JCB

Dbnl and β-catenin promote pro-N-cadherin processing to maintain apico-basal polarity

Antonio Herrera, ANGHARA MENENDEZ, BLANCA TORROBA, ANDREA OCHOA, and Sebastián Pons

Corresponding Author(s): Sebastián Pons, CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS

Review Timeline:

Submission Date:	2020-07-10
Editorial Decision:	2020-09-10
Revision Received:	2021-02-15
Editorial Decision:	2021-03-03
Revision Received:	2021-03-11

Monitoring Editor: Marianne Bronner

Scientific Editor: Dan Simon

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1083/jcb.202007055

September 10, 2020

Re: JCB manuscript #202007055

Dr. Sebastián Pons
MOLECULAR BIOLOGY INSTITUTE OF BARCELONA
Department of Cell Biology
C/ Baldiri Reixac, 10-12
Edificio Cluster, Parc Cientific de Barcelona
Barcelona, Barcelona 08028
Spain

Dear Dr. Pons,

Thank you for submitting your manuscript entitled "Debrin-like (Dbnl) and β-catenin promote pro-N-cadherin processing to maintain apico-basal polarity". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. Overall, the reviewers were enthusiastic about the study and we invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that Reviewer #1 asks for an additional experiment to demonstrate that N-cadherin maturation by β-catenin requires Dbnl (pt#1) and also questions the conclusion that aPKC interacts with N-cadherin exclusively in the AC domain (pt#2). Reviewer #2 requests IPs of endogenous N-cadherin & β-catenin to rule out effects of exogenous proteins (pt#1). Both of these reviewers also request additional controls as well as clarifications to text and figures. Reviewer #3 notes that data distinguishing phospho-pro-N-cadherin from processed phospho/unphospho-forms of N-cadherin is not convincing (pt#1) and that β-catenin's transcriptional roles on N-cadherin processing have not been conclusively ruled out (pt#2). We feel these requests and concerns are reasonable and every effort should be made to address them with more definitive data or with revised conclusions and discussion. We understand that endogenous IPs may be difficult to perform but there are also other approaches that can be used to show endogenous binding, such as Proximity Ligation Assays. We agree that the question of how Dbnl mediates N-cadherin/ β-catenin trafficking through the Golgi (reviewer#3 pt#3) is interesting but feel that addressing this is beyond the scope of this paper.

Please be sure to also include a point-by-point rebuttal for all the items raised by the reviewers.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the

policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Marianne Bronner, Ph.D.
Monitoring Editor
Journal of Cell Biology

Dan Simon, Ph.D.
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This manuscript analyses the processing of N-cadherin during the formation of apical junction in the chick neural tube. The authors had previously published that b-catenin plays an important role on apical junction maintenance in the neural tube. Here the aim is to identify the mechanism of this b-

catenin activity. They show that b-catenin promote the transition from pro-N-cadherin to mature N-cadherin in the Golgi apparatus. They identify the actin binding protein Dbnl as important b-catenin partner in this process.

Most of the experiments shown in this manuscript are conclusive and beautifully illustrated. The maturation of adherens junction proteins, and in particular N-cadherin, are poorly understood, especially in an in vivo context, as it is the case in this manuscript. The authors show a novel role for b-catenin, independently of its structural role in the adherens junction or its transcriptional role on Wnt signalling. This findings will be important for cell, developmental and cancer biologists, and therefore I recommend publication of this work in JCB, after the following issues are addressed:

1. The authors performed elegant experiments in vivo and in vitro with in all kind of combinations, but there was one experiment that was missing which it will allow to address the question whether the promotion of N-cadherin maturation by b-catenin requires Dbnl. The authors could transfect b-catenin in control versus Dbnl depleted cells, and analyse the effect on N-cadherin maturation.

2. The authors conclude that aPKC interacts with N-cadherin exclusively in the AC domain; however the AC is the only region where aPKC is detected in Fig 3, therefore it would be impossible to find a co-localization elsewhere.

3. The characterization of the new pro-N-cadherin antibody is incomplete. How do the authors know that it recognizes N-cadherin?

4. If the antibody recognizes the endogenous pro-N-cadherin, why did they not use it to analyse the effect of b-catenin on the endogenous N-cadherin processing, instead of transfected N-cadherin (Fig 5b)?

5. Fig 6F: a sh control is missing for comparison.

6. The authors claim that "Dbnl immunostaining, was mainly observed in the Golgi and in the space between the Golgi and the AC"; however I see Dbnl immunostaining almost everywhere, and definitely in the basal side of the neural tube (Fig 6G).

Minor comments

-no loading control for the western blots?
-the code used in Fig 2 of "structural and transcriptional up or down" is confusing and it should be made more clear in text and figure.
-Fig 5B: why the pro-N-cad band looks smaller in the b-catenin lane compared with the pCIG lane?

Reviewer #2 (Comments to the Authors (Required)):

Summary:
This manuscript shows that N-cadherin interacts with β-catenin in its phosphorylated full-length form (pro-N-cadherin) in the Golgi prior to being processed by Furin and inserted into the apical membrane. Interaction with Dbnl is necessary for the maturation of N-cadherin via Furin and the formation of adherens junctions in the posterior neural tube (early spinal cord). Using high resolution imaging and multiple gain and loss of function experiments in addition to Mass Spectrometry, the

authors demonstrate nascent knowledge about the molecular mechanisms involved in in vivo processing of N-cadherin, and show that excess pro-N-cadherin leads to a loss of the apical adherens junctions causing abnormal neural tube morphology and integrity. Overall, this paper provides strong mechanistic information about the intracellular post-translational processing and function of adherens junction proteins during early trunk neural tube development. The experiments and results in the manuscript are clearly a significant amount of work, and I believe that the paper will move the field forward with an understanding of cell-cell adhesion proteins, differential processing and function, identification of new protein-protein interactions in vivo, and understanding how those molecular events affect the development of epithelial tissues.

Minor/Moderate revisions:
1. The manuscript would benefit from grammatical editing. There are a number of small (adherents vs. adherens, acting vs. actin, etc.) and moderate (sentence structure) language and spelling errors that should be corrected prior to resubmission.

2. It would be helpful if the authors can either add letters to individual sections in figures or put boxes around related data because it is hard to follow each figure as they are currently laid out. They seem to follow both left to right and top to bottom directions in single figures. Additionally, in Figure 2, the panels are so sparsely labeled (there are 36 different panels and the figure is literally separated only in the 2A, B, C and D) it is difficult to link the section text with the appropriate results.

3. In figure 1H the top labels do not correspond with the lanes in the gel. These should be corrected.

4. Methods: The authors should add references for any vectors they used in the study that were not generated in the lab.

5. Please add more rationale for why specific treatments are considered "structural" or "transcriptional".
a. For Figure 2A, are there papers published that describe a preference for TCF3 by β-catenin over cadherin proteins? If so, please add reference. If not, please indicate in manuscript why excess constitutively active TCF3 would increase the transcriptional activity of β-catenin.

