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Binding of APC and dishevelled mediates Wnt5a-regulated focal adhesion dynamics in migrating cells

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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 10 December 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see while referee 1 is not in favour of publication of the study here the other two referees consider the study as interesting and significant in principle. Still, both of them feel strongly that a significant amount of additional work will be required. Taking together all three reports we have come to the conclusion that we would be able to consider a revised version of the manuscript. However, it will be important to strengthen the study considerably along the lines suggested to the full satisfaction of referees 2 and 3. I would like to specify that it will be important to address all points put forward by referee 2 in the list of "additional points ..." and in particular the first point will need to be addressed experimentally in depth. Furthermore, in our view the data on microtubule stabilisation should not be removed, but the conclusions should be toned down. An additional important issue that we feel needs to be addressed is the presentation of the line of evidence and the conclusions in the manuscript text as pointed to by referees 1 and 2. This will be crucial for the reader to follow and appreciate the study more easily.

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 as well as on the final assessment by referees 2 and 3.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors report that Dvl and APC interact and that both are involved in regulating microtubule plus end dynamics. Wnt5a and the Fz2 receptor are found at the leading edge of migrating cells and interact with integrins, FAK and paxillin. Finally Wnt5A acting through APC/Dvl regulates focal adhesion turnover at the leading edge.

Wnt5 contributes to many polarity and morphogenetic programs during development, although the mechanisms involved are not well understood. The topic of this paper is therefore important and of wide general interest. However, this was a very difficult paper to read and the overall conclusions are vague. No striking new mechanistic insights into how Wnt5a regulates cell morphology or migration clearly emerge.

1. The data in Fig.1 seem a little confusing. Knockdown of Dvl inhibits adhesion (A), yet it promotes focal adhesion stability (C), which might be expected to increase matrix adhesion.
2. Overall, this is a very difficult paper to read - many cell types are used and multiple assays of adhesion, migration, focal complex assembly and microtubule dynamics. It lacks a clear direction as you read the paper.
3. The final section on 3D morphogenesis appears to be thrown into the mix to add some relevance to the observations. It is a superficial study that does not belong in this paper.
4. After having read the paper several times, I am still not sure what the conclusion is.

Referee #2 (Remarks to the Author):

This is a thorough and carefully conducted study of the subcellular localization and function of Dvl and APC in polarized mammalian cells, based on state-of-the-art cell-biological and biochemical analysis. The main results centre on Dvl and APC: these proteins, as well as Fz2 receptor, are highly localized at the leading edge of migrating cells, and Dvl mediates the recruitment of APC to Fz2 and APC via direct binding. Evidence is also provided that Dvl and APC are both required for FAK activation, and that they interact with FAK and paxillin, respectively, thus affecting the dynamics of focal adhesions (as judged by the monitoring of paxillin staining). The interaction between Dvl and APC appears to be stimulated slightly by Wnt5a (though see below), consistent with earlier findings by the same group (Kurayoshi et al, 2006) that this ligand of Fz2 stimulates FAK, and is required for paxillin turnover and cell migration. These core data are convincing and support the title claim of the paper, which is of general interest, and thus merit publication in the EMBO Journal.

The main short-coming of this paper is its discussion, and model, neither of which are very clear. The model in Fig. 8 and its legend are vague, and the authors fail to specify the sequence of molecular interactions. In particular, the domains that mediate the Dvl>APC interaction also mediate the interactions with FAK and paxillin, respectively (see below): do these interactions occur simultaneously, or sequentially? What are the small coloured circles in Fig. 8 meant to depict?

Perhaps the weakest part of the paper is the issue of microtubule stabilization, whose documentation is rather preliminary: for example, the effect of Dvl depletion on microtubules (Fig. 1E) appears very subtle (possibly because it is indirect), and is far less convincing than the block of FAK activation due to Dvl and APC depletion (Fig. 1B, S4B). It therefore seems more likely that Dvl and APC act primarily through FAK activation rather than microtubule stabilization. This part should probably be removed from the paper (including the relevant sentences in the abstract, and in the

final paragraph of the discussion).

In summary, the paper would benefit significantly from focussing on the most compelling results (which are clearly and succinctly summarized at the end of the Introduction), and on a more explicit and testable model.

Additional points the authors might wish to consider:

(1) Protein interactions: it is somewhat curious that the Dvl DEP domain mediates two distinct interactions (with APC and FAK), as does the APC Arm domain (with Dvl and paxillin). It would be reassuring if the authors could exclude the possibility of artefacts due to unfolding or stickiness; e.g. if they could show that the reported interactions depended on specific surface residues (note that point mutations in such residues of both domains have been obtained in genetic approaches, and are known to inactivate the functions of Dvl and APC). Secondly, competition experiments would help to clarify whether the described interactions (Dvl>APC, Dvl>FAK, APC>paxillin) are simultaneous, or sequential. Finally, the only data documenting direct binding are those between APC Arm and full-length Dvl1, however, a high fraction of the latter are unstructured sequences, which could produce artefacts. It would be far preferable to use recombinant DEP domain to demonstrate direct binding to APC Arm.

(2) Involvement of Wnt5a: this Fz2 ligand stimulates the Dvl>APC interaction only slightly (Fig. 3C), and Dvl and APC are already highly localized at the leading edge in the absence of Wnt5a. Clearly, the phenomenology described in this paper does not require Wnt stimulation, which should probably be stressed in the Discussion.

(3) Could the anteriorly-directed membrane trafficking (providing insertion of new membrane material at the leading edge) be responsible for the accumulation of Fz2 at the leading edge (which in turn recruits Dvl and APC)? Perhaps worth considering for the discussion (or even for testing).

(4) The authors may also wish to consider whether the Axin complex is co-recruited with APC to the Fz2-associated Dvl at the leading edge (which seems highly likely, given the well-documented interactions between Axin and APC, and Axin and Dvl). If so, activation of FAK might be due to Axin-associated kinases?

(5) The more speculative parts for the Discussion (top of p13, middle of page 14) could probably be removed.

Referee #3 (Remarks to the Author):

Non canonical Wnt signalling has specific functions in cell migration processes but the molecular pathways are only loosely described. This paper studies the interplay of Wnt5a/Frizzled2, Dishevelled, APC/Microtubules in the control of focal adhesion structures and cell migration. Previous studies have studied these components one by one or in certain combinations but this is the first time that a direct functional pathway is tried to establish. It is suggested that stimulation of frizzled receptors by Wnt5a leads to polarized recruitment of dishevelled -Apc complexes to the cell cortex which in turn stabilize microtubules and associate with focal adhesion constituents (paxillin/FAK) and activate these. This remodelling of focal adhesion is thought to be important for cell migrations, and in a tubular branching model, Wnt5A, Dvl, and APC are required for production of cell extensions. While the paper does not address direct molecular mechanism of how dishevelled and APC activate focal adhesion dynamics (except of their interactions with FAK and paxillin) it provides an important framework for further studies by linking Dvl to APC. However, there are some gaps to fill by the authors to give a coherent picture of the pathway.

1 Fig. 1 shows that Dvls are required for efficient cell substrate adhesion, activation of FAK, remodelling of focal contacts and microtubule dynamics at the leading edge. Similar results are shown for APC in the supplement. It should be tested whether all of these parameters are also dependent on Wnt5A.

2 Similarly, in Fig. 3 it is shown that Wnt5A stimulates interaction of Dvl with APC and promotes APC localization at cell protrusions. Does it also stimulate Dvl localization at the same structures? Is the APC localization dependent on Dvl?

3 In Fig. 6 E, F it is shown that Wnt5a increases numbers of focal adhesions, dependent of Fz2. Is this also dependent on Dvl and APC?

Minor points

1 Images of APC Western blots are identical in Figs 2E and 5F, presumably because the same IP has been blotted for Dvl2 and paxillin and results presented in two different figures. In principle no problem, but it might be better to show separate experiments or combine the data in one figure.

2 Several Anti-APC antibodies are known to give unspecific signals in IF. It would worth to discuss and analyse the specificity of endogenous APC staining shown in Figs 3,5, and 7. Because of the faint nature of staining for Dvl in Fig. 3B this should also be analysed for specificity using Dvl knockdown.

1st Revision - authors' response 25 January 2010

Our responses to the referees' comments for EMBO J-2009-73144

Referee #1

(1) The referee said that the data in Fig. 1 seems a little confusing.

Figure 1A and B showed that Dvl is required for initial cell adhesion and adhesion-dependent activation of FAK, because these assays were performed within 1 h after the cells were seeded to collagen- or fibronectin-coated dishes. Figure 1C demonstrated that focal adhesion areas were enlarged but focal adhesion numbers are reduced (see Figure 6F) in Dvl-knockdown cells at 96 h after the seeding. In this condition, FAK activity was decreased as compared with control cells (data not shown). This reflects the inhibition of focal adhesion dynamics by Dvl depletion. Therefore, the individual sizes of focal adhesions are not always correlated with cell-to-substrate adhesion activity. Our results do not indicate that knockdown of Dvl increases adhesion activity.

(2) The referee criticizes that many cell types and multiple assays are used.

We used HeLaS3 cells in most experiments and Vero cells in experiments to show cell polarization, because it was easy to polarize Vero cells. To confirm that the phenotypes by Dvl knockdown are common between HeLaS3 and Vero cells, we further demonstrated that knockdown of Dvl in Vero cells suppresses cell-substrate adhesion and FAK activation. The results are shown in Supplementary Figure S3 and described in the text (page 5, line 3 from the bottom though page 6, line 1). HPPL cells were used to observe the effects of Wnt5a, Dvl and APC on epithelial branching, because HeLaS3 or Vero cells did not induce branching in 3D culture. Thus, major results were obtained from the experiments using HeLaS3 and Vero cells.
Cell migration is a complex cellular behavior including adhesion, spreading, focal adhesion dynamics, and microtubule dynamics. Therefore, we performed multiple assays to understand which cellular responses are affected by this new Wnt5a signaling pathway.

(3) The referee commented that 3D morphogenesis study in Fig. 7 is superficial.

From the experiments using knockout mice, Wnt5a has been suggested to be involved in developmental processes including epithelial morphogenesis in vivo. Epithelial morphogenesis is a complex multi-cellular process involving cell polarization, migration, and cell-substrate adhesion. Therefore, we would like to have examined whether the new Wnt5a signaling pathway identified in

monolayer culture is involved in epithelial morphogenesis in 3D culture. As pointed out by the referee, the results might be preliminary. However, the results showing that knockdown of Wnt5a, Dvl, and APC affects the formation of epithelial branching processes indicate that this new Wnt5a pathway regulates tubulogenesis. These results suggest this pathway plays an important role in epithelial morphogenesis in vivo. We will clarify the detailed mechanisms of Wnt5a-dependent tubulogenesis in the near future.

Referee #2

(1) The referee requested more detailed analyses of protein interactions between Dvl, APC, paxillin, and FAK.

The DEP domain of Dvl and the armadillo repeats of APC are known to bind to multiple proteins. As pointed out by the referee, it is important to exclude the possibility of artifacts due to unfolding or stickiness in our findings concerning protein interactions. The referee suggested to use point mutations in these domains, but we used several deletion mutants due to the reasons described below.
The Dvl(DEP+) construct contained the DEP domain with the extended N- and C-terminal regions (see Figure 2C). In COS7 cells, the binding activity of GFP-Dvl2(ΔDEP) or GFP-Dvl2(1-509) to APC was similar to that of GFP-Dvl2, but GFP-Dvl2(506-736) did not form a complex with APC. Therefore, either the DEP domain or the C-terminal region after the DEP domain is necessary for the binding of Dvl to APC, but the C-terminal region alone is not sufficient for it. GFP-Dvl2(ΔDEP) and GFP-Dvl2(506-736) associated with FAK as well as GFP-Dvl2, but GFP-Dvl2(1-509) did not. Therefore, the DEP domain is not necessary for the binding of Dvl to FAK. The C-terminal region after the DEP domain of Dvl is necessary and sufficient for the binding of Dvl to FAK. These results suggest that Dvl forms a complex with APC and FAK at different sites although they might be overlapped partially. The study using Dvl(ΔDEP) showed that the DEP domain itself is not essential for the binding of Dvl to APC or FAK. Therefore, we did not use Dvl2(K446M) mutant, which is known to be located to the DEP domain and lose the Dvl activity in Wnt/PCP pathway, in this study. The results are shown in Supplementary Figure S14 and described in the text (page 10, lines 4 through 12 from the bottom).
The APC armadillo construct contained seven Arm repeats with the extended N- and C-terminal regions (APC(Arm+)) (see Supplementary Figure S15). Paxillin formed a complex with EGFP-APC(Arm) with the similar efficiency with EGFP-APC(Arm+), but the binding of Dvl to EGFP-APC(Arm) was weaker than that of Dvl to EGFP-APC(Arm+). In addition, EGFP-APC(Arm1) or EGFP-APC(Arm4) was sufficient for the binding to paxillin, but neither of them bound to Dvl. These results suggest that APC forms a complex with Dvl and paxillin at different sites.
APC(K507M) mutant is known to prevent the interaction of APC with Asef. This point mutation is located to the second armadillo repeat, which was not required for the interaction of APC with Dvl. Therefore, we did not use APC(K507M) in this study. The results are shown in Supplementary Figure S15 and described in the text (page 10, line 3 from the bottom through page 11, line 3).
Whether the Dvl and APC complex binds to FAK or paxillin was also examined. The complex formation between Dvl and APC at endogenous levels was inhibited by the overexpression of EGFP-APC(Arm+) or HA-Dvl1(DEP+). However, under the same conditions the interaction of Dvl with FAK was not inhibited by EGFP-APC(Arm+). The complex formation of APC with paxillin was not suppressed by HA-Dvl1(DEP+), either. Taken together, these results suggest that Dvl binds to APC and FAK simultaneously and that APC also binds to Dvl and paxillin simultaneously. The results are shown in Supplementary Figure S15 and described in the text (page 11, lines 4 through 11).
The referee also asked to show the direct binding of the C-terminal region of Dvl and the Arm repeats of APC using recombinant proteins. According to the referee's comment, we purified GST-Dvl1(395-670) and MBP-APC(Arm+) from E. coli and carried out their binding assays. The results are shown in Supplementary Figure S7 and described in the text (page 7, lines 11 and 12 from the bottom).

(2) The referee commented that we should stress the findings that the interaction between Dvl and APC does not require Wnt5a stimulation.

As pointed out by the referee, Dvl and APC were already localized at the leading edge without the addition of Wnt5a in Vero cells. However, endogenously secreted Wnt5a might be involved in their

localization. Wnt5a indeed induced the accumulation of APC at the cell periphery of HeLaS3 cells. Therefore, it seems that Dvl and APC bind each other without Wnt5a stimulation, but Wnt5a might determine the localization of APC at the cell periphery through Dvl. These statements are described in the text (page 13, line 7 from the bottom through page 14, line 1).

(3) The referee suggested that we should discuss the possible involvement of the anterior-directed membrane trafficking in Fz2 accumulation at the leading edge.

It is thought that anterior-directed membrane trafficking of focal adhesion components including integrins is an important process during cell migration. Although how Fz2 is accumulated at the leading edge is not known at present, anterior-directed membrane trafficking might be one possible mechanism. According to the referee's suggestion, the possibility is discussed (page 14, lines 4 through 13 from the bottom). We would like to clarify the molecular mechanism by which Fz2 is located to the leading edge of polarized and migrating cells in the near future.

(4) The referee asked whether the Axin complex is recruited with APC to the Fz2-associated Dvl2 at the leading edge.

According to the referee's comment, we examined whether Axin is recruited to the cell cortex by the expression of Dvl or Fz2. HA-Axin was observed as cytoplasmic fine punctate structures in Vero cells. When HA-Axin and FLAG-Dvl2 were coexpressed, HA-Axin was colocalized with cytoplasmic FLAG-Dvl2, but not with cell-surface FLAG-Dvl2. Overexpression of FLAG-Fz2 did not affect the distribution of HA-Axin, either. Therefore, it is conceivable that Axin is not recruited with APC to the Fz2-associated Dvl. The results are shown in Supplementary Figure S12 and described in the text (page 9, lines 8 through 14 from the bottom).

(5) The referee said that the more speculative parts for the Discussion could be removed.

According to the referee's suggestion, we removed the speculative parts of Discussion.

(6) The referee pointed out that the model in Figure 8 and its legend are vague.

We improved the model in Figure8 and described the sequence of molecular interactions (page 26, lines 2 through 9).

Referee#3

(1) The referee requested to show phenotypes induced by knockdown of Wnt5a.

We already reported that focal adhesion dynamics and migration are suppressed in Wnt5a-knockdown cells (Kurayoshi, M. et al, Cancer Res. 66, 10439-10448, 2006; Kurayoshi, M. et al., Biochemistry, 402, 515-523, 2007). Therefore, we tested the effect of Wnt5a knockdown on cell-substrate adhesion, activation of FAK, and the microtubule dynamics at the cell periphery. The results are shown in Supplementary Figure S5 and described in the text (page 6, lines 2 through 7 from the bottom).

(2) The referee asked whether Wnt5a promotes Dvl localization at cell protrusions and the APC localization is dependent on Dvl in Fig. 3.

According to the referee's comments, we tested the possibilities. Wnt5a-dependent localization of APC to the cell periphery was reduced in Dvl knockdown HeLaS3 cells, suggesting that Dvl is required for Wnt5a-dependent localization of APC at the cell periphery. The results are shown in Supplementary Figure S10 and described in the text (page 8, lines 10 though 12).
Endogenous Dvl was observed as cytoplasmic puncta mainly in HeLaS3cells. Unlikely of APC, it was difficult to see Dvl localization at the cell periphery in response to Wnt5a. This might be due to the sensitivity of anti-Dvl antibody. Since we speculate that Wnt5a recruits Dvl to Fz2 on the membrane, leading to the localization of APC at the cell periphery, we would like to prove this model experimentally by preparing good antibodies for Dvl. Therefore, I ask the referee to understand that we do not mention Wnt5a-dependent localization of Dvl to the cell periphery in this study.

(3) The referee asked whether Wnt5a-induced increase in focal adhesion numbers is depend on Dvl and APC in Figure 6E and F.

Knockdown of Dvl or APC suppressed Wnt5a-dependent increase in focal adhesion numbers. The results are shown in Figure 6F and described in the text (page 12, lines 6 and 7).

(4) The referee pointed out that images of APC blot are identical in Figures 2E and 5F.

We appreciate the referee's kind suggestions. Because we have already tested the interaction of Dvl2 or paxillin with APC in several different experiments, the separate blots of APC are used in Figures 2E and 5H of the revised manuscript.

(5) The referee said that the specificity of anti-APC and anti-Dvl antibodies should be tested in immunofluorescence studies.

Depletion of APC or Dvl by siRNA reduced their staining at the leading edge, indicating that these findings are not simply due to non-specific signals by antibodies. The results are shown in Supplementary Figure S8A and B and described in the text (page 7, lines 5 though 7 from the bottom).

2nd Editorial Decision 09 February 2010

Thank you for sending us your revised manuscript. Our original referees 2 and 3 have now seen it again, and you will be pleased to learn that in their view you have addressed all criticisms in a satisfactory manner. They both have no further comments to the authors. The paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

