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Dual functions of DP1 promote biphasic Wnt-on and Wnt-off states during anteroposterior neural patterning

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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 21 February 2012

Thank you for the submission of your manuscript to The EMBO Journal. We have just now received the full set of reports from the referees, which I copy below.

As you will see below, all three referees agree in the interest of your manuscript and consider that revision should be granted. As you will see below, the most important concern is raised by referee #1 concerning the interpretation of your data. S/he proposes a different model of DP1 function and suggests a number of experiments to distinguish between the two options.

I understand that given the enormous amount of experimental data provided, probably as a result of a previous review somewhere else as referee #2 points out, it would be out of the scope of this study to further ask for an extensive revision. On the other hand, the data needs to be conclusive enough, either supporting your model or the new model proposed by referee #1. In order to properly address this issue, I think that two of the in vivo experiments proposed by referee #1 would help strengthen your data. The main problem with the interpretation of the in vivo evidence lies in the multiple effects of Wnts during embryonic development. According to referee #1 this should be circumvented by testing the effects of DP1 early enough in ectopic dorsalizing centers and using DNA transfection instead of mRNA for DP1 expression to avoid effects on the maternally activated pathway. These experiments should also add to the physiological significance of your study and thus address some of the concerns of referee #3.

Naturally, any further data that you would like to include in order to address the remaining concerns would only be in your best interest, but will not be absolutely required for the acceptance of your manuscript. Do not hesitate to contact me at any time along the revision process in case you need further clarifications.

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, please visit our website: http://www.nature.com/emboj/about/process.html

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

This is a very interesting manuscript that analyzes the role of DP1 in the Wnt pathway. The author propose that DP1 has two opposed roles: In the cytoplasm, where DP1 would bind Dishevelled and Axin, thus preventing Dvl inhibitory action on Axin, with as consequence increased bcat degradation. In the nucleus, it would bind the kinase NLK and inhibit/compete NLK phosphorylation of the transcription factors Lef/TCF. The result would be a decrease of the NLK-dependent dissociation of the bcat-Lef/TCF complex.

The topic is of high interest, as it 1) describes a putative "sharpening mechanism", where DP1 would further shut off low signals, while boosting strong signals, 2) it proposes a new mode of regulation of an intracellular cascade by a single protein that would act on two very different steps along the pathway, 3) more would solve the discrepancy between previous contradictory reports. The possible conceptual impact goes beyond Wnt signaling.

I should start by stating that I am impressed by this effort to figure out a complex system. This type of work is unfortunately too rare in the field. The study of the Wnt pathway, in particular, has generated hundreds of data sets but with often little or no attempt to reconcile divergent results and build a coherent picture.

The present work is outstanding by its comprehensiveness, as it reports and dissects three new interactions (DP1-Dvl, DP1-Axin, DP1-NLK), including characterization of interaction domains, gain and loss of functions, effect on the pathway measured in culture cells and in embryos by a whole range of parameters.

With a few exceptions, the execution of the experiments is close to perfection, yielding crystal clear data.

I like very much the gradient of active bcat and DP1 distribution along the A-P axis of the embryo, and the related loss-of-function experiments. The differences between the manipulated and control halves are very convincing.

However, I found unfortunately a series of rather serious caveats, which, if remaining unsolved, would compromise the confidence in these results and cast doubts about some of the main

conclusions. Considering the huge amount of data, the thoroughness of the analysis, and the intriguing model proposed,

I feel rather harsh to ask for additional experiments, but this is in my view necessary. Leaving these issues open would defeat the purpose of the comprehensiveness of this study and would make the whole picture rather more confused than clearer.

PROBLEMS AND CAVEATS
I would summarize the main issue in one word: "timing". In both the cell culture and embryo systems, some of the key results presented in this manuscript can be interpreted either as reflecting a "dual" activity (positive AND negative regulator of the Wnt pathway) as suggested by the authors, OR as due to one single action (for example always positive), but with the output changing according to the timing of the activation, or its length (short versus long term). These experiments, as they stand, cannot be safely interpreted. The specific cases explained below should make my point clearer:

1) IMMEDIATE VERSUS LONG TERM EFFECTS. The authors claim that DP1 has a dual role, positive for cells stimulated by Wnt, negative in the absence of Wnt ligand. For the cell culture experiments (Fig.1), the argument is based on the experiments evaluating the effect of gain and loss-of-function (siRNA depletion) of DP1 using a reporter assay. The comparison is made between cells stimulated with Wnt conditioned medium and cells transfected with Dvl or bcat. The caveat is here that these conditions are NOT comparable: Wnt treatment is short term, transfections produce chronic upregulation of the pathway.

In the case of direct stimulation with Wnt, DP1 boosts the signal, and siDP1 dampens it, as expected. In the case of Dvl/bcat transfection, the authors find the opposite result, implying that it plays then the role of a repressor of the pathway. However, an equally possible interpretation is that DP1 is exclusively a positive regulator, but during a LONG TERM activation of the pathway (transfection), the DP1 would cooperate with bcat or Dvl to produce more of Axin2. This direct Wnt target would on the long run efficiently downregulate the pathway, as previously proposed in Drosophila.

One condition presented in the manuscript where DP1 appears to work as positive regulator when the pathway is stimulated by transfection, i.e. expression of stabilized S45A bcat (Fig. 3D+F). This could support that the negative role observed in the case of wt bcat and Dvl expression is real and not an indirect effect of long term stimulation. However, S45A bcat is here downstream of Axin, thus trivially insensitive to the feedback produced by stimulation of Axin2 expression.

To sort out whether DP1 has really a dual role, it would be essential to use comparable conditions for Wnt, Dvl and bcat. It would be easy to test Wnt-transfections (long term experiments), as well as LiCl treatment as a way to stabilize endogenous bcat over a short period of time, thus comparable to induction by Wnt conditioned medium. This type of experiments may well solve the discrepancy already observed between Drosophila embryo and culture cell experiments.

2) WHAT IS THE EFFECT OF DP1 ON AXIN FUNCTION? The authors state that binding of DP1 to Axin and Dvl results in stimulation of bcat degradation and thus DP1 is a negative regulator of the pathway. The argument is based on pretty good arguments: DP1 can bind both Axin and Dvl, DP1 siRNA increases Dvl - Axin interaction, while DP1 overexpression blocks this interaction (Fig. 2). Furthermore, the levels of ubiquitinated bcat are strongly increased by DP1 overexpression (Fig. 2). However, THIS MODEL IS IN CLEAR CONTRADICTION WITH FIGURE 1 PANEL E, which shows that DP1 depletion decreases bcat levels both in resting and in Wnt-stimulated cells, clearly demonstrating a POSITIVE role of DP1 in the pathway. The whole manuscript is built of the proposal that the positive role of DP1 is DOWNSTREAM of bcat, i.e. DP1 strengthens bcat-TCF interaction by inhibiting NLK, while the "NEGATIVE" ROLE IS PLACED UPSTREAM of bcat, as it would prevent Dvl to inhibit Axin activity. If this would be the case, DP1 depletion would be expected to cause an INCREASE in bcat levels, even though it would decrease the amount of bcat bound to TCF.

The data suggest a very different possibility: the Axin binding site for DP1strikingly overlaps with the GSK3 and bcat binding sites. From the IP in Fig. 2, GSK3 seems still bound to Axin in the

presence of DP1, but what about bcat? Verifying that bcat can bind to Axin in the presence of DP1 is an easy (in vitro pull down using DP1 as a bait, to make sure that Axin is binding simulatenously DP1 and bcat) and important experiment.

If Axin function is indeed inhibited as I expect, all the other results that are used to support of negative role of DP1 could be readily re-interpreted based on the timing issue mentioned in point 1 and, for the embryo experiment, in the next point.