6. In the section titled "β-catenin is associated to N-cadherin in the Golgi apparatus" the authors refer to Figures A, B, D, and E prior to referring to C. Please either re-arrange the figure or text so that text matches the figure order.

7. In the sentence, "We observed a similar Golgi distribution in the NSCs of the developing neural tube (Fig. 3 C), and calculated the colocalization index of N-cadherin/β-catenin in the Golgi, in the AC-to-Golgi and in the AC regions (Fig. 3, D and E)." It should be specified that the noted expression is in the trunk/posterior neural tube in contrast from previous findings in the developing brain cortex.

8. It would be helpful in Figure 3D if the authors included the grayscale/black and white images for the N-cadherin/β-catenin /golgi staining because it is difficult to see the overlap using the red/green/blue. Also, if possible, the authors should use more color-blind friendly color combinations in any figures that are currently red/green.

9. It is unclear in Figure 3H whether the IHC in the panels is for N-cadherin or if they are just showing the localization of the N-cadherin-GFP fusion construct. They should add a panel showing apico-

basal localization of endogenous N-cadherin in addition to the images shown.

10. To see the Dbnl expression more clearly in Fig. 6G, please add a Dbnl/GM130 only overlay as well.

11. The staging used in figure 5 is unclear. The text says that the lysates were isolated from HH12 chick neural tubes, but the figure shows that the experiment was performed at HH12 and the neural tubes were isolated 24 hours later. Please clarify the actual stage used for all experiments in the text as HH12+ 24 hours could mean a number of stages depending on egg quality, temperature incubated, etc. Either use somite stage or HH stage as the tissues are so dynamic it is important to note the actual stage of tissue fixation or collection.

12. In figure 5E the authors stage that AP treatment, "turned the two bands into one, showing that the molecular weight difference between the two pro-N-cadherin bands was due to phosphorylation (Fig. 5 E)." But there are still two bands. The language should be changed to reflect that the higher MW band seems to become more dense/darker and the smaller lighter/less dense suggesting that there is less dephosphorylation occurring, but it is not eradicated by the treatment.
a. Please indicate stage of embryo in figure legend for 5B.

13. The labels in Figure 7A and 7C are different. A says proNcad/Ncad/GFP while C says proNcadh/Ncadh/GFP. Is this intentional?

14. Supplemental figures:
a. In figure S4, the authors show IHC for N-cad/Pro-N-cad in embryos injected with shRNAs to Golgi proteins, but it is impossible to tell if there are actual differences in the levels/localization of N-cadherin since they only show the overlay. Please include the individual channel images.
b. Figure S5B uses Top-Flash as a representation of β-catenin transcription, but Top-Flash is actually a canonical Wnt-signaling β-catenin mediated transcriptional reporter. If the authors want to represent changes in β-catenin transcription after injection of the N-cadherin constructs, they should analyze β-catenin transcript expression. Otherwise, they should re-label the figure to read β-catenin-mediated transcription for the output of that assay.

Moderate revisions
1. In figure 1, the authors overexpressed tagged expression constructs coding for N-cadherin, β-catenin, and aPKC and performed co-IP assays to identify potential interacting proteins. They demonstrate that the tagged N-cadherin pulls down "very little" β-catenin. See related comments below.
a. Can the authors perform the experiments in Figure 1 using pull downs with endogenous proteins? Minimally, they should comment on similar experiments that were previously published (Rogers et al., Mech of Dev. 2018)- comparing the methodological differences with their own results. Their data are solid, but there is always the fear with overexpression pull downs that the interactions can be caused by artifacts of exogenous proteins.
b. Additionally, the authors should either change the language in the text or quantitate the bands because it is difficult to understand what they mean by "very little" β-catenin, as the bands in that gel do not seem markedly less robust than the other blots they show.
c. Similarly, in Figure 6: Since they have created antibodies specific to the pro-N-cadherin, processed N-cadherin and the ST domain, it seems feasible to use endogenous proteins for basic demonstration of interactions while tagged mutated forms are necessary to demonstrate lack of interaction (Fig. 6H panel 2 for example).

2. In figure 2, all transcripts that are knocked down or overexpressed should be verified by in situ hybridization or some other method. Figure S1 shows overexpression controls, and Figure S4 has knockdown controls in vitro (chick embryonic fibroblasts) and in vivo for the Golgi protein knockdowns, but 1) in vitro cells may not have the same response as the in vivo neural tube, and 2) the manuscript is lacking controls for the knockdowns from Figure 2. Therefore, it is necessary to confirm that the shRNA constructs reduce expression in vivo in the chick neural tube.

3. In figure 2, the change in cell states are characterized by structural up/down or transcriptional up/down, but without IHC to verify the changing membrane (structural) or nuclear (transcriptional) localization of β-catenin , it is difficult to make those conclusions about the treatments. I would recommend either confirming the changed intracellular localization of β-catenin using IHC, referencing papers that show these data for the treatments that are used, or altering the description/titles to something that can be confirmed.

4. In Figure 4A (or in supplemental), the authors should perform IHC/ICC in the HEK cells using an antibody against N-cadherin paired with the staining for the ST tag to verify that the localization of the protein matches that of the tagged version to ensure co-localization with golgi/ER.

5. The western blots in Figure 5A and B should include a loading control. Without a loading control it is impossible to tell that, "Notably, β-catenin knockdown severely reduced mature N-cadherin band, whereas β-catenin expression selectively erased the upper pro-N-cadherin band (Fig. 5 B)," because the pro-n-cadherin band was also reduced by β-catenin knockdown compared to the sh-scramble.

6. The phenotype in Figure 7G caused by overexpression of the uncleavable N-cadherin seems to show cells migrating out of the neuroepithelium. The authors should probe for a neural crest marker (Pax7, Sox9, Sox10, etc) to ensure that these cells are not changing fate and delaminating/migrating as neural crest and to confirm that the neural tube progenitors/precursors are expanding. Recent work from the Sauka-Spengler lab (Williams et al., Dev. Cell., 2019) identified that premigratory NC cells express Sox2 along with NC specifiers.

Reviewer #3 (Comments to the Authors (Required)):

Rationale/context: This group previously found that forced expression of an oncogenic/degradation-resistant form of bCat led to enlarged apical junctional complexes (AJCs) in neuroepithelial stem cells using the chick neural tube as a system (Herrera et al., 2014, Nat. Comm). The current study aims to address the molecular means by which over-expressed bCat promotes this AJC expansion, distinguishing between bCat's direct role in binding/trafficking of cadherins versus its established transcriptional role in this process.

