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Modulating F-actin organization induces organ growth by affecting the Hippo pathway.

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Corresponding author: Georg Halder, Baylor College of Medicine

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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 04 January 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees express significant interest in your work, and are broadly in favour of publication. However, there are a couple of important concerns raised that would need to be addressed for the study to be potentially suitable for EMBOJ. Firstly, referee 1 questions whether the effects of actin on the Hippo pathway are autonomous or non-autonomous. A clear answer to this question is critical, and if there is non-autonomy, some discussion as to how this may be relayed would be important (although we would clearly not expect a mechanistic explanation at this stage). Secondly, referee 3 raises concerns as to whether the observed effects in Drosophila genuinely represent a direct consequence of altered actin polymerisation, and makes a number of constructive suggestions to make the study more definitive in this regard. Finally, I would strongly encourage you to follow the suggestion of referee 2, and to include the complete data from the RNAi screen as supplementary information.

In the light of the referees' positive recommendations, I would therefore 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.

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 (Remarks to the Author):

This manuscript describes a link between the F-actin cytoskeleton and the Hippo signaling pathway. The authors show that RNAi-mediated knockdown of actin capping proteins (cpa or cpb), or expression of an activated form of Diaphanous, inhibits the activity of the Hippo pathway - known downstream targets of the pathway are upregulated, growth is increased, and the increased growth is suppressed by reduction of yorkie. They document the effect of F-actin accumulation on Hippo signaling in imaginal discs, and in both Drosophila and mammalian cultured cells. The mechanism by which F-actin affects Hippo signaling remains unknown, but through genetic experiments, they provide evidence that the influence of F-actin is likely to be upstream of Warts. The effect of F-actin on Hippo signaling is sure to be of broad interest, and in general the results are well documented and the analysis is thorough.

The one significant issue that I think the authors need to address before publication is that the effects of their F-actin manipulations on cell proliferation (eg BrdU labeling, Fig. 2) and target genes (eg ex-lacZ, Fig 3) appear to be non-autonomous, which would imply that it's an indirect effect. Yet the authors never comment on or discuss this issue. Is the effect autonomous or non-autonomous, and if its non-autonomous what are the possible models that would explain a non-autonomous effect of F-actin accumulation of Hippo signaling?

Minor points
-In Fig 5, I can see that there has been a suppression by wts, and not by hpo or ex, based on growth/folding/distortion of the dpp stripe, but its hard to tell that there is an effect on ex-lacZ. Perhaps the result would be more clear with BrdU labeling?

- Fig S7A,B is supposed to show us that there is no effect on Fat or Mer protein localization , but it looks like the stains didn't work, as I can't see the expected apical localization in wild-type cells here. I think these either need to be improved, or if this is not possible, the result should be deleted from the manuscript as at present they do not convincingly support the authors claims here.

Referee #2 (Remarks to the Author):

Sansores-Garcia et al., use an S2 cell based screen to identify novel regulators of the Hippo pathway. Interestingly they recovered several regulators of F-actin as genes that can influence Yki activity. These findings were validated in vivo with respect to tissue growth and reporters of Hippo pathway activity, as well as by studying Diaphanous, an actin nucleation factor. The authors show that Wts overexpression, but not Ex or Hpo, can rescue the increased Yki activity in tissues lacking cpa or cpb. The authors also use mammalian tissue culture to provide a hint that this mechanism might be conserved throughout evolution. The scene is now set for interesting future studies where the precise mechanism by which actin and the Hippo pathway communicate to modulate tissue size. Given the novelty of the findings presented here and the rigour with which the study has been executed I recommend publication in EMBO. The only suggestion is that the authors might wish to

include a detailed summary and/or raw data from their RNAi screen as was recently done by Ribeiro et al., (Mol Cell 2010) in a similar S2 cell-based RNAi screen for Hippo pathway regulators.