I find difficult to interpret the observed changes in levels of ubiquitinated bcat. Would one expect proteasomal degradation downstream of the ubiquitination machinery to be limiting? Normally these experiments are performed in the presence of proteasome inhibitors. Yet I agree that this particular experiment definitely argues for a negative role of DP1, but a direct demonstration that DP1 does not affect Axin function is needed.

3) MULTIPLE WNT SIGNALS IN THE EMBRYO: The experiments in Xenopus have also a caveat related to the timing of the Wnt stimulation:
Several consecutive Wnt signals occur during embryonic development, with distinct, and indeed opposite, effects on axis formation:
- The maternal pathway induces first the dorsalizing center, which is responsible to build the anterior and dorsal structures of the body.
- Shortly afterwards, the first zygotic (wnt8-induced) pathway inhibits formation of these very same anterior/dorsal structures. In this case, Wnt signaling impairs indirectly neural induction, by reducing or even blocking the source of the neural inducing signal, i.e. the underlying dorsal mesoderm, which is.
-Finally, another Wnt pathway patterns the anterior-posterior axis of the neural tube (high Wnt posterior).

Interpreting a late phenotype is thus here a problem, because the manipulations (e.g. mRNA or Morpholino injections) can affect any of these various Wnt signals. For instance, reduced anterior structures could be due to downregulation of the maternal pathway, upregulation of the Wnt8 pathway (and thus decrease in inducing signals emanating from the dorsal mesoderm that pattern the overlying neuroderm), or the direct patterning of the neuroderm itself.
I honestly could not predict with confidence which condition will stimulate more or less one or the other of these three successive Wnt signals. The case is complex, depending on levels and stability of the proteins, as well as on various regulatory circuits still poorly characterized.

A further complication stems from the fact that the early endogenous maternal Wnt/bcat pathway cannot be inhibited upstream of Axin/GSK3: It is well known that interference with Wnts, with their receptors, or with Dishevelled cannot be efficiently achieved by mRNA/morpholino injections in the embryo, probably because these manipulations act too late. The only efficient way is to deplete these maternal components in the oocyte.
One way to circumvent this problem and still use the early signal as an assay is the induction of a secondary dorsalizing center in the ventral side, which is sensitive to manipulations at all levels of the pathway. This assay is used in this manuscript, but is scored at late stages, which complicated the interpretation (see below).

With these facts in mind, the experiments need to be thoroughly revisited. Here are some examples:
- For instance: the general embryo phenotype of DP1 depletion (Figure 4B) can be interpreted in three ways: The authors propose a specific effect on neural crest migration. This is a fair interpretation, but other equally plausible explanations would be a weak inhibition of the maternal pathway or weak over- activation of the Wnt8 pathway. Eye reduction is indeed the first effect of reducing anterior-dorsal structures. The use of markers can hardly be sufficient here to discriminate between these possibilities: a) late anterior neural and/or neural crest markers will be equally sensitive to the status of earlier patterning. b) earlier dorso-ventral markers may not show much change, since the phenotype is very weak on the scale of early D-V patterning .

This is an important issue: if the effects of DP1-MO on the anterior neural structures (Fig.4E, 5A) are not due to interference with the neural patterning, but with early maternal signaling, DP1 would then play the role of a positive regulator of the pathway. The "dual" function may then be an "illusion" caused by the fact that in the dorsal/anterior side of the embryo the Wnt maternal pathway

is ON, and thus "sensitive" to DP1 depletion, while it is OFF on the ventral-posterior side, thus there DP1 MO have no early action, but act only later on the ventralizing/posteriorizing zygotic signals.

One possible way to circumvent this problem and demonstrate that the effect of DP1 MO in the anterior region reflect a bona fide negative role of DP1 in this region would be to attempt rescue with DP1 plasmid DNA rather than mRNA. DNA would not be transcribed before late blastula-early gastrula, thus bypassing the early maternal signal. DNA injections have the disadvantage to lead to mosaic expression, but at least partial rescue should be achievable. Obviously the same rescue would be attempted in the posterior side, to control for the efficiency of the treatment.

Another possible test would be to stimulate bcat stabilization downstream of Wnt and Dvl by LiCl. This can be performed at different times: early treatment (stage 32 cells) hits the maternal pathway, treatment in the late blastula hits the zygotic signals.

- Another similar problem: In Fig.1H, again, the conditions Wnt Dvl and bcat cannot be properly compared, because the initial activity is very different, with Wnt basely inducing some very weak double-axis, while Dvl and bcat making complete axis. This experiment can be easily fixed by proper mRNA titration. Again, one would like to see what happens with lower levels of Dvl/bcat, and with higher levels of Wnt mRNAs. Because one cannot predict which condition stimulates more or less one or the other, the safest is a) to use comparable stimulations, and b) look by RT-PCR at early targets in the blastula (e.g. Siamois) to avoid the later complications.

Reporter gene constructs, including the one containing the Siamois promoter (Fig. 5D), should be avoided in the embryo, because they may start to be expressed as early as transcription starts (blastula), and one measures their accumulation over several hours, i.e. over a period spanning the multiple consecutive endogenous signals, with again the same above-mentioned caveats.

- Because there is no guarantee that DP1 depletion/interference would inhibit the endogenous maternal pathway if really DP1 works at the level of Dvl, I would test in a more definitive way DP1 function on Wnt-induced double Axis, as done on Fig. 1H. In addition of the titration explained here above, I would also complete the experiment, so far based on DP1 overxpression, with a loss-of-function. It is quite plausible that DP1 may not be sufficiently depleted at these early stages (see other issue here below), in which case the use of a deletion mutant with predicted dominant negative activity would be quite justified. RT-PCR analysis of endogenous Siamois levels in the injected ventral side would be the best read-out.

4) FUNCTION IN THE NUCLEUS OR IN THE CYTOPLASM? The use of a NLS-DP1, largely concentrated in the nucleus, to demonstrate that nuclear DP1 activity is sufficient to rescue Twist expression is quite compelling, but one would wish to see the complementary experiment, i.e. that rescue could not be achieved by a cytoplasmic from of DP1. A "NES-DP1" mentioned in legend of Fig. S6 but no data are shown. Also, an NES-DP1 would still reach the nucleus, and would actually be expected to deplete NLK from the nucleus. An alternative experiment would be to produce a myristylated or palmitoylated form that would be sequested to the plasma membrane.

5) EFFECT ON BCAT-TCF/LEF INTERACTION UNCLEAR. While the data show convincingly that DP1 "competes" with TCF for NLK, resulting in decreased phosphorylation of TCF, the key consequence of this effect, i.e. an increase in TCF/bcat interaction is not shown.
The manuscript is so rich that it would take pages to dissect all the different experiments, but the above remarks should be sufficient to serve as guide for a careful revision of the most crucial points that need to be validated.

CONCLUSION
Again, the work is overall really nice, with about everything one would wish in terms of controls for the biochemical as well as microscopy data. But unfortunately this mass of experiments is missing some crucial points that are absolutely required for the data to make sense. I believe that a few well targeted experiments will conclusively back up the proposed model.

MINOR POINTS:

Drosophila data should be presented in the result section.

Lef/TCF kinase assay in Suppl. Figure S5H not convicing: there is still a migration shift in the last lane (+ DP1). The fact that the shift looks smaller compared to the 2nd lane and the highest band is not seen could simply due to the significantly weaker signal.

Referee #2:

In this interesting study Kim et al. introduce a new regulator, DP1, in Wnt signalling. It was previously reported (though pretty much hidden in the literature unless systematically researched, as done by the authors), that DP1 could function either as a positive or negative Wnt regulator. By an elegant combination of biochemical, cell biological and in vivo studies encompassing cell culture, Xenopus embryos and Drosophila they provide compelling evidence for a dual role of DP1. Their key mechanistic finding is that the cytoplasmic and nuclear function of DP1 are different and that the protein switches from negative to positive in the presence of Wnt ligand. The study is comprehensive, in general well executed, and the paper is well written. I had previously reviewed this ms for another "TOP" journal and the authors have dealt satisfactorily with my past comments by direct experimentation.