Findings: The authors present evidence that over-expressed bCat enhances N-cadherin precursor processing along the biosynthetic pathway by directly binding the N-cadherin bCat-binding domain. This model is mostly based on analysis of N-cadherin constructs lacking the bCat binding domain, expressed in either chick neural tube or HEK293 cells. These data are well-presented but largely confirm previous studies showing the requirement of bCat to cadherin processing and cell surface trafficking (e.g., Hinck et al., 1994, Chen et al., 1999, McEwen, 2014). Evidence that N-cadherin processing is enhanced by bCat lacking its C-terminal transcriptional transactivation domain is used to rule out over-expressed bCat enhancing trafficking through up-regulating transcriptional targets

of bCat- but this interpretation is not definitive given that bCat's N-terminus also contains transcriptional co-activator functions via Pygopus/Bcl9. The most novel aspect of the study is identification of proteomic partners of the N-cadherin/bCat complex, one of which, Dbnl, is validated by knock-down to block N-cadherin/bCat trafficking. However analysis of the Dbnl/bCat/N-cadherin interaction and how it drives anterograde trafficking is under-developed. Other data are presented which seem to address lingering questions from this group's previous work-e.g., N-cadherin/bCat is required for aPKC localization to apical junctions to a sub-region more apical than where the N-cadherin-adherens junctions are localized. Evidence that Pro-N-cadherin is anti-adhesive was well documented by David Colman's lab years ago, although it is nice to see this observation reinforced in the context of the neuroepithelial stem cell system (especially designing a non-Pro-cleavable form, FXa-Ncad). Lastly, the authors claim that bCat binds phosphorylated, pro-N-cadherin and promotes its conversion into mature N-cadherin, but the data are more suggestive than decisive, interpreted largely from gel shifts post-phosphatase treatment.

Assessment: Overall, this is thoughtful and largely well-presented study addressing how overexpressed bCat drives AJC expansion and N-cadherin targeting. Much of the conclusions reinforce previous studies, and the most novel aspect of the study (how Dbnl promotes N-cadherin trafficking) is insufficiently developed.

Specific Criticisms:

1. Figure 5F- I am not sure that one can confidently assign/distinguish Phospho-pro from processed phospho/unphospho-forms of N-cadherin from gel-shift analyses alone (schematic). I am particularly skeptical that one can distinguish fully processed N-cadherin phosphor-from un-phospho-N-cadherin, especially given evidence from Bill Weis's group that a cadherin tail without bCat binding is unstable. Moreover, one might expect that forced expression of bCat would force association with and stabilize the unmodified cadherin-tail, but in fact, Figure 5F does not show this.

2. The authors claim that forced expression of bCat expands the AJC through direct binding to N-cadherin rather than any nuclear signaling activity, based on evidence that a bCat missing its C-terminal "transactivation domain" phenocopies WT bCat. However, it is also known that bCat's N-terminus has transactivation function- via Pygopus and possibly other interactors (ref). So the authors have not really ruled out bCat's capacity as a transcriptional co-activator here.

3. Dbnl knock-down experiment showing elevation of the Pro-form of N-cadherin and loss of AJs is interesting, and is certainly consistent with the model that Dbnl contributes to N-cadherin/bCat trafficking through the Golgi (especially given known roles of Dbnl in Golgi anterograde trafficking), but clear evidence this model is lacking. Said another way, there are many perturbations that could lead to increased N-cadherin biosynthesis and detection of the Pro-form, or reduced cell-cell adhesion and slowed trafficking. For a paper titled Debrin-like (Dbnl) and bCat promote pro-N-cadherin processing to maintain apico-basal polarity, I think that further analysis of how Dbnl achieves this is expected.

Minor Issues:

1. Stylistic: The first half of the Discussion is thoughtful and well-integrates this group's findings with the studies that have come before. The second section speculating whether bCat tumor suppressor activity may be due to its ability to promote N-cadherin processing in the context of Wnt-driven medulloblastomas seems more appropriate for a review than a central discussion point in this manuscript.

202007055R Response to Reviewers

Reviewer #1

1. The authors performed elegant experiments in vivo and in vitro with in all kind of combinations, but there was one experiment that was missing which it will allow to address the question whether the promotion of N-cadherin maturation by b-catenin requires Dbnl. The authors could transfect b-catenin in control versus Dbnl depleted cells, and analyze the effect on N-cadherin maturation.

We want to thank the reviewer, following his/her advice we have done the proposed experiment that has greatly reinforced our previous results about the implication of DBNL on b-catenin mediated maturation of N-cadherin. Although immunochemistry quantification of Pro-N-cadherin and N-cadherin after shRNA transfection resulted a very useful tool to scan the effect of the different Golgi proteins shown in Fig 6, to really measure the effect of DBNL depletion on b-catenin mediated maturation of N-cadherin we needed to quantify the ratio between N-cadherin and pro-N-cadherin by a more exact method. Moreover, although treatment with shRNA against DBNL induced a detectable increase of pro-N-cadherin, we recently learned that the dominant negative mutant of DBNL (DBNL-2F) used in an interesting work where the authors demonstrate that DBNL regulates the amount of N-cadherin expressed on the plasma membrane of the cortical neurons (Inoue et al., 2019), was the perfect tool to perform the experiment suggested by the reviewer. Therefore, using DBNL-2F we have studied the ratio of N-cadherin maturation by western blot using purified Streptactin fractions. As expected, DBNL-2F expression induced Pro-N-cadherin accumulation, in addition, expression of β-catenin incremented the ratio of mature N-cadherin over pro-N-cadherin, but notably, the effect of β-catenin disappeared when DBNL-2F and β-catenin were transfected together. Therefore, β-catenin requires DBNL to promote N-cadherin maturation. This new experiment is now Fig 7D.

2. The authors conclude that aPKC interacts with N-cadherin exclusively in the AC domain; however the AC is the only region where aPKC is detected in Fig 3, therefore it would be impossible to find a co-localization elsewhere.

We absolutely agree with the reviewer in that. In chicken neural tube at the stages studied, aPKC is exclusively detected in the AC. Of course, it does not mean that no aPKC exists out of the AC, it means that with the available reagents we only detect it at the AC, in spite that this particular antibody is very good. For example, b-Catenin antibody is also very good, at the AC we detect b-catenin and aPKC more or less with the same intensity. However, although we detect abundant b-catenin also in the vesicular system we do not detect aPKC out of the AC. The fact that we only detect aPKC at the AC tells us that aPKC in mostly accumulated at the AC and if it were present in the same

vesicles as N-Cadherin and b-Catenin in the same proportion as they are, with this antibody we should detect it. Therefore, we believe that the images are clear enough to affirm that if aPKC forms complexes with b-Catenin and N-Cadherin out of the AC, it is necessarily in a very low proportion.

3. The characterization of the new pro-N-cadherin antibody is incomplete. How do the authors know that it recognizes N-cadherin?

We cannot agree with the referee on this. I will now enumerate several evidences that in our opinion fully confirm that anti-Pro-N-Cadherin antibody mostly recognizes Pro-N-cadherin but not N-Cadherin or nonspecific bands.
1- Western blots in Fig 5A and Fig 5B show that anti-Pro-N-Cadherin recognizes two bands that run just above mature N-Cadherin, either in a blot of untransfected lysates or in a blot where N-cadherin-ST has been transfected and purified through streptactin affinity columns. Of course the mentioned two bands could be non-specific in the total lysates, but it is very unlikely that this same two bands appear in a highly purified sample if they do not contain the ST tag. Yes, although very unlikely the two bands could be some protein that binds to N-cadherin and are recognized by the anti-Pro-N-cadherin antibody. To rule out this possibility we have the evidence number two.
2- In Fig 5B we now show that the two bands detected with the anti-pro-N-cadherin antibody in overexpressed and column purified N-Cadherin-ST, are also detected with the anti ST antibody.

4. If the antibody recognizes the endogenous pro-N-cadherin, why did they not use it to analyse the effect of b-catenin on the endogenous N-cadherin processing, instead of transfected N-cadherin (Fig 5b)?

Transient transfection of chicken embryonic neural tubes has demonstrated qualities unmatchable in many aspects because its versatility, immediacy and accuracy compared to transgenic mouse models. However, it has the inconvenience that only a part of the cells are transfected. The percentage of transfected cells vary from one embryo to another but we calculated that it reaches at best 50%, being more often closer to 25%. In addition, in our experience is impossible to be consistent dissecting similarly transfected pieces of neural tube. Therefore, when we evaluate endogenous Pro-N-cadherin and N-cadherin expression in neural tubes where b-catenin has been expressed or suppressed, our sample will contain only about 30% of the cells where b-catenin expression has been modified, the rest are just untransfected cells, with the aggravating circumstance that this percentage is not constant. A high proportion of untransfected cells in the sample may not be a problem if what we investigate is the appearance of a protein that is not endogenously expressed, but in our case the variable amounts of Pro-N-cadherin and N-cadherin coming from the untransfected cells would make unreliable any observed variation. By electroporation and purification of ST tagged N-cadherin we ensure that this N-cadherin has been produced exclusively in the same cells that also express the other constructs used to modify the conditions (either ShRNAs or proteins), as a

result, the reliability of the experiment is mostly independent of the percentage of transfection. For cDNA expression arrays we have successfully used cell sorting of transfected cells, unfortunately the sorting process requires trypsin treatment that is not compatible with plasma membrane protein studies by western blot. Therefore, we believe ST tagged proteins transfected at low concentrations is the most reliable and realistic method to study N-cadherin processing in the developing neural tube.

5. Fig 6F: a sh control is missing for comparison.

Thank you, we have added the requested control. Please, notice that due to new data added in this section we decided to split the old Fig 6 in Fig 6 and Fig 7, the requested control is now in Figure 7B.

6. The authors claim that "Dbnl immunostaining, was mainly observed in the Golgi and in the space between the Golgi and the AC"; however I see Dbnl immunostaining almost everywhere, and definitely in the basal side of the neural tube (Fig 6G).

We absolutely agree with the referee that in agreement with its multi functionality, in neural tube, Dbnl presents a wide-spread localization as in other tissues where has been studied. We have changed the previous sentence mentioned above by:
“In the neural tube, Dbnl presented a wide-spread localization including the Golgi region and the space between the Golgi and the AC”. Please notice that the referred Fig 6G is now Fig 7A.

Minor comments

-no loading control for the western blots?

Loading controls are required when the intensity of a protein is related to the total amount of protein loaded in each lane and the different lanes are compared to each other. None of the westerns presented in this work is used for that purpose. We consider that in the purification experiments the correct controls are the starting lysate, the last wash and the elution fractions that tells it is an specific interaction. If a purified protein is used to do different treatments, we assume the only difference in the amount of protein loaded must be the pipetting error, but again no loading control is applicable here. In the cell fractioning experiments, we used compartment markers to demonstrate that the fractioning was correct. In fig 5 and 7 we use the western to calculate the ratio of pro-N-cadherin and N-cadherin within each lane, and then we compare the ratios, therefore each lane acts as an individual experiment. Because this is an important issue to as, we will enumerate all the westerns shown in this work to show that all have the correct controls:

Westerns in Figure 1 are all elutions of affinity column purifications. In each purification the input lysate lane is shown.

Western in Figure 5A, evaluates de signal using increasing amounts of protein. (increasing embryos), just to see the antibody works at different proteins concentrations.
Westerns in Figure 5 B-I and K we use purified proteins to study the ratio between two bands within the same lane or to study the apparent molecular weight of this bands.
Western in Figure 5 J, as in Fig1 are elutions of affinity column purifications and the input material is shown.
Western in Figure 7C are also elutions of affinity column purifications.
Westerns in Figure 7D as in Fig5 we use purified proteins to study the ratio between pro-N-cadherin and N-cadherin within the same lane.
Western in Figure 8B,D, are also elutions of affinity column purifications.
Western in Figure S2E compares the amount of ST (N-Cadherin or N-Cadherin delta b-Cat) in each cell fractions in the presence or not of b-Catenin.

-the code used in Fig 2 of "structural and transcriptional up or down" is confusing and it should be made more clear in text and figure.

Sorry about that, we have now added a box in Figure 2 where the different elements used to describe the expected effect of the constructs used on structural and transcriptional activity have been clearly exposed. We have also added a paragraph in results to clearly explain what we me mean by Structural and Transcriptional activities of b-catenin.

-Fig 5B: why the pro-N-cad band looks smaller in the b-catenin lane compared with the pCIG lane?

We do not believe it looks smaller. In in this particular lane the bands have an smile shape, also evident in N-cadherin band. When this happens the belly of the smile runs lower than it should. Maybe the lanes in the gel did not follow a perfect straight line too. These situations are common with minigels, but we prefer not to rotate parts of a gel separately. We believe the quality of this particular western is quite good. In any case in the western blot the mentioned lower Pro-N-Cadherin band is located just above the mature N-cadherin band that is where we normally observe it.

Reviewer #2 (Comments to the Authors (Required)):

Minor/Moderate revisions:

1. The manuscript would benefit from grammatical editing. There are a number of small (adherents vs. adherens, acting vs. actin, etc.) and moderate (sentence structure) language and spelling errors that should be corrected prior to resubmission.

Thank you. We have now deeply screened for spelling typos and the language has been reviewed at www.biomedred.com professional proofing service.

2. It would be helpful if the authors can either add letters to individual sections in figures or put boxes around related data because it is hard to follow each figure as they are currently laid out. They seem to follow both left to right and top to bottom directions in single figures. Additionally, in Figure 2, the panels are so sparsely labeled (there are 36 different panels and the figure is literally separated only in the 2A, B, C and D) it is difficult to link the section text with the appropriate results.

Thank you. We have now named each panel with a different letter where it was possible and we have boxed together all the result groups.

3. In figure 1H the top labels do not correspond with the lanes in the gel. These should be corrected.

Thank you. It has been corrected.

4. Methods: The authors should add references for any vectors they used in the study that were not generated in the lab.

Thank you. We have added references for all the constructs not generated in the laboratory and also for the cloning vectors used that were not from commercial origin.

5. Please add more rationale for why specific treatments are considered "structural" or "transcriptional".

In the introduction section of our manuscript we say:

“β-catenin mediates canonical WNT signalling, stimulating Tcf dependent transcription (Grigoryan et al., 2008; Nelson and Nusse, 2004). However, β-catenin also plays important roles in epithelial cell polarity, for example associating with classic cadherins through its armadillo domains and thereby contributing to Adherens Junction (AJ) formation (Baum and Georgiou, 2011).” The concept of structural and transcriptional activities of b-catenin is based on the fact that b-catenin on one hand acts at the AJs binding N-cadherin with components of the actin cytoskeleton (Structural because works on cell structure) and on the other, binds to Tcf transcription factors displacing Groucho

(that is a repressor) and consequently acting as a transcription activator, in fact it is a transcription co-activator because by itself does not regulate transcription, for that it will always need the Tcf factors (Transcriptional activity because regulates transcription).The interaction of b-catenin with N-cadherin and Tcf is in both cases mediated by the armadillo domains. It is curious that the same domain is used to carry out two such disparate activities. As the reviewer suggested, we have modified the text in the first part of results for Fig2 to make all these concepts clearer, the new text is as follows:

“As mentioned above, β-catenin binds N-cadherin at the AP linking the AJs with the actin cytoskeleton, but also binds to Tcf transcription factors displacing Groucho, a transcriptional repressor. We named these two activities of β-catenin as structural and transcriptional because they regulate cell structure and gene transcription, respectively. Notably, the interaction of β-catenin with N-cadherin and Tcf is mediated in both cases by the armadillo domains of β-catenin. Therefore, changes in β-catenin expression will necessarily affect both activities. In contrast, the expression Tcf3·Vp16, a constitutive activator of TCF binding motives that does not bind β-catenin, would induce a β-catenin-like transcription activation. In addition, N-cadΔβcat, a truncated form of N-cadherin that does not bind β-catenin, displaces the endogenous N-cadherin inhibiting its structural activity but not affecting its capacity to interact with Tcf.”

a. For Figure 2A, are there papers published that describe a preference for TCF3 by β-catenin over cadherin proteins? If so, please add reference. If not, please indicate in manuscript why excess constitutively active TCF3 would increase the transcriptional activity of β-catenin.

We want to apologize for this misunderstanding, the Tcf3·Vp16 construct is the fusion of the Vp16 transcriptional activator with the HMG (DNA binding domain) box of Tcf3, it does not have the b-catenin binding domain. Therefore, no competition occurs between N-Cadherin and Tcf to bind b-catenin. Tcf3·Vp16 is a pure transcriptional activator of TCF sites, the only sites used by b-catenin. A TOP-Flash reporter assay (TCF dependent activity) comparing the activity of b-Catenin and Tcf3·Vp16 is shown in Fig S1N and Herrera et al 2014.

6. In the section titled "β-catenin is associated to N-cadherin in the Golgi apparatus" the authors refer to Figures A, B, D, and E prior to referring to C. Please either re-arrange the figure or text so that text matches the figure order.

Thank you. It has been corrected.

7. In the sentence, "We observed a similar Golgi distribution in the NSCs of the developing neural tube (Fig. 3 C), and calculated the colocalization index of N-cadherin/β-catenin in the Golgi, in the AC-to-Golgi and in the AC regions (Fig. 3, D and E)." It should be specified that the noted expression is in the trunk/posterior neural tube in contrast from previous findings in the developing brain cortex.

Thank you. It has been corrected.

8. It would be helpful in Figure 3D if the authors included the grayscale/black and white images for the N-cadherin/β-catenin /golgi staining because it is difficult to see the overlap using the red/green/blue. Also, if possible, the authors should use more color-blind friendly color combinations in any figures that are currently red/green.

We absolutely agree with the reviewer; it is difficult to see the degree of overlapping in the three channel picture. Because we believe the false color images are still informative, we decided to keep them and in addition add three more panels where we combined b-Catenin/N-Cadherin, N-Cadherin/GM130 and b-Catenin/GM130 using green/purple palette. We believe in this case it is worth to show the three combinations of two channel to clearly appreciate the overlapping areas.

9. It is unclear in Figure 3H whether the IHC in the panels is for N-cadherin or if they are just showing the localization of the N-cadherin-GFP fusion construct. They should add a panel showing apico-basal localization of endogenous N-cadherin in addition to the images shown.

We are sorry if it was not clear before. Figure 3G shows the distribution of endogenous N-cadherin (green)/phalloidin(red) and Figure 3H their apico basal distribution. In addition, Figure 3I and Figure 3J shows GFP signal of transfected N-Cadherin·GFP fusion protein. We have changed the label of this panel and made it clearer in the figure legend.

10. To see the Dbnl expression more clearly in Fig. 6G, please add a Dbnl/GM130 only overlay as well.

We added two new images of DBNL/N-Cadherin and DBNL/GM130 using green/purple palette. Please note that Fig 6G is now Fig 7A.

11. The staging used in figure 5 is unclear. The text says that the lysates were isolated from HH12 chick neural tubes, but the figure shows that the experiment was performed at HH12 and the neural tubes were isolated 24 hours later. Please clarify the actual stage used for all experiments in the text as HH12+ 24 hours could mean a number of stages depending on egg quality, temperature incubated, etc. Either use somite stage or HH stage as the tissues are so dynamic it is important to note the actual stage of tissue fixation or collection.

Thank you, absolutely agree with the reviewer, it was very confusing. We have changed them all. Now we primarily indicate the final HH stage at which the experiment was done and then we also indicate the stage at which the embryos were electroporated and for how long.
In our conditions, HH12 embryos electroporated for 24 and 48 h, normally reached stages HH18 and HH23, respectively. Embryos that did not develop to the expected stages were discarded. To stage the different embryos we used the images published in:

https://embryology.med.unsw.edu.au/embryology/index.php/Hamburger_Hamilton_Stages. Thus, we have now added (in the text and in the figures), in addition to the electroporation time, the stage of the embryos used in all experiments. In addition, we have also added a paragraph in methods (in "Chick embryo *in ovo* electroporation") explaining this question.

12. In figure 5E the authors stage that AP treatment, "turned the two bands into one, showing that the molecular weight difference between the two pro-N-cadherin bands was due to phosphorylation (Fig. 5 E)." But there are still two bands. The language should be changed to reflect that the higher MW band seems to become more dense/darker and the smaller lighter/less dense suggesting that there is less dephosphorylation occurring, but it is not eradicated by the treatment.

We apologize if a misunderstanding occurred with these blots (C,D,E). As you observed, in addition to the two upper bands which we labelled with blue (upper) and purple (lower) arrows, a very faint band running just below 135 KDa was also observed, we always considered this band as non-specific or a degradation product because its size was not modified by any of the treatments. Thus this band, although very weak, it was observed in the untreated lanes of C, D and in E and in the treated lane of E, in treated lanes of C and D it was masked because the deglycosylated pro-N-cadherin runs on top of it. However, it is by far less intense than the two upper ones. What we defend is that the two labelled bands (blue and purple arrows) turn into one (purple), we defend that because the purple band significantly increases its intensity after AP treatment. On the contrary the non-specific band (that runs under 135) does not significantly change in size or intensity. To make the blots clearer we have added an non-specific label (NS) to this extra band. We added the correspondent information in the figure legend.

a. Please indicate stage of embryo in figure legend for 5B.

Thank you, it has been done.

13. The labels in Figure 7A and 7C are different. A says proNcad/Ncad/GFP while C says proNcadh/Ncadh/GFP. Is this intentional?

Thanks a lot, it was a mistake. It has been corrected. Please note that Fig 7 is now Fig 8.

14. Supplemental figures:
a. In figure S4, the authors show IHC for N-cad/Pro-N-cad in embryos injected with shRNAs to Golgi proteins, but it is impossible to tell if there are actual differences in the levels/localization of N-cadherin since they only show the overlay. Please include the individual channel images.

Sorry about that, we have now included the N-cadherin and Pro-N-cadherin channels in grey scale palette for each ShRNA tested.

b. Figure S5B uses Top-Flash as a representation of β-catenin transcription, but Top-Flash is actually a canonical Wnt-signalling β-catenin mediated transcriptional reporter. If the authors want to represent changes in β-catenin transcription after injection of the N-cadherin constructs, they should analyze β-catenin transcript expression. Otherwise, they should re-label the figure to read β-catenin-mediated transcription for the output of that assay.

Thank you, we changed de label.

Moderate revisions
1. In figure 1, the authors overexpressed tagged expression constructs coding for N-cadherin, β-catenin, and aPKC and performed co-IP assays to identify potential interacting proteins. They demonstrate that the tagged N-cadherin pulls down "very little" β-catenin. See related comments below.
a. Can the authors perform the experiments in Figure 1 using pull downs with endogenous proteins? Minimally, they should comment on similar experiments that were previously published (Rogers et al., Mech of Dev. 2018)- comparing the methodological differences with their own results. Their data are solid, but there is always the fear with overexpression pull downs that the interactions can be caused by artifacts of exogenous proteins.

We agree with the reviewer in that expressed proteins may give false interactions if not used properly. Co-immunoprecipitation (CoIP) of endogenous proteins has long been used to describe new interactions among proteins, however, not all described interactions using this technique have been proven true. The main advantage of using endogenous proteins is that the physiologic molar ratio among the tested proteins is always correct. On the other hand, it fully depends on the existence of the appropriate antibodies. Not all antibodies IP properly or are specific enough, but all these problems can be solved finding the right ones. However, there are several problems that cannot be solved because they are intrinsic to the IP itself. 1) The CoIP requires a strong interaction that has to be maintained during the CoIP process, even when the environment in which this interaction was created is disrupted. 2) The recognition epitope of de IP antibody cannot be masked by the interaction. 3) The stringency that can be applied (ionic detergents and caotropics) during the CoIP has to be maintained low to preserve the interaction of the molecule with the antibody and between the two molecules that are CoIP. 4) Finally, the problem that created most of the artefacts in CoIPs, the proteins are liberated from the purification bed with a caotropic (SDS or Urea) which stringency is much higher than the washes. Of course this last inconvenience can be solved carrying the proper controls. We began using ST3-Streptactin affinity purification to describe N-cadherin associated proteins by mass spectroscopy, the study was intended to find direct and indirect interactions and we were especially interested in interactions that were environment specific or transitory, consequently, we looked for a method that would allow us to stabilize the interactions and at the same time allow us to apply great stringency during purification washes. We followed the recommendations published in (Klockenbusch and Kast, 2010) to mildly cross-link the complexes and applied the protocols developed in (Kubben et al., 2010) to purify these cross-linked complexes using the ST3-Streptactin purification system. The biggest

advantages of ST3-Streptactin system are: first, due to the extremely high affinity of Streptactin for the ST3-tag, the complexes to be purified can be dissolved in relatively high amounts of caotropics, ensuring the access to cellular compartments that otherwise remain insoluble. Second, but not less important, the elution is carried out at the same or less stringency than the washes, because it is an affinity elution, done with biotin (a competitor of the ST3-tag). Of course the complexes can be latter de-crosslinked for their characterization either by mass spectroscopy or western blot. The fact that most of the known interactions of N-caherin were detected in our mass spectroscopy study using the ST3 method, convinced us of the potentials of this method. Before finding ST3 method, we planned to use CoIPs of endogenous proteins, however, we were very concerned whether our antibodies against N-Cadherin would recognize with the same affinity the N-Cadherin present in the different cellular compartments and whether the different cellular compartments would be soluble in the maximum stringency allowed. Unfortunately, we discovered that N-Cadherin antibodies recognized mainly the mature N-cadherin, even worst, we found out that using the maximum stringency allowed in a normal CoIP, most of the immature forms of N-cadherin remained insoluble. Therefore, we realized that to discover and study new interactions of N-cadherin that occurred away from the plasma membrane the classical CoIP technique was not appropriate. To maximize the similarity of our study to physiology, we transfected low concentrations of DNA for only 24h. We firmly believe that this technology is at the present one of the best to answer the questions we tried to solve in this work, we agree that proximity ligation is also very useful to detect interactions but again in that case very specific antibodies are required and is not a discovery tool. On the other hand, similar results could have been obtained with Bio-Id vicinity labeling, but again it requires transfection and at least in our hands ST3-streptactin technique is superior. I want to make it clear that although not appropriate for our work, CoIP is a great technique to describe stable interactions as the one between N-Cadherin and E-Cadherin, described in the interesting paper that the reviewer kindly suggested for discussion (Rogers et al., 2018). We have now added the methods references and more rational about why we used the ST3 and cross-linking and discussed our results in the context of Rogers et al paper.

b. Additionally, the authors should either change the language in the text or quantitate the bands because it is difficult to understand what they mean by "very little" β-catenin, as the bands in that gel do not seem markedly less robust than the other blots they show.

Sorry about that, it was a copy paste mistake the “very little” should not be there, it initially referred to the amount of aPKC that was later described as “almost no aPKC”. It has been corrected.

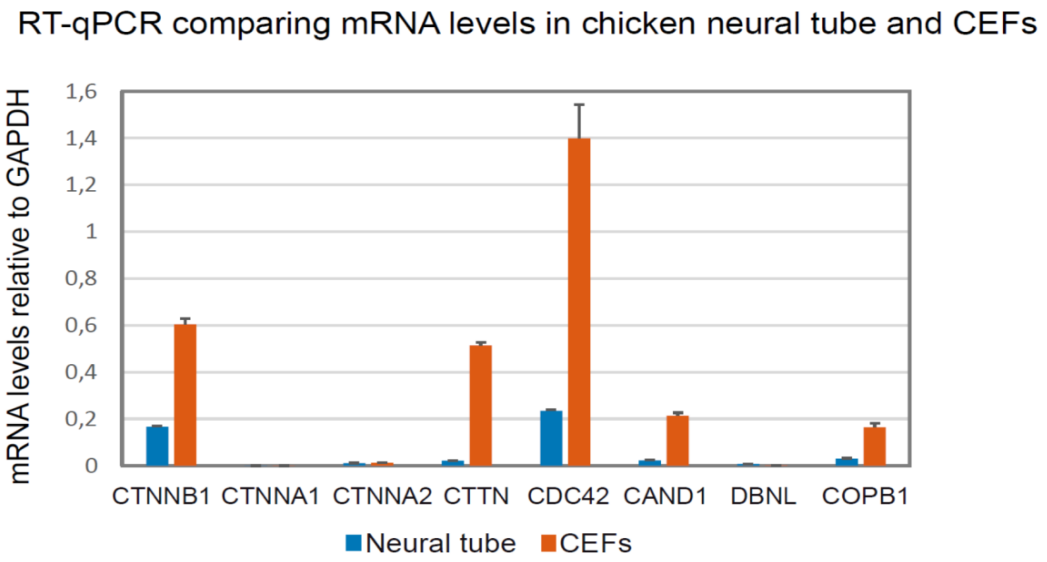
c. Similarly, in Figure 6: Since they have created antibodies specific to the pro-N-cadherin, processed N-cadherin and the ST domain, it seems feasible to use endogenous proteins for basic demonstration of interactions while tagged mutated forms are necessary to demonstrate lack of interaction (Fig. 6H panel 2 for example).

As we extensively exposed in the point Moderate 1, the main problem we found to describe N-cadherin interactions that occurred in the intracellular compartments was the solubility of that N-cadherins. At certain point we tried to IP with the Pro-N-cadherin antibody, and it resulted difficult to say whether it was a bad antibody for IP or that we had to increase the stringency of the IP buffer too much to make the pro-N-cadherin soluble. It is true that some antibodies resist high stringency but it is not very common and it mostly depends on the sequence against which it was created, in this case we had no options the antibody had to be generated against the propeptide that is not particularly immunogenic. In any case we have to admit that the experiment the reviewer proposes was not possible with this antibody.

2. In figure 2, all transcripts that are knocked down or overexpressed should be verified by in situ hybridization or some other method. Figure S1 shows overexpression controls, and Figure S4 has knockdown controls in vitro (chick embryonic fibroblasts) and in vivo for the Golgi protein knockdowns, but 1) in vitro cells may not have the same response as the in vivo neural tube, and 2) the manuscript is lacking controls for the knockdowns from Figure 2. Therefore, it is necessary to confirm that the shRNA constructs reduce expression in vivo in the chick neural tube.

In Fig 2 we knocked down b-catenin (CTNNB1) and over expressed b-catenin, N-CadΔbCat and Tcf3·Vp16. In fig S1E'' we show that the level of b-Catenin expression is specifically decreased in the cells that express the Sh-CTNNB1(arrows), in addition we have now added RT-PCR results done in CEFs cultures showing that the messenger is also reduced (Fig S1M) but most importantly we have also added a Wnt pathway reporter assay (TOP-Flash) done in chicken neural tube (Fig S1N) showing that the transcriptional activity mediated by b-catenin is reduced by Sh-CTNNB1. In fig S1C'' we show evidences of the overexpression of b-catenin, and in Fig 2F' and Fig S1L'evidences of the of N-CadΔbCat overexpression. We do not show the overexpression of Tcf3-Vp16 here, however we reported it in Herrera et al 2014 and here we have added new data showing that Tcf3-Vp16 greatly increased TOP-Flash transcription (Fig S1N), moreover, we also show in Fig S1N that neither transfected b-catenin (from human origin) nor Tcf3-Vp16 activity are affected by Sh-CTNNB1. In addition, we observed the expected phenotype consisting in small invaginations and the accumulation of cytoplasmic aPKC Fig S1H, Fig S1H'' typical of Tcf3-Vp16 expression. In our hands in situ hybridization has resulted to be a very unreliable method to test the efficiency of knock down constructs, mostly because the in situ procedure affects very negatively to GFP detection and therefore to the discrimination of the cells that are transfected, similarly, checking ShRNA constructs efficiency by neural tube transfection followed by RT-qPCR, has demonstrated to be very inefficient due to the overwhelming dilution effect that the untransfected cells exert. We agree that CEFs and neural tubes are not the same, and the mRNA may be differently processed, however, we have now measured in neural tube and in CEFs by RT-qPCR the levels of the different mRNAs against which we generated shRNAs and normalized them with GAPDH to be sure that the levels of a particular gene was not much higher in neural tube than in CEFs (See image

below). Notably, we observed that the levels for all the measured genes were higher in CEFs than in neural tube, we understand that this is not a 100% guaranty that if a shRNA is good in CEFs will be good in neural tube, but at least guaranties that the amount of mRNA that a particular shRNA will have to knock down is lower in neural tube than in CEFs. In any case, at least in our hands, CEFs have proven to be the best method to choose the most efficient shRNAs constructs.



3. In figure 2, the change in cell states are characterized by structural up/down or transcriptional up/down, but without IHC to verify the changing membrane (structural) or nuclear (transcriptional) localization of β-catenin, it is difficult to make those conclusions about the treatments. I would recommend either confirming the changed intracellular localization of β-catenin using IHC, referencing papers that show these data for the treatments that are used, or altering the description/titles to something that can be confirmed.

As we have explained in point 5 of minor revision, the concept of structural and transcriptional does not mean that the referred treatment translocates b-catenin to the membrane or to the nucleus. The concept of structural and transcriptional activities of b-catenin is based on the fact that b-catenin on one hand acts at the AJs binding N-cadherin with components of the actin cytoskeleton (Structural because works on cell structure) and on the other, binds to Tcf transcription factors displacing Groucho (that is a repressor) and consequently acting as a transcription activator. In Herrera et al 2014 we showed that b-catenin increases AJs size by binding N-Cadherin and also stimulate TOP flash dependent transcription. Thus if b-catenin has separated structural and transcriptional activities, overexpression of b-catenin will increase both, and Knock down will decrease both. As we explained in point 5, the Vp16-

Tcf3 constructs directly stimulates TCF binding sites, but does not bind b-catenin, therefore it emulates b-catenin but only in its transcriptional component. Finally, the N-cadherin mutant that does not bind b-catenin will affect only the activity that b-catenin carries out at the adherent junctions not inferring with transcription. As we explained above we have added new text in results section to make all these concepts clearer. We also refer to Herrera et al 2014, for the effect of b-catenin expression on AJs and TCF dependent transcription, and added a new panel (Fig S1N) showing the effect of the different treatments on TCF dependent transcription.

4. In Figure 4A (or in supplemental), the authors should perform IHC/ICC in the HEK cells using an antibody against N-cadherin paired with the staining for the ST tag to verify that the localization of the protein matches that of the tagged version to ensure co-localization with golgi/ER.

As we mentioned above the anti N-cadherin antibody mainly recognizes the mature form of N-cadherin that is found at the AJs. On the contrary, the anti-Pro-N-cadherin antibody mostly recognizes immature forms in internal compartments. In Fig 5F and now in Fig 5B we show that the anti-ST antibody specifically recognizes all forms of ST tagged N-cadherin. Thus in our opinion the best way to evaluate where mature and immature forms of N-cadherin are located is using the anti ST antibody. In Fig 4O is shown very clearly that transfected N-cadherin-ST (HEK cells have no endogenous N-cadherin) generates mature N-cadherin in the AJs and Pro-N-Cadherin in more internal compartments. Moreover, this N-cadherin–ST can be seen as Pro-N-cadherin colocalizing with golgin97. Consequently, double labelling of Pro or mature N-cadherin with anti-ST would be only partially coincident and in our opinion does not add any valuable information to this experiment.

5. The western blots in Figure 5A and B should include a loading control. Without a loading control it is impossible to tell that, "Notably, β-catenin knockdown severely reduced mature N-cadherin band, whereas β-catenin expression selectively erased the upper pro-N-cadherin band (Fig. 5 B)," because the pro-n-cadherin band was also reduced by β-catenin knockdown compared to the sh-scramble.

Western in Figure 5A, evaluates de signal using increasing amounts of protein (more embryos) just to check the performance of the two antibodies under different protein concentrations. Our intention was not to accurately compare the lanes, only check that with more protein we had more signal. In fact, we only wanted to see whether both antibodies worked in a wide range of proteins concentrations.

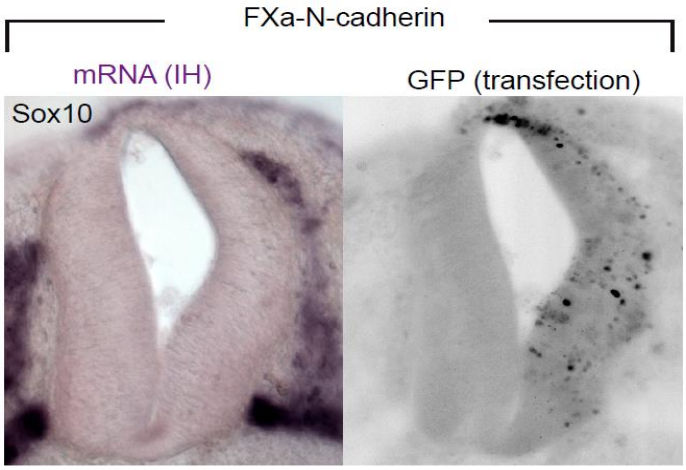
When samples coming from different purifications are analysed, this is the case in Fig 5B, the signal from two different purifications cannot be directly compared and because it is a purification, no loading control really exists. However, in Fig 5B what we studied was the ratio between two different signals (Pro and mature N-cadherin) within a lane (a purification). In other words, each lane acts as an

individual experiment. However, we absolutely agree with the reviewer that to be able to say that the amount of mature N-cadherin changes respect to Pro-N-cadherin (Ratio), the ratios from different experiments need to be calculated. Therefore, we have now calculated the ratio between N-cadherin and Pro-N-cadherin from individual purifications where b-catenin was either knocked down or over-expressed. The mean and the SD of these experiments is now shown at the bottom of the western panel in figure Fig 5B

6. The phenotype in Figure 7G caused by overexpression of the uncleavable N-cadherin seems to show cells migrating out of the neuroepithelium. The authors should probe for a neural crest marker (Pax7, Sox9, Sox10, etc) to ensure that these cells are not changing fate and delaminating/migrating as neural crest and to confirm that the neural tube progenitors/precursors are expanding. Recent work from the Sauka-Spengler lab (Williams et al., Dev. Cell., 2019) identified that premigratory NC cells express Sox2 along with NC specifiers.

We did not check for NC markers before because in our experience cells that change fate and migrate as NC into the mesenchyme show a very particular morphology and way to penetrate the mesenchyme that is not present after FXa-N-cadherin transfection. However, we agree with the reviewer that it was a possibility that had to be ruled out. Thus, we have transfected FXa-N-cadherin and 48 hpe checked for Sox10 expression by ISH. Although GFP signal was a little bit blurred by the ISH process, it clearly showed that the transfected areas did not show increased Sox10 mRNA expression.

HH23 chicken neural tubes 48 hpe with FXa-N-cadherin



Reviewer #3 (Comments to the Authors (Required)):
Specific Criticisms:

1a. Figure 5F- I am not sure that one can confidently assign/distinguish Phospho-pro from processed phospho/unphospho-forms of N-cadherin from gel-shift analyses alone (schematic).

We have to disagree with the reviewer in this point, because while it is true that the final read out is the position occupied by each band in the gel, the molecular assignment that we do to the different Pro and N-cadherin bands comes from a combination of immunological and biochemical properties of the different bands. However, to make the issue clearer we have now added to Fig 5B the immunoblot with anti-ST antibody (reveals all bands that contain the ST tag), it is shown in grey scale. In brief, in Fig 5B we show that N-cadherin ST transfection generates 3 main bands (blot ST), the two upper ones are recognized by the Pro-N-cadherin antibody, and the lower by the mature N-cadherin antibody (blot pro-N-cadherin and N-cadherin). In Fig 5E we demonstrate that the upper band of the two Pro-N-cadherin bands, is phosphorylated. Finally, in Fig 5F we demonstrate that the band of mature N-cadherin can also be dephosphorylated. I have to insist that we do not see unphosphorylated mature N-cadherin in normal conditions, just when it has been dephosphorylated by AP treatment.

1b. I am particularly skeptical that one can distinguish fully processed N-cadherin phosphor-from un-phospho-N-cadherin.

I am afraid the reviewer misunderstood the message we try to transmit in Fig 5F. In this figure we do not try to distinguish between phosphorylated and un-phosphorylated mature N-cadherin, in fact we believe that in normal conditions unphosphorylated mature N-cadherin either do not exist or its abundance is very low. In Fig 5F what we show is a dephosphorylation experiment, that causes a band shift of the mature N-cadherin (a very clear shift), and from there we deduce that mature N-cadherin is mostly phosphorylated.

1c. especially given evidence from Bill Weis's group that a cadherin tail without bCat binding is unstable.

Absolutely agree with the reviewer, Weis's group demonstrated that the N-cadherin that does not bind b-catenin is very unstable. We have shown here that un-phosphorylated N-cadherin does not bind b-catenin, therefore, in a cellular environment it shall be degraded very quickly. However, what we show in Fig 5F is an in vitro dephosphorylation experiment using purified N-cadherin-ST fractions, moreover, the digestion buffer contains abundant protease inhibitors that may slow down its degradation.

1d. Moreover, one might expect that forced expression of bCat would force association with and stabilize the unmodified cadherin-tail, but in fact, Figure 5F does not show this.

The ratio between dephosphorylated N-cadherin and Pro-N-cadherin bands shown in Fig 5F increases from 0.54 to 0.88 after b-catenin transfection, therefore, forced b-catenin expression in fact does stabilize the un-phosphorylