA and GIPZ non-silencing control shRNAs were acquired from Open Biosystems. The GIPZ TPMα shRNA (Open Biosystems, Catalog Number RMM4431-98754654, Clone ID V2LMM_25293 shRNA) is targeted to the following sequence: CACTACATATGTAATTGGT. When blasted, the shRNA matches TPM transcript variants 2, 3, 4, 6, and 7. The GIPZ TPMβ shRNAs (Open Biosystems) target the following sequences: #1-AAATACGAAGAAGAGATCA (#1), CGGACAAGTATTCCACCAA (#2), and CCGAGCAGAGTTTGCTGAA (#3). The Open Biosystems TPMα shRNA targets a sequence in exon 9D of TPMα that is predicted to knockdown isoforms Tm6, Tm2, Tm3, Tm5a, and Tm5b. This is not predicted to knockdown any TPMβ isoforms, including Tm1."

4. We also added the following paragraph to the Methods section: “NIH3T3 cells express Tm1,
Tm2, Tm3, Tm6, Tm5a, Tm5b, Tm5NM1, and Tm5NM2 (Percival et al., Cell Motility and the Cytoskeleton 2000, 47: 189–208; Schevzov et al., J Histochem Cytochem. 2005, 53: 557-570).

5. We added the following statement to the Discussion: “Tm2 in the mammalian cells is not the same as TmII in Drosophila. Therefore, future studies will be necessary to establish more precisely the molecular link between Tm2 in Drosophila and in mammals…” We also relabeled Drosophila Tm2 as TmII, according to the paper by Erdelyi et al, Nature, 1995, 377: 524-527, to make this even clearer.

6. We now state in the revised Methods section that the wound healing assays in Fig. 6G, H were done 2 times with 3 independent samples each time, thus the total n = 6.

7. We now mention in the revised Methods section that all tropomyosin constructs used in the present study were cloned into pFLAG-CMV-5 vectors, therefore the tags are on the C-terminus and do not interfere with the critical function of the N-terminal domain.

8. We added the following information to the revised legend of Figure 4E: “The first lane shows coinfection with the non-silencing control shRNA (con) and the empty pLentix control vector. The second lane shows coinfection with the TPMα shRNA and the empty pLentix control vector. The third lane shows co-infection with the TPMα shRNA and FLAG-tagged wild type RhoA. The last lane show co-infection with the TPMα shRNA and FLAG-tagged Smurf-1 resistant RhoA (K6,7R).

9. We now point out more clearly in the Results section that fibroblasts do not express endogenous synaptopodin but only Tm. Thus there is no redundancy between synaptopodin and Tm in fibroblasts, and therefore we can find the loss of RhoA and stress fibers in Tm knockdown fibroblasts.

10. We also made it now clearer that similar to fibroblasts, MDA MB 231 cells do not express endogenous synaptopodin. Thus lactacystin cannot act through synaptopodin stabilization in MDA MB 231 cells. We also mention now that synaptopodin is not a target of proteasomal degradation but instead is degraded by cathepsin L mediated cleavage (Faul et al,. Nature Med, 2008).

11. We rewrote the text to make it clear that flies do not express a Synpo orthologue.