Referee #3 (Remarks to the Author):

In this manuscript Halder and colleagues through a RNAi screen in Drosophila S2 cells to identify new regulators of the Salvador-Warts-Hippo (SWH) tissue growth control pathway, discover a function for the actin polymerisation regulators, the capping proteins A (Cpa) and B (Cpb), Capulet and Twinstar, in regulation of tissue growth via the SWH pathway. In Drosophila wing disc epithelial tissues, they show that knockdown of Cpa or Cpb results in increased F-actin and promotes increased tissue growth without affecting apico-basal cell polarity or Hedgehog (Hh) or Decapentaplegic (Dpp - TGFb) signaliing. They also show that a constituatively active mutation in Diaphanous (Dia-CA), which also increases actin polymerisation, has the same effect on tissue growth. They provide evidence for a link of Cpa/b downregulation and Dia upregulion to the SWH pathway through analysis of targets and the requirement for the transcriptional coactivator, Yki, for the tissue growth effects. Through epistasis analysis they show that the effect on Yki activity occurs at the level of the Wts protein kinase and independent or in parallel to the SWH pathway regulators, Expanded and Hpo. They also show that F-actin levels affect the activity of the Yki homolog, Yap, in mammalian (Hela) cells. They show that increasing F-actin via expression of activated mDia increases active Yap, but inhibition of actin polymerisation using the drug CytochalasinD leads to decreased Yap activity. This is the strongest evidence that it is F-actin levels per se that are linked to the regulation of the SWH pathway. It would have been nice to see an experiment with CytochalasinD in Drosophila S2 cells or tissues.

Overall the quality of the data is high and the novelty of this work makes it highly suitable for publication in Embo J. My only reservation is the validity of their conclusion that in Drosophila it is F-actin levels per se that result in deregulation of Yki activity. They show that upregulation of F-actin levels leads to increased Yki activity, but did they identify anything in their screen where decreased F-actin results in decreased Yki activity? Does F-actin levels correlate with the degree of tissue overgrowth? ie They state the Dia-CA leads to greater tissue growth than with knocking down the Capping proteins, but does this correlate with different degrees of F-actin accumulation? Could it be that there is another function provided bt increasing Dia activity or decreasing Capping protein activity that affects Yki activity? This point needs to be addressed experimentally by data from the screen or by using CytochalasinD, or the argument for the link between F-actin levels to Yki activity stated more clearly in the results.

It was curious that knockdown of Actin genes, Actin5C and Actin87E were initially identified in the screen, but ruled out on the secondary screen. A comment should be made about this. Would decreasing Actin gene levels be expected to lead to less F-Actin, and if so how would this fit with the hypothesis that increased F-Actin results in increased Yki activity?

In the analysis of the effect of knocking down the capping proteins on the localasation of apico-basal polarity regulators, although it is clear from the images that epithelial structure is maintained, to be complete basal-lateral determinants, such as Dlg or the basal markers, B-Integrin or Dystroglycan, should be examined, even if the data isnt shown. Does Dia-CA also show no effect on epithelial structure or Dpp or Hh signaling? - A comment should be made about this.

Minor corrections:
Dia* is an unusual way to indicate constituatively active Dia - it would be better to refer to it as Dia-CA.

Consistency is needed in refering to Hippo - should be defined by the abbreviated gene name (Hpo) when first mentioned and then the abbreviation used from then onwards

p12 - ref Yu et al needs a publication date.

1st Revision - authors' response 14 April 2011

I am happy to resubmit our manuscript entitled "Modulating F-actin organization induces organ growth by affecting the Hippo pathway" to the *EMBO Journal.* In our revised version we addressed all of the reviewers' comments as explained below in our point-by-point response. We added new data as requested and also added unrequested data and analyses that makes the manuscript significantly stronger. Most importantly, we have (1) added new data in Figure 3 showing that the effects of modulating actin organization on the Hippo pathway are cell autonomous as requested by reviewer 1, (2) we included the data of the S2 cell RNAi screen as a supplementary table as requested by reviewer 2, (3) we added an experiment in Figure 1 showing that directly decreasing F-actin levels by cytochalasinD treatment cause a reduction of Yki activity in S2 cells as requested by reviewer 3. Thus increasing or decreasing F-actin levels cause an increase or decrease respectively, of Yki activity both in fly as well as in mammalian cells. Finally, (4) we have semi-quantified the epistasis experiments presented in Figure 5 and added that analysis to that figure. Together, with our other additions and modifications to the paper, these new data further strengthen our conclusion that Cpa and Cpb act as tumor suppressors and that changing actin organization affects growth by affecting the Hippo pathway. We hope that our revised manuscript is acceptable to you and the reviewers. Thank you for your interest in our work!

Point by point response to comments

Comments made by the editor:

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three referees express significant interest in your work, and are broadly in favour of publication. However, there are a couple of important concerns raised that would need to be addressed for the study to be potentially suitable for EMBOJ. Firstly, referee 1 questions whether the effects of actin on the Hippo pathway are autonomous or non-autonomous. A clear answer to this question is critical, and if there is non-autonomy, some discussion as to how this may be relayed would be important (although we would clearly not expect a mechanistic explanation at this stage).

To address the question of cell-autonomy, we generated clones of cells that had knock down of Cpa function (Dia^{CA} overexpressing clones were lethal even at 17°C). In order to prevent the death of cpa^{RNAi} clones, which are eliminated by JNK dependent apoptosis (Janody et al. 2006), we coexpressed bsk^{DN} to inhibit JNK signaling. We found that such clones showed clear cell autonomous upregulation of *ex-lacZ*, while control clones only expressing bsk^{DN} did not show effects on *ex-lacZ* expression. These new data are presented in Figure 3I and Figure S5A.

We performed these new experiments because assessing cell autonomy in wing discs in which Dia^{CA} was overexpressed by *dpp-* or *30A-Gal4* is complicated due to the fact that Gal4 in these driver lines is not stably expressed in cell lineages but is lost when progenitor cells move away from the domain where Gal4 expression was induced. Because of that, and because bGal has a long half live that may be significantly longer than that of GFP, "non"-GFP expressing cells may show upregulated *ex-lacZ* due to earlier Gal4 expression driving Dia^{CA}. Thus, seemingly non-autonomous effects may still be due to cell autonomous action.

Secondly, referee 3 raises concerns as to whether the observed effects in Drosophila genuinely represent a direct consequence of altered actin polymerisation, and makes a number of constructive suggestions to make the study more definitive in this regard.

We addressed the comments of the referee in two ways. First, we tested the effect of loss of F-actin more directly by treating S2 cells with the F-actin destabilizing drug cytochalasinD and measured Yki activity as suggested by reviewer 1. We found that cytochalasinD treatment resulted in a significant reduction in luciferase expression driven by the Yki responsive promoter 3xSd2-luc and Yki, but had no effect on the Renilla luciferase control plasmid, indicating a reduction in Yki activity. We included these data in Figure 1. Second, we identified in the S2 cell screen genes that are required for the formation of F-actin and whose knockdown reduced Yki activity (WASp and Arc-p20). We now mention these in the text. Together, these new data show that a decrease in F-actin levels causes reduced Yki activity.

Finally, I would strongly encourage you to follow the suggestion of referee 2, and to include the complete data from the RNAi screen as supplementary information.

We have added the data of the screen as table S1.

Referee #1 (Remarks to the Author):

This manuscript describes a link between the F-actin cytoskeleton and the Hippo signaling pathway. The authors show that RNAi-mediated knockdown of actin capping proteins (cpa or cpb), or expression of an activated form of Diaphanous, inhibits the activity of the Hippo pathway - known downstream targets of the pathway are upregulated, growth is increased, and the increased growth is suppressed by reduction of yorkie. They document the effect of F-actin accumulation on Hippo signaling in imaginal discs, and in both Drosophila and mammalian cultured cells. The mechanism by which F-actin affects Hippo signaling remains unknown, but through genetic experiments, they provide evidence that the influence of F-actin is likely to be upstream of Warts. The effect of F-actin on Hippo signaling is sure to be of broad interest, and in general the results are well documented and the analysis is thorough.

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In the revised manuscript, we now include four new panels in Figure 3 showing that clones of cells with knockdown of *cpa* cause cell autonomous upregulation of *ex-lacZ.* Control clones had no effect on *ex-lacZ* expression.

Minor points

-In Fig 5, I can see that there has been a suppression by wts, and not by hpo or ex, based on growth/folding/distortion of the dpp stripe, but it's hard to tell that there is an effect on ex-lacZ. Perhaps the result would be clearer with BrdU labeling?
We expanded our analysis and repeated the stainings, and now show clearer images in Figure 5. In addition, we quantified the suppression by categorizing the discs depending on the size of the expression domain. This quantification confirmed our result that Wts but not Hpo or Ex expression strongly suppresses Dia^{CA} induced overgrowth phenotypes. We have included this analysis in Figure 5.

- Fig S7A,B is supposed to show us that there is no effect on Fat or Mer protein localization , but it looks like the stains didn't work, as I can't see the expected apical localization in wild-type cells here. I think these either need to be improved, or if this is not possible, the result should be deleted from the manuscript, as at present they do not convincingly support the authors' claims here.
We repeated the stainings for Fat and Merlin and now show a new picture for Mer that has been added to Fig S9A. Unfortunately, we were unable to improve our Fat stainings and we have thus opted to remove it from the manuscript as suggested.

Referee #2 (Remarks to the Author):

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executed I recommend publication in EMBO. The only suggestion is that the authors might wish to include a detailed summary and/or raw data from their RNAi screen as was recently done by Ribeiro et al., (Mol Cell 2010) in a similar S2 cell-based RNAi screen for Hippo pathway regulators.
We have added the data of the primary S2 cell screen in a supplementary table (Table S1), similar as to what Ribeiro et al. did.

Referee #3 (Remarks to the Author):

In this manuscript Halder and colleagues through a RNAi screen in Drosophila S2 cells to identify new regulators of the Salvador-Warts-Hippo (SWH) tissue growth control pathway, discover a function for the actin polymerization regulators, the capping proteins A (Cpa) and B (Cpb), Capulet and Twinstar, in regulation of tissue growth via the SWH pathway. In Drosophila wing disc epithelial tissues, they show that knockdown of Cpa or Cpb results in increased F-actin and promotes increased tissue growth without affecting apico-basal cell polarity or Hedgehog (Hh) or Decapentaplegic (Dpp - TGFb) signaling. They also show that a constitutively active mutation in Diaphanous (Dia-CA), which also increases actin polymerisation, has the same effect on tissue growth. They provide evidence for a link of Cpa/b downregulion and Dia upregulion to the SWH pathway through analysis of targets and the requirement for the transcriptional coactivator, Yki, for the tissue growth effects. Through epistasis analysis they show that the effect on Yki activity occurs at the level of the Wts protein kinase and independent or in parallel to the SWH pathway regulators, Expanded and Hpo. They also show that F-actin levels affect the activity of the Yki homolog, Yap, in mammalian (Hela) cells. They show that increasing F-actin via expression of activated mDia increases active Yap, but inhibition of actin polymerisation using the drug CytochalasinD leads to decreased Yap activity. This is the strongest evidence that it is F-actin levels per se that are linked to the regulation of the SWH pathway. It would have been nice to see an experiment with CytochalasinD in Drosophila S2 cells or tissues.

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We added new evidence supporting that the levels of F-actin are the factor that affects the Hippo pathway. First, we mention that actin modulators that are necessary for the formation of F-actin were retrieved in our S2 cell RNAi screen as hits whose knockdown caused a reduction in Yki activity, such as Wasp and Arc-p20. Second, we added new data to Figure 1 showing that cytochalasinD treatment of S2 cells reduced the activity of Yki. Thus, as in mammalian cells, also in *Drosophila* cells, increase and decrease of F-actin affect the activity of the Hippo pathway in an opposite manner. These data thus support that it is indeed the levels of F-actin that affect Hippo signaling activity. We mention in the text that Dia^{CA} induced overgrowths were stronger than those induced by cpa^{RNAi}. In both situations we saw an increase in Phalloidin staining. However, given the very abnormal morphology of the affected tissues (especially in the case of Dia^{CA} expression), we refrained from commenting on a correlation between F-actin levels and the strength of the overgrowths, although it did indeed appear that the amount of extra Phalloidin staining was higher in response to Dia^{CA} than cpa^{RNAi} expression.

It was curious that knockdown of Actin genes, Actin5C and Actin87E were initially identified in the screen, but ruled out on the secondary screen. A comment should be made about this. Would decreasing Actin gene levels be expected to lead to less F-Actin, and if so how would this fit with the hypothesis that increased F-Actin results in increased Yki activity?
The primary screen was performed using pAC5.1 as the vector to express Yki-GDBD, Ex and Renilla. pAC5.1 contains an actin promoter which responds to actin levels. We therefore used the metallothionein gene promoter in the secondary screen to retest the hits identified in the primary

screen, such that we could exclude possible hits due to effects on the actin promoter. Actin5C and Actin87E did not test positive in this assay. We added this discussion to the main text.

In the analysis of the effect of knocking down the capping proteins on the localization of apico-basal polarity regulators, although it is clear from the images that epithelial structure is maintained, to be complete basal-lateral determinants, such as Dlg or the basal markers, B-Integrin or Dystroglycan, should be examined, even if the data isnt shown. Does Dia-CA also show no effect on epithelial structure or Dpp or Hh signaling? - A comment should be made about this.
We examined Dlg localization in *hh-Gal4, UAS-cpa*^{RNAi} discs and we saw no significance difference in localization. These data were added to Figure S7E. In addition, we analyzed E-Cad, Patj, Crb and Dlg localization in *30A-Gal4, UAS-Dia*^{CA} discs but saw no significant changes in their localization in most cells, although some cells appeared to have left the epithelium and lost expression of these membrane markers. We also analyzed Ci and pMad expression as readouts for Hh and Dpp signaling as suggested. The gradients of both Ci and pMad are still visible in the regions that overexpress Dia^{CA}. We included the new data from the *30A-Gal4, UAS-Dia*^{CA} discs in a new Figure S8.

Minor corrections:

Dia is an unusual way to indicate constituatively active Dia - it would be better to refer to it as Dia-CA.*
We changed the text and figures accordingly.

Consistency is needed in referring to Hippo - should be defined by the abbreviated gene name (Hpo) when first mentioned and then the abbreviation used from then onwards.
We use the abbreviated form "*hpo*" when we refer to the *hippo* gene or protein. We use the full name "Hippo" when we refer to the pathway (Hippo signaling).

p12 - ref Yu et al needs a publication date.
We corrected this mistake.

2nd Editorial Decision 21 April 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-76780R. It has now been seen again by referee 3, whose comments are enclosed below. As you will see, he/she finds the manuscript substantially improved and is fully supportive of publication - pending a few final changes as detailed. From the editorial side, I would also ask you to remove the Supplementary Table with the screen results from the main Supplementary Information file, and to upload it instead as a separate excel (or similar) file (I realise that we had asked you earlier to put all the supplementary information together in a single file - I'm sorry that we didn't make it clear that this shouldn't include the dataset).

Once you have made these last few revisions, we will then be able to accept the manuscript for publication in EMBOJ.

Many thanks for choosing to publish this study with us: it's a really great piece of work, and I'm very happy to be publishing it!

I look forward to receiving the final version of your manuscript.

REFEREE REPORTS

Referee #3 (Remarks to the Author):

In this revised version of this manuscript, Halder and colleagues have addressed all the reviewers' concerns and supplied additional data, which substantially improves the paper.
I therefore recommend publication.

Some minor points to consider:
Fig2 - given the apparant non-cell autonomous effects on BrdU labelling, which the authors argue in the cover letter is due to GFP being turned off in the expression domain, for the benefit of the readers it would be pertinent to add this explanation to the Fig2 legend. The same would be helpful in the leg for Fig3.

In Supp Table 1 - what does NA stand for?

In the manuscript and supplementary information, there are some inconsistencies with the genetic nomeclature - all genetic elements should be italicised and the gene format in flybase should be used.

Also there are some typos that need correcting in the main and supplementary information. The phrase on page 8 "These results made our day" should be removed, even though I can appreciate the excitement associated with this result!

2nd Revision - authors' response 26 April 2011

We are pleased to submit the final version of our paper titled “Modulating F-actin organization induces organ growth by affecting the Hippo pathway”. We are happy about this paper being accepted for publication in EMBO journal.

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-76780R. It has now been seen again by referee 3, whose comments are enclosed below. As you will see, he/she finds the manuscript substantially improved and is fully supportive of publication - pending a few final changes as detailed. From the editorial side, I would also ask you to remove the Supplementary Table with the screen results from the main Supplementary Information file, and to upload it instead as a separate excel (or similar) file (I realise that we had asked you earlier to put all the supplementary information together in a single file - I'm sorry that we didn't make it clear that this shouldn't include the dataset). Once you have made these last few revisions, we will then be able to accept the manuscript for publication in EMBOJ.

We have uploaded the files separately.

Many thanks for choosing to publish this study with us: it's a really great piece of work, and I'm very happy to be publishing it! I'd also like to let you know that we are commissioning a "Have You Seen?" on your article - to highlight it further and for the wider audience. I look forward to receiving the final version of your manuscript.

Best wishes,

Referee #3 (Remarks to the Author): In this revised version of this manuscript, Halder and colleagues have addressed all the reviewers' concerns and supplied additional data, which substantially improves the paper. I therefore recommend publication.

Some minor points to consider:
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We have added the explanation of the figure legend as suggested.

In Supp Table 1 - what does NA stand for?

We have added the explanation for NA (not annotated) in the table legend.

In the manuscript and supplementary information, there are some inconsistencies with the genetic nomeclature - all genetic elements should be italiced and the gene format in flybase should be used. Also there are some typos that need correcting in the main and supplementary information.

We have gone through the entire manuscript and corrected typos and made the genetic nomenclature consistent throughout the paper.

The phrase on page 8 "These results made our day" should be removed, even though I can appreciate the excitement associated with this result!

We were very excited indeed, and in our excitement we used the words of Harry Callahan (Sudden Impact, 1983). Anyway, we have removed the movie quote form the article and left the science in.