I recommend publication of the ms as is.

Minor:
p.8 “truck region" should be “trunk"

Referee #3:

Kim et al address the role of E2F-independent functions of DP1, focussing on the Wnt/b-catenin signalling pathway. The study extends and consolidates previous reports on the importance of the E2F pathway in Wnt signalling. The significance of the present study relates to the dual role of DP1, independent of E2F that acts negatively or positively depending on the level of Wnt signalling. The authors suggest that this binary role reflects the cyplasmic or nuclear location of DP1. This is quite an interesting study which suggests a novel regulatory circuit in Wnt signalling, and highlights new roles for DP1 which remains a poorly described E2F subunit. However, there are some shortcomings, both technical and otherwise, which prevent the manuscript being published. In no particular order of priority:

1) The DP1 siRNA (1B) does not appear to be very efficient. I would like to see complete knockdown, rather than partial. Ectopic/endogenous protein levels should be shown throughout for example (1D, 1F, 1G, most of SI Fig 1 etc). I think "epistasis analysis", used in the context of co-expressing different ectopic proteins (1F), is not the correct scientific description.
2) In 2A, I am not entirely clear what the common band in IP Flag/IB GFP is? The endogenous IP (2B) is not entirely convincing; please confirm that the co-IP band is DP1, and improve the axin blot. The colocalization of endogenous DP1 and Dvl/2 should be shown (SI 2C has been performed with ectopic Dvl). Fig 2D lacks the anti-Axin control treatment. Fig 2E lacks antibody controls too. As a control, I would like to see the effect of DP1 on catenin ubiquitination in plus and minus Wnt3a-CM conditions. It is not entirely clear why (2F and 2G) the altered level of B catenin ubiquitination is not reflected in protein levels?
3) Throughout the data in Figure 2, I do not think that he authors can unequivocally rule out that nuclear DP1 and/or DP1 bound to E2F plays a role in the results. The authors should use appropriate DP1 mutants (for example no dimerization domain) to exclude these possibilities.
4) The competition between Dv1, E2F and DP1 (S3G) should be confirmed under physiological conditions, say by regulating endogenous Dv1, and monitoring E2F activity.
5) The evidence that DP1 interacts with NlK1 (3B) is not convincing. The efficiency and specificity of the interaction is very low under physiological conditions (is this level meaningful), and the effect of Wnt3a on the interaction should be documented under physiological conditions (3C is with ectopic protein). It should be shown that the NLK bound to DP1 is enzymatically active, to fit with the authors model. Further, it is not clear whether the putative NLK phosphorylation sites (S5D) in

DP1 are real physiological sites, which needs to be addressed. The data in 3G needs a comparison to DP1 and HD.
6) Fig 4A needs repeating; it is not clear how the effect of xDP1-MO is compared to the control. The images in Fig 6A are unclear; positive controls for nucle: and cytoplasmic staining are required. The data in S6K are not complete; where is NES-DP1, plus control DP1? In general, Fig 6 data should address expression levels of ectopic proteins (DP1, NLS, DB etc). The nuclear cytoplasmic arguments should be supported by biochemistry, rather than rely totally on imagining.

Overall, this manuscript requires a much more robust analysis of the authors model under physiological conditions. Many of the data are derived from ectopic protein conditions and/or suffer from over-interpretation.

1st Revision - Authors' Response 15 May 2012

Referee #1:

This is a very interesting manuscript that analyses the role of DP1 in the Wnt pathway. The author propose that DP1 has two opposed roles: In the cytoplasm, where DP1 would bind Dishevelled and Axin, thus preventing Dvl inhibitory action on Axin, with as consequence increased bcat degradation. In the nucleus, it would bind the kinase NLK and inhibit/compete NLK phosphorylation of the transcription factors Lef/TCF. The result would be a decrease of the NLK-dependent dissociation of the bcat-Lef/TCF complex. The topic is of high interest, as it 1) describes a putative "sharpening mechanism", where DP1 would further shut off low signals, while boosting strong signals, 2) it proposes a new mode of regulation of an intracellular cascade by a single protein that would act on two very different steps along the pathway, 3) more would solve the discrepancy between previous contradictory reports. The possible conceptual impact goes beyond Wnt signalling.

: We truly appreciate for the reviewer’s valid evaluation for the significance of our manuscript.

I should start by stating that I am impressed by this effort to figure out a complex system. This type of work is unfortunately too rare in the field. The study of the Wnt pathway, in particular, has generated hundreds of data sets but with often little or no attempt to reconcile divergent results and build a coherent picture. The present work is outstanding by its comprehensiveness, as it reports and dissects three new interactions (DP1-Dvl, DP1-Axin, DP1-NLK), including characterization of interaction domains, gain and loss of functions, effect on the pathway measured in culture cells and in embryos by a whole range of parameters.

With a few exceptions, the execution of the experiments is close to perfection, yielding crystal clear data. I like very much the gradient of active bcat and DP1 distribution along the A-P axis of the embryo, and the related loss-of-function experiments. The differences between the manipulated and control halves are very convincing. However, I found unfortunately a series of rather serious caveats, which, if remaining unsolved, would compromise the confidence in these results and cast doubts about some of the main conclusions. Considering the huge amount of data, the thoroughness of the analysis, and the intriguing model proposed, I feel rather harsh to ask for additional experiments, but this is in my view necessary. Leaving these issues open would defeat the purpose of the comprehensiveness of this study and would make the whole picture rather more confused than clearer.

: Thank you for providing thoughtful and constructive suggestion. We think all concerns raised by the reviewer were really valid and resolving the concerns truly strengthens our claims. Especially we thank for the reviewer’s specific experimental suggestion which makes us to complete revision relatively easy and improves the comprehensiveness of our study.

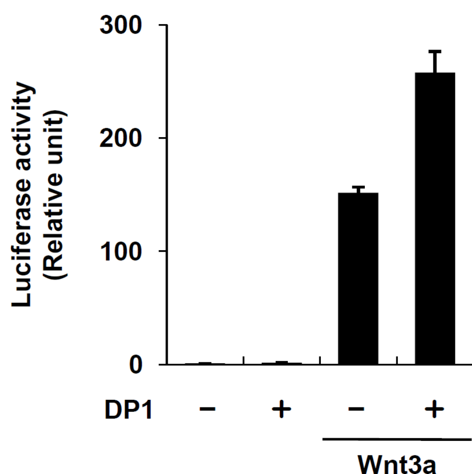
PROBLEMS AND CAVEATS

I would summarize the main issue in one word: "timing". In both the cell culture and embryo systems, some of the key results presented in this manuscript can be interpreted either as reflecting a "dual" activity (positive AND negative regulator of the Wnt pathway) as suggested by the authors, OR as due to one single action (for example always positive), but with the output changing according to the timing of the activation, or its length (short versus long term). These experiments, as they stand, cannot be safely interpreted. The specific cases explained below should make my point clearer:

1) IMMEDIATE VERSUS LONG TERM EFFECTS. The authors claim that DP1 has a dual role, positive for cells stimulated by Wnt, negative in the absence of Wnt ligand. For the cell culture experiments (Fig.1), the argument is based on the experiments evaluating the effect of gain and loss-of-function (siRNA depletion) of DP1 using a reporter assay. The comparison is made between cells stimulated with Wnt conditioned medium and cells transfected with Dvl or bcat. The caveat is here that these conditions are NOT comparable: Wnt treatment is short term, transfections produce chronic upregulation of the pathway.
In the case of direct stimulation with Wnt, DP1 boosts the signal, and siDP1 dampens it, as expected. In the case of Dvl/bcat transfection, the authors find the opposite result, implying that it plays then the role of a repressor of the pathway. However, an equally possible interpretation is that DP1 is exclusively a positive regulator, but during a LONG TERM activation of the pathway (transfection), the DP1 would cooperate with bcat or Dvl to produce more of Axin2. This direct Wnt target would on the long run efficiently downregulate the pathway, as previously proposed in Drosophila.

One condition presented in the manuscript where DP1 appears to work as positive regulator when the pathway is stimulated by transfection, i.e. expression of stabilized S45A bcat (Fig. 3D+F). This could support that the negative role observed in the case of wt bcat and Dvl expression is real and not an indirect effect of long term stimulation. However, S45A bcat is here downstream of Axin, thus trivially insensitive to the feedback produced by stimulation of Axin2 expression.

To sort out whether DP1 has really a dual role, it would be essential to use comparable conditions for Wnt, Dvl and bcat. It would be easy to test Wnt-transfections (long term experiments), as well as LiCl treatment as a way to stabilize endogenous bcat over a short period of time, thus comparable to induction by Wnt conditioned medium. This type of experiments may well solve the discrepancy already observed between Drosophila embryo and culture cell experiments.



: We agree with reviewer's point that duration of signaling activation may differently affect the function of DP1. In our study, cells were treated with Wnt3a-CM for about 20 hr to investigate the effect of DP1 on the Wnt mediated reporter activity. We added treatment period of Wnt3a-CM in Figure legend of revised manuscript. To further resolve the reviewer's concern, we performed the experiment suggested by the reviewer. We investigated whether DP1 is still able to positively regulate Wnt3a-mediated reporter activity when DP1 was co-transfected together with Wnt3a plasmids. As shown in left Figure, we observed consistent data that DP1 could significantly enhance the reporter activity induced by Wnt3a-transfections. We included this data in Supplementary Figure S1A.

The reviewer suggested us to test LiCl treatment as a way to stabilize endogenous b-catenin over a short period of time. However, our proposed negative roles of DP1 against b-catenin mediated

pathway activation is originated from its inhibitory effect on the Dvl, whose function is to inhibit GSK3 mediated phosphorylation of b-catenin by binding to Axin and interfering the destruction complex. LiCl is a potent chemical inhibitor of GSK3b, therefore if administered, it stabilizes b-catenin no matter whether Dvl is inhibited or not. With this notion in consideration, we think it is not plausible to use LiCl in our experiments to address the timing issues that the reviewer raised.

2) WHAT IS THE EFFECT OF DP1 ON AXIN FUNCTION? The authors state that binding of DP1 to Axin and Dvl results in stimulation of bcat degradation and thus DP1 is a negative regulator of the pathway. The argument is based on pretty good arguments: DP1 can bind both Axin and Dvl, DP1 siRNA increases Dvl - Axin interaction, while DP1 overexpression blocks this interaction (Fig. 2). Furthermore, the levels of ubiquitinated bcat are strongly increased by DP1 overexpression (Fig. 2). However, THIS MODEL IS IN CLEAR CONTRADICTION WITH FIGURE 1 PANEL E, which shows that DP1 depletion decreases bcat levels both in resting and in Wnt-stimulated cells, clearly demonstrating a POSITIVE role of DP1 in the pathway. The whole manuscript is built of the proposal that the positive role of DP1 is DOWNSTREAM of bcat, i.e. DP1 strengthens bcat-TCF interaction by inhibiting NLK, while the "NEGATIVE" ROLE IS PLACED UPSTREAM of bcat, as it would prevent Dvl to inhibit Axin activity. If this would be the case, DP1 depletion would be expected to cause an INCREASE in bcat levels, even though it would decrease the amount of bcat bound to TCF.

: The reviewer argued that our model is in clear contradiction with Fig 1E. However, we feel that the reviewer might overlook the small (insignificant) difference presented in Fig 1E. In this experiment, our main observation was made to the difference between control and si-DP1 (#1 and #2) when Wnt3a-CM is applied, thus we originally concluded that DP1 is required for the Wnt-induced b-catenin accumulation, in accordance with the observation made in *Xenopus* embryo (Fig. 5G). However, we do not know how it increases b-catenin level in the presence of Wnt, thus we left the mechanism aside for the future investigation. Please note that we have provided two different mechanisms of the positive roles of DP1 on the Wnt signaling, one is to increase b-catenin level by unknown mechanism and the other is to inhibit NLK kinase activity in the nucleus which is described in a more detail throughout the Fig 3. Nevertheless, we also noticed some differences in controls of Fig. 1E, as the reviewer carefully pointed out. In fact, we utilized two different siRNAs in Fig. 1E and one of them slightly increased (#2) whereas the other slightly decreased (#1) the b-catenin level. However, we felt that these slight changes of b-catenin were not significant enough to conclude that DP1 knockdown either increases or decreases the b-catenin level. Moreover, the two different siRNAs should have different activities toward Wnt signaling if these slight changes were to be significant enough, which was always not the case (Fig. 1C and data not shown; we used siRNA (#1) in Fig 1D, G, 2F, G). Therefore, we would conclude that DP1 knockdown does not affect b-catenin level in a resting state. Also, we would not make the prediction to be that DP1 knockdown should increase b-catenin level in a resting state in 293T cells because in other data, knockdown of DP1 always had no effect on the signaling in a resting state (Fig. 1C, D, G and data not shown). We speculated that the negative function of DP1 is always meaningful when Dvl or b-catenin is abnormally over-expressed or over-stabilized than the normal resting level. We already included this discussion in the figure legend of Fig. 7. Then, why in *Xenopus*, knockdown of DP1 increased activated b-catenin level in the anterior neural plate (Fig. 5G), where endogenous Wnt signaling is kept low. We speculate that it is one of the mechanisms which inhibit Wnt-ligand-independent pathway activation by Dishevelled in this specific developmental context. In fact, Dishevelled is strongly expressed in this region (Gray et al., 2009, Diversification of the expression patterns and developmental functions of the dishevelled gene family during chordate evolution. Dev Dyn, 238, 2044-2057) and there are other known regulatory mechanisms to inhibit the Dishevelled mediated pathway activation in this developmental context (Angers et al., 2006, The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-b-catenin pathway by targeting Dishevelled for degradation. Nat Cell Biol, 8, 348-357). Therefore, we would add our suggested DP1-mediated negative regulatory function as a new layer of Dishevelled regulation for the proper anterior development.

The data suggest a very different possibility: the Axin binding site for DP1 strikingly overlaps with the GSK3 and bcat binding sites. From the IP in Fig. 2, GSK3 seems still bound to Axin in the presence of DP1, but what about bcat? Verifying that bcat can bind to Axin in the presence of DP1 is an easy (in vitro pull down using DP1 as a bait, to make sure that Axin is binding simultaneously DP1 and bcat) and important experiment. If Axin function is indeed inhibited as I expect, all the

other results that are used to support of negative role of DP1 could be readily re-interpreted based on the timing issue mentioned in point 1 and, for the embryo experiment, in the next point.

: When we performed experiments for the data shown in Fig 2D, the interaction between Axin and b-catenin in the presence of DP1 was examined. As shown in the revised Fig 2D, the interaction between Axin and b-catenin was not significantly changed in the presence of DP1.

I find difficult to interpret the observed changes in levels of ubiquitinated bcat. Would one expect proteasomal degradation downstream of the ubiquitination machinery to be limiting? Normally these experiments are performed in the presence of proteasome inhibitors. Yet I agree that this particular experiment definitely argues for a negative role of DP1, but a direct demonstration that DP1 does not affect Axin function is needed.

: We are sorry for not having included the detailed experimental procedures for the ubiquitination assays in the previous manuscript. When we examined the effect of DP1 on the poly-ubiquitination of β-catenin, HEK293T cells were incubated with proteasomal inhibitor MG132 for 4-6 hr before harvesting cells. That description was included in the revised Figure legend.

The reviewer also suggested the possibility that DP1 might inhibit Axin function, by which it activates the Wnt signaling in short term by inhibiting Axin-mediated destruction of b-catenin and in long-term inhibits the signaling by producing more Axin2. If it's the case, we should expect enormous increase of b-catenin in the absence of Wnt, when DP1 is overexpressed, because Axin acts primarily at the resting state to restrict b-catenin level. However, our observation was not the case (Fig 1A and data not shown). Therefore, we think that DP1 does not act at the level of Axin.

In addition, according to the reviewer's interpretation, the long-term effects DP1 on Dsh or b-catenin overexpression might be due to the increased expression of Axin2 as a negative feedback product of the signaling. However, we already showed in the Supplementary Figure S1D that the interesting prediction made by the reviewer is not true. Even suppose the prediction is true that DP1somehow cooperates with Dvl or b-catenin to increase Axin2 expression, there is still no reason that the newly produced Axin2 cannot be inhibited by the DP1. In this case, the predicted outcome should be very fluctuating, while in reality, it was always consistent to be negative on the signaling.

3) MULTIPLE WNT SIGNALS IN THE EMBRYO: The experiments in Xenopus have also a caveat related to the timing of the Wnt stimulation:
Several consecutive Wnt signals occur during embryonic development, with distinct, and indeed opposite, effects on axis formation:
- The maternal pathway induces first the dorsalizing centre, which is responsible to build the anterior and dorsal structures of the body.
- Shortly afterwards, the first zygotic (wnt8-induced) pathway inhibits formation of these very same anterior/dorsal structures. In this case, Wnt signalling impairs indirectly neural induction, by reducing or even blocking the source of the neural inducing signal, i.e. the underlying dorsal mesoderm, which is.
-Finally, another Wnt pathway patterns the anterior-posterior axis of the neural tube (high Wnt posterior).

Interpreting a late phenotype is thus here a problem, because the manipulations (e.g. mRNA or Morpholino injections) can affect any of these various Wnt signals. For instance, reduced anterior structures could be due to downregulation of the maternal pathway, upregulation of the Wnt8 pathway (and thus decrease in inducing signals emanating from the dorsal mesoderm that pattern the overlying neuroderm), or the direct patterning of the neuroderm itself.
I honestly could not predict with confidence which condition will stimulate more or less one or the other of these three successive Wnt signals. The case is complex, depending on levels and stability of the proteins, as well as on various regulatory circuits still poorly characterized.

A further complication stems from the fact that the early endogenous maternal Wnt/bcat pathway cannot be inhibited upstream of Axin/GSK3: It is well known that interference with Wnts, with their receptors, or with Dishevelled cannot be efficiently achieved by mRNA/morpholino injections in the

embryo, probably because these manipulations act too late. The only efficient way is to deplete these maternal components in the oocyte.
One way to circumvent this problem and still use the early signal as an assay is the induction of a secondary dorsalizing centre in the ventral side, which is sensitive to manipulations at all levels of the pathway. This assay is used in this manuscript, but is scored at late stages, which complicated the interpretation (see below).

With these facts in mind, the experiments need to be thoroughly revisited. Here are some examples:
- For instance: the general embryo phenotype of DP1 depletion (Figure 4B) can be interpreted in three ways: The authors propose a specific effect on neural crest migration. This is a fair interpretation, but other equally plausible explanations would be a weak inhibition of the maternal pathway or weak over- activation of the Wnt8 pathway. Eye reduction is indeed the first effect of reducing anterior-dorsal structures. The use of markers can hardly be sufficient here to discriminate between these possibilities: a) late anterior neural and/or neural crest markers will be equally sensitive to the status of earlier patterning. b) earlier dorso-ventral markers may not show much change, since the phenotype is very weak on the scale of early D-V patterning .

This is an important issue: if the effects of DP1-MO on the anterior neural structures (Fig.4E, 5A) are not due to interference with the neural patterning, but with early maternal signalling, DP1 would then play the role of a positive regulator of the pathway. The "dual" function may then be an "illusion" caused by the fact that in the dorsal/anterior side of the embryo the Wnt maternal pathway is ON, and thus "sensitive" to DP1 depletion, while it is OFF on the ventral-posterior side, thus there DP1 MO have no early action, but act only later on the ventralizing/posteriorizing zygotic signals.

One possible way to circumvent this problem and demonstrate that the effect of DP1 MO in the anterior region reflect a bona fide negative role of DP1 in this region would be to attempt rescue with DP1 plasmid DNA rather than mRNA. DNA would not be transcribed before late blastula-early gastrula, thus bypassing the early maternal signal. DNA injections have the disadvantage to lead to mosaic expression, but at least partial rescue should be achievable. Obviously the same rescue would be attempted in the posterior side, to control for the efficiency of the treatment.

Another possible test would be to stimulate bcat stabilization downstream of Wnt and Dvl by LiCl. This can be performed at different times: early treatment (stage 32 cells) hits the maternal pathway, treatment in the late blastula hits the zygotic signals.

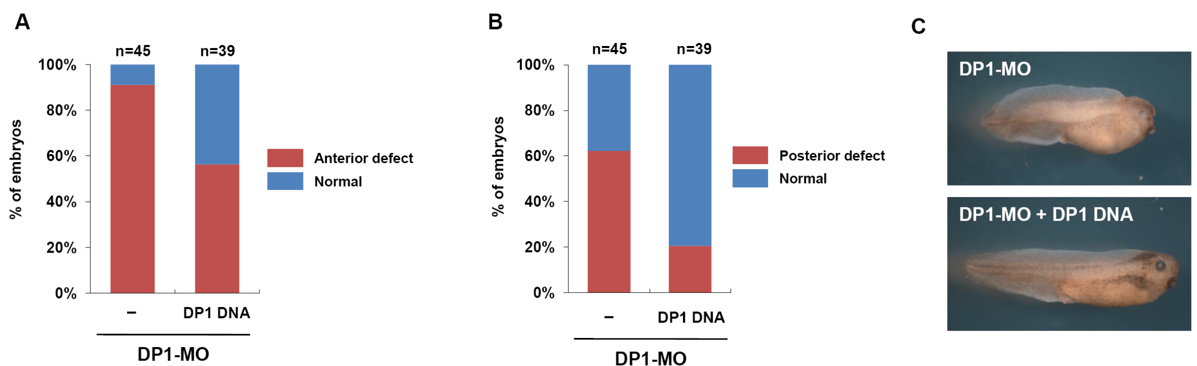
- Another similar problem: In Fig.1H, again, the conditions Wnt Dvl and bcat cannot be properly compared, because the initial activity is very different, with Wnt basely inducing some very weak double-axis, while Dvl and bcat making complete axis. This experiment can be easily fixed by proper mRNA titration. Again, one would like to see what happens with lower levels of Dvl/bcat, and with higher levels of Wnt mRNAs. Because one cannot predict which condition stimulates more or less one or the other, the safest is a) to use comparable stimulations, and b) look by RT-PCR at early targets in the blastula (e.g. Siamois) to avoid the later complications.

Reporter gene constructs, including the one containing the Siamois promoter (Fig. 5D), should be avoided in the embryo, because they may start to be expressed as early as transcription starts (blastula), and one measures their accumulation over several hours, i.e. over a period spanning the multiple consecutive endogenous signals, with again the same above-mentioned caveats.

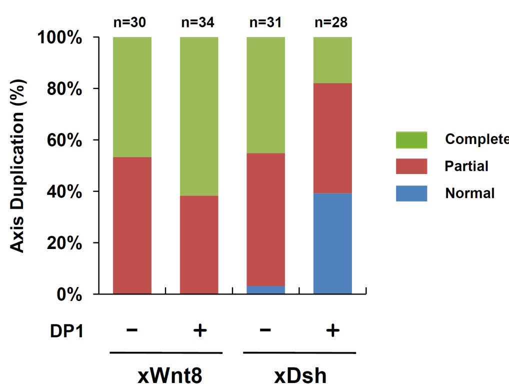
- Because there is no guarantee that DP1 depletion/interference would inhibit the endogenous maternal pathway if really DP1 works at the level of Dvl, I would test in a more definitive way DP1 function on Wnt-induced double Axis, as done on Fig. 1H. In addition of the titration explained here above, I would also complete the experiment, so far based on DP1 overexpression, with a loss-of-function. It is quite plausible that DP1 may not be sufficiently depleted at these early stages (see other issue here below), in which case the use of a deletion mutant with predicted dominant negative activity would be quite justified. RT-PCR analysis of endogenous Siamois levels in the injected ventral side would be the best read-out.

: We already showed in the previous manuscript several lines of evidence that the dual function of DP1 is directly on the Wnt signaling during anteroposterior neural patterning, but not an "illusion" caused by its effect on the maternal Wnt signaling. At first, DP1-MO had no effects on the Gsc expression (Supplementary Fig. S6C), suggesting that DP1 is not required for the maternal Wnt signaling. Second, sox2 expression was never decreased, whereas anterior neural markers were decreased on the DP1-MO injected side of neurula embryo (Fig 5A, B), suggesting that maternal Wnt-mediated dorso-anterior development is not perturbed (sox2) whereas zygotic Wnt mediated A-P patterning is inhibited by DP1-MO. Third, TOP-FLASH activity is increased in the anterior but decreased in the posterior region by DP1 depletion (Fig 5D), which cannot be achieved by the inhibition of maternal Wnt signaling but can be explained only by our suggested dual-role model. Fourth, activated b-catenin level is increased in the anterior neuroectoderm by DP1-MO injection (Fig 5G) demonstrating that the Wnt signaling is indeed increased in the anterior neural plate at this stage by DP1-MO.

Nevertheless, we understand the reviewer's point that these are not enough to completely rule out the possibility that the observed phenotypes of DP1 morphants (especially the anterior defects) were due to weak perturbation of the maternal Wnt signaling. Therefore, we tested whether DP1 DNA can rescue DP1-MO induced defects both in the anterior and the posterior tissues, as the reviewer suggested. As shown below, DP1 DNA rescued DP1-MO induced anterior and posterior defects. We included this result in Supplementary Figure S6G and H in the revised manuscript.

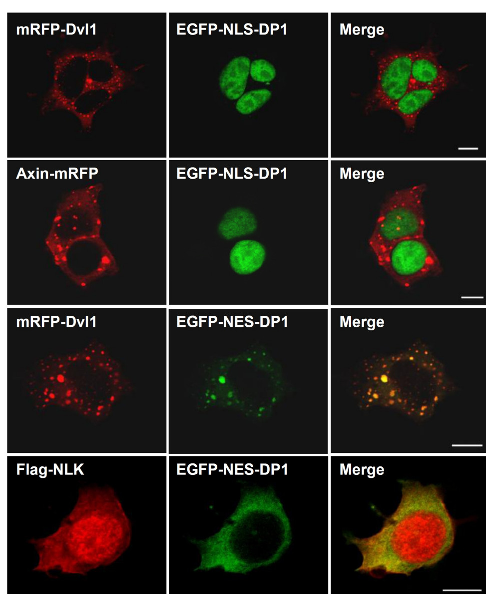


The reviewer's point is true for the axis duplication assay data in Fig. 1H. Initially, we made a number of axis duplication assays with various titrations. In our previous attempts, DP1 always enhanced Wnt activity of axis duplication even with higher doses of Wnt (the data are shown in left). Likewise, DP1 inhibited Dvl- or b-catenin-mediated axis duplication even with the lower doses of Dvl or b-catenin. However, the differences were not too significant when we used higher Wnt because the axis inducing activity of higher Wnt is strong enough to be less sensitive to the DP1 overexpression than that of the lower Wnt. Therefore we had to titrate Wnt concentration to a lower level and finally optimized this experiment with lower level of Wnt mRNA, to best represent the positive role of DP1 on the Wnt signaling.



4) FUNCTION IN THE NUCLEUS OR IN THE CYTOPLASM? The use of a NLS-DP1, largely concentrated in the nucleus, to demonstrate that nuclear DP1 activity is sufficient to rescue Twist expression is quite compelling, but one would wish to see the complementary experiment, i.e. that rescue could not be achieved by a cytoplasmic from of DP1. A "NES-DP1" mentioned in legend of Fig. S6 but no data are shown. Also, an NES-DP1 would still reach the nucleus, and would actually be expected to deplete NLK from the nucleus. An alternative experiment would be to produce a myristylated or palmitoylated form that would be sequested to the plasma membrane.

: While we were preparing for previous manuscript, we did not remove “NES-DP1” from the Figure legend by mistake. Because we do not know how the localization of DP1 is determined, we decided not to include NLS/NES data in previous manuscript. When we performed experiments with NES-DP1 we also had the same concerns that the reviewer raised. Therefore we added two copies of the NES sequences to the N-terminus of DP1 in order to clearly exclude nuclear localization. Using myristylated or palmitoylated form of DP1 is another good idea, however it might cause unexpected results such as mis-localization of Axin or Dvl to the membrane which may lead to perturbation of Wnt signaling.



As shown in left Figure, NLS-DP1 couldn’t interact with Dvl1 or Axin unlike wild-type DP1. We also observed that NES-DP1 colocalized with Dvl1 in a similar pattern that wild-type DP1 did, whereas it has no influence on the localization pattern of NLK. These results strongly suggest that these DP1 constructs were usable for further experiments.

We included this data in Supplementary Figure S7B in the revised manuscript.

With these constructs we further investigated the role of DP1 according to its differential localizations. As expected, NLS-DP1 had a stronger ability to augment Wnt3a-mediated TOP-FLASH activation than that of wild-type DP1 whereas NES-DP1 did not enhance the reporter activity (Supplementary Figure S7C). Moreover, NES-DP1 still impeded the β-catenin-induced reporter activation (Supplementary Figure S7D). Consistent with our hypothesis NLS-DP1 did not induce the poly-ubiquitination of β-catenin (Supplementary Figure S7E). We next asked whether NLS-DP1 and wild-type DP1 differentially regulate Dvl- or β-catenin-induced reporter activity. Surprisingly, NLS-DP1, unlike wild-type DP1, enhanced Dvl- or β-catenin-induced reporter activity (Supplementary Figure S7F). To further confirm these results, we injected sub-optimal doses of xWnt8 or xDsh mRNA into the ventrovegetal region of the early *Xenopus* embryo. Although DP1 or NLS-DP1 alone did not induce the secondary axis (data not shown), NLS-DP1 exerted a stronger positive effect on xWnt8- or xDsh-mediated axis duplication than wild-type DP1 did (Supplementary Figure S7G). Overall, these findings suggest that the dual roles of DP1 in Wnt/β-catenin signaling are determined by its differential nucleocytoplasmic localizations.

As the reviewer suspected, NES-DP1 was exclusively absent in the nucleus of HEK293T cells but could be comparably well detected in the nucleus of *Xenopus* animal cap as wild-type DP1 could. Therefore, we could not use NES-DP1 in *Xenopus* assays to rescue DP1-MO induced A-P markers changes.

Also, as the reviewer pointed out, nuclear DP1 activity is sufficient to rescue Twist expression. However, at the same time, NLS-DP1 was not very sufficient to rescue En2 expression (Fig. 6D). Twist and En2 were used to assay positive roles of DP1 on the Wnt signaling since they are Wnt target genes at this stage. Therefore, we speculate that nuclear DP1 function cannot explain all the positive roles of DP1 on the Wnt signaling. Indeed, throughout the manuscript, we suggested two different positive regulatory mechanisms of DP1 on the signaling. One is the inhibition of NLK kinase activity, which must be dependent on the nuclear DP1 and the other is the increase of b-catenin level in the presence of Wnt, which we do not know the detailed mechanism. Perhaps the latter might be the function of cytoplasmic or membraneous DP1. Comparing membraneous DP1 (myristylated or palmitoylated DP1) and nuclear DP1 might be potentially very interesting, but we believe that this is out of the scope of the current manuscript. Our demonstration that nucleocytoplasmic localization of DP1 dictates its dual function during *Xenopus* A-P patterning is largely based on the finding that NLS-DP1 cannot rescue anterior neural markers (Fig. 6D). Therefore, we now realized that our statement in the manuscript should be changed a little while. We initially concluded from the Fig. 6 that “nuclear DP1 is REQUIRED for the activation of Wnt/b-

catenin signaling ~" (page 14 line 3-4 in the previous manuscript). We change the word "required for" to "sufficient to" in this conclusion, because nuclear DP1 is not solely responsible for the positive role of DP1 on the Wnt signaling.

5) EFFECT ON BCAT-TCF/LEF INTERACTION UNCLEAR. While the data show convincingly that DP1 "competes" with TCF for NLK, resulting in decreased phosphorylation of TCF, the key consequence of this effect, i.e. an increase in TCF/bcat interaction is not shown.

: In Fig. 3G, we tried to show that DP1 inhibits NLK autophosphorylation activity, which leads to inhibit NLK kinase activity. We do not claim that DP1 competes with TCF for NLK. NLK has been known to induce the dissociation of β-catenin/TCF complex from DNA, but not regulate the interaction between β-catenin and TCF (Ishitani et al., Nature, 1999; Ishitani et al., Mol Cell Biol, 2003). Therefore we think it is not necessary to examine the TCF/b-catenin interaction.

The manuscript is so rich that it would take pages to dissect all the different experiments, but the above remarks should be sufficient to serve as guide for a careful revision of the most crucial points that need to be validated.

CONCLUSION
Again, the work is overall really nice, with about everything one would wish in terms of controls for the biochemical as well as microscopy data. But unfortunately this mass of experiments is missing some crucial points that are absolutely required for the data to make sense. I believe that a few well targeted experiments will conclusively back up the proposed model.

: We are deeply indebted to the reviewer who suggested well targeted experiments to resolve the potential problems. Performing these experiments truly enhances the completeness of our manuscript.

MINOR POINTS:

Drosophila data should be presented in the result section.

: Yes. We presented this data in the result section.

Lef/TCF kinase assay in Suppl. Figure S5H not convincing: there is still a migration shift in the last lane (+ DP1). The fact that the shift looks smaller compared to the 2nd lane and the highest band is not seen could simply due to the significantly weaker signal.

: We repeated this experiment again and got clear data. Supplementary Figure S5H was replaced with new data.

Referee #2:

In this interesting study Kim et al. introduce a new regulator, DP1, in Wnt signalling. It was previously reported (though pretty much hidden in the literature unless systematically researched, as done by the authors), that DP1 could function either as a positive or negative Wnt regulator. By an elegant combination of biochemical, cell biological and in vivo studies encompassing cell culture, Xenopus embryos and Drosophila they provide compelling evidence for a dual role of DP1. Their key mechanistic finding is that the cytoplasmic and nuclear function of DP1 are different and that the protein switches from negative to positive in the presence of Wnt ligand. The study is comprehensive, in general well executed, and the paper is well written. I had previously reviewed this ms for another "TOP" journal and the authors have dealt satisfactorily with my past comments by direct experimentation.

I recommend publication of the ms as is.

Minor:
p.8 „truck region" should be „trunk"
: Thank you. It is fixed.

Referee #3:

Kim et al address the role of E2F-independent functions of DP1, focussing on the Wnt/b-catenin signalling pathway. The study extends and consolidates previous reports on the importance of the E2F pathway in Wnt signalling. The significance of the present study relates to the dual role of DP1, independent of E2F that acts negatively or positively depending on the level of Wnt signalling. The authors suggest that this binary role reflects the cyplasmic or nuclear location of DP1. This is quite an interesting study which suggests a novel regulatory circuit in Wnt signalling, and highlights new roles for DP1 which remains a poorly described E2F subunit. However, there are some shortcomings, both technical and otherwise, which prevent the manuscript being published. In no particular order of priority:

1) The DP1 siRNA (1B) does not appear to be very efficient. I would like to see complete knockdown, rather than partial. To introduce siRNA efficiently, I sometime transfect siRNA 2 or 3 times.
: We transfected siRNAs 2 times for 72hour, and then knockdown (KD) efficiencies of two siDP1 were about over 70%. We presented our data as it is, however, if we adjust contrast and bright a little bit, the data will be as the reviewer want to see (compare original data shown in Fig 1B (left) with adjusted data (right)). However, it is not what we should do.

More importantly, we clearly showed that these efficiencies were sufficient to reduce Wnt-induced signaling activity, including TOPflash activity, the level of cytosolic b-catenin and Wnt target gene expression. In addition, since DP1 together with E2F is one of cell cycle regulators, complete knockdown of DP1 might impede the progression of cell cycle which leads to inhibition of cell proliferation.

Ectopic/endogenous protein levels should be shown throughout for example (1D, 1F, 1G, most of SI Fig 1 etc).

: It is possible to show the ectopic protein levels, however as we know people usually do not show them in the data of luciferase assays published in decent scientific journals. In addition, no one ever show the level of Wnt3a when they used Wnt3a CM (Fig 1D).

I think "epistasis analysis", used in the context of co-expressing different ectopic proteins (1F), is not the correct scientific description.

: "To further investigate the underlying mechanisms by which DP1 regulates Wnt/β-catenin signaling, we performed *epistasis analysis*." was changed to "To further investigate the underlying mechanisms by which DP1 regulates Wnt/β-catenin signaling, we performed *more luciferase analysis with different Wnt signalling components*."

2) In 2A, I am not entirely clear what the common band in IP Flag/IB GFP is?

: It is IgG heavy chain. We marked the common band with an asterisk in Fig 2A.

The endogenous IP (2B) is not entirely convincing; please confirm that the co-IP band is DP1, and improve the axin blot.

: We purchased two commercial anti-DP1 antibodies from SantaCruz (monoclonal) and Abcam (polyconal) which have been used widely. These antibodies recognize a specific band corresponding to the size of endogenous DP1 and the level of band was specifically reduced by siDP1s, which strongly support the specificity of DP1 antibodies.

It has been shown that the level of Axin is extremely low compared to Dvl (0.02 nM vs. 100 nM in *Xenopus* extract; Lee et al. (2003) The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. PLoS Biol. 1(1):E10.). Therefore it is extremely difficult to show very strong co-IPed Axin band in the endogenous immuno-precipitated complex using anti-Dvl antibody. We wish the reviewer understand the technical difficulty. In addition, because we used the Axin/Dvl interaction, which has been generally accepted, as a positive control for the interaction between Dvl and DP1, we think that it is not crucial to perform again in this revision.

The colocalization of endogenous DP1 and Dvl/2 should be shown (SI 2C has been performed with ectopic Dvl).

: We understand the reviewer's concern. Therefore we have tried to show the interactions at the endogenous level whenever it was technically possible. However, the commercially available antibody for Dvl2 is good for western blot but not for indirect immunofluorescence (IF) analysis and anti-Dvl1 antibody works for neither western blot nor IF. The data shown in Supplementary Figure S2C is the best when we consider the technical limitation.

Fig 2D lacks the anti-Axin control treatment. Fig 2E lacks antibody controls too.

: In Fig 2D and E we tried to show the effect of presence/absence of DP1 on Dvl/Axin interaction. Although we did not perform IP with control IgG, we used myc-GFP as a negative control in Fig 2D. We do not agree that antibody control is necessary in Fig 2E.

As a control, I would like to see the effect of DP1 on b-catenin ubiquitination in plus and minus Wnt3a-CM conditions.

: We expect the level of b-catein ubiquitination in the presence of Wnt3a-CM will be very low and the effect of DP1 may not be detectable. It is interesting to see, however, we think that it may not add anything more to support or exclude our claim. Therefore we did not include in our revision.

It is not entirely clear why (2F and 2G) the altered level of B catenin ubiquitination is not reflected in protein levels?

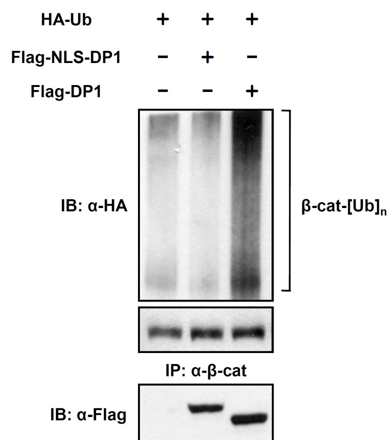
: To elucidate the effect of DP1 on the poly-ubiquitination of β-catenin, HEK293T cells were incubated with proteasomal inhibitor MG132 for 4-6hr just before harvest cells. It was a typical method to distinguish the status of protein ubiquitination. Therefore, the change of β-catenin protein level was not observed in our condition.

3) Throughout the data in Figure 2, I do not think that the authors can unequivocally rule out that nuclear DP1 and/or DP1 bound to E2F plays a role in the results. The authors should use appropriate DP1 mutants (for example no dimerization domain) to exclude these possibilities.

: As we showed that the heterodimerization domain (HD) of DP1 is required for both Axin and E2F binding, using DP1ΔHD construct to resolve reviewer's concern was not appropriated. Thus, we performed an alternative experiment to exclude the possibility raised by the reviewer. As shown in left Figure, NLS-DP1, which is incapable of interacting with Axin, does not induce the poly-ubiquitination of β-catenin. This finding suggests that negative function of DP1 in Wnt/β-catenin signaling is dependent of its cytoplasmic localization but not nuclear DP1 or DP1 bound to E2F. We presented this data in Supplementary Figure S7E in the revised manuscript.

4) The competition between Dv1, E2F and DP1 (S3G) should be confirmed under physiological conditions, say by regulating endogenous Dv1, and monitoring E2F activity.

: To clarify the reviewer's concern knockdown of *all* Dvl (Dvl1/2/3) may be required, however, it is very difficult to perform. We wish the reviewer understand the technical limitation. In addition, we only provide additional evidence in S3G to support the finding that Dvl and E2F share the same binding site of DP1, it is not worthy to perform the suggested experiment by overcoming that difficulty.



5) The evidence that DP1 interacts with NlK1 (3B) is not convincing. The efficiency and specificity of the interaction is very low under physiological conditions (is this level meaningful), and the effect of Wnt3a on the interaction should be documented under physiological conditions (3C is with ectopic protein).

: Similar as we discussed above, because the available antibodies for NLK are not that good it was hard to present clear blot as the reviewer expected. However this experiment was repeated several times with consistent data. Therefore we think our data is fairly convincing.

It should be shown that the NLK bound to DP1 is enzymatically active, to fit with the authors model.

: It might be mistyping. The reviewer must say that it should be shown that NLK bound DP1 enzymatically "inactive". Because our point is that DP1 inhibits the phosphorylation activity of NLK to Tcf/Lef1, which was clearly shown in Fig 3G and Supplementary Figure 5H, it is not an important issue whether NLK bound DP1 is enzymatically inactive or not.

Further, it is not clear whether the putative NLK phosphorylation sites (S5D) in DP1 are real physiological sites, which needs to be addressed.

: It is interesting to examine, however it does not strengthen our points and is out of the scope of the current manuscript.

The data in 3G needs a comparison to DP1 and ΔHD.

: It is good to perform experiment as the reviewer suggested. However, we used GST as a negative control in Fig 3G, and the same issue was supported in other reporter assay (Fig 3F).

6) Fig 4A needs repeating; it is not clear how the effect of xDP1-MO is compared to the control.

: The level of DP1 might not be completely reduced due to maternal DP1. However, subsequent experiments clearly suggest that xDP1-MO caused clear phenotypic changes and these were rescued by co-injection of the morpholino-insensitive mouse DP1, confirming the efficiency and specificity of xDP1-MO.

The images in Fig 6A are unclear; positive controls for nucle: and cytoplasmic staining are required.

: We think it is generally accepted that Hoechst is sufficient as the nuclear control and the remaining region that was not stained by Hoechst can be considered as cytoplasm.

The data in S6K are not complete; where is NES-DP1, plus control DP1? You must be able to show them easily.

: Thank you for careful reading. While we were preparing for our manuscript, we did not remove this from the Figure legend by mistake. We added more NES and NLS data in order to resolve concerns raised by the reviewer #1 in Supplementary Figure S7A-G.

In general, Fig 6 data should address expression levels of ectopic proteins (DP1, NLS, ΔDB etc).

The nuclear cytoplasmic arguments should be supported by biochemistry, rather than rely totally on imagining.

: In *Xenopus* developmental biology field the way of presenting data in our manuscript is generally accepted. We believe our data are clear enough to support our hypothesis. To answer the reviewer's comments we basically need to repeat all experiments in Fig 6. This is too much work and does not strengthen our data that much.

Overall, this manuscript requires a much more robust analysis of the authors model under physiological conditions. Many of the data are derived from ectopic protein conditions and/or suffer from over-interpretation.

: We agree that we have used many ectopic/overexpression experiments when they were necessary. However, we have tried to compensate the problems with knockdown experiments in cell culture and morpholino in Xenopus systems to present data under physiological condition as possible as we can.

2nd Editorial Decision 06 June 2012

Thank you for the re-submission of your manuscript to The EMBO Journal and please accept my apologies for the delay in responding. I have received the report from on of the original referees, who is favorably impressed by the improvements you made to the manuscript and now considers that it is ready for publication.

That being said, some minor details need to be addressed before we can proceed with the formal acceptance of your study. Browsing through the manuscript myself I have noticed that most of the micrographs in your figures lack scale bars, which we require for clarity. In addition, the statistical analysis of the results is not properly described. As a guide, statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply and will include a definition of the error bars used and the number of independent experiments performed – not replicates. If the number of independent experiments is less than three, use of error bars is not appropriate and one representative experiment should be provided, clearly indicating this fact. Along these lines, albeit not absolutely necessary, we recommend the use of statistical significance analysis tools to further strengthen the interpretation of the results. The statistical significance analysis tool chosen must be also clearly stated. Please keep in mind that this applies to supplementary information as well. Regarding supplementary information, I would like to add that we have noticed that some parts of the file use a red font, which should be corrected as well.

As a novel initiative in The EMBO Journal, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this initiative do not hesitate to contact me.

Thank you very much for your patience and congratulations in advance on a successful publication. Once these minor changes suggested are incorporated into the manuscript, you will receive an official acceptance letter with further instructions on how to proceed with that publication process.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The revision of this manuscript is in my view fully satisfactory.

The authors have answered most reviewers' comments. They have added two crucial experiments : the DNA rescue and the analysis of NLS/NES fusions. Both gave results that fully confirmed the previous conclusions, which is a strong test for their model. While some aspects of this regulation remain obscure, this is not surprising considering the complexity of the system. I commend the effort to face this complexity, which has been so far waved away by too many studies in the field, leaving us with a huge pile of disparate data, each of them seemingly clear and simple in isolation, but intelligible when put together. In this regards, the present study is largely above the accepted standards.

Specific comments about answers to reviewer #3:

- I support the author's conclusion for a DP1-NlK1 interaction, although it is weak. Significance cannot be inferred by the amount immunoprecipitated, first because of expected dissociation due to the huge dilution during extraction/precipitation/washes, secondly because of the possible existence of multiple pools, some of which may not be involved in this process. Significance is here best supported by the combination of evidence for physical interaction, gain/loss-of function and structure-function analysis.
- About nuclear/cytoplasmic localization, even though immunofluorescence is not a perfect criterion, it is still the cleanest demonstration at the moment. I am not aware of a convincing method for biochemical separation of nuclear (nucleosolic?) and cytoplasmic proteins in the frog embryonic cells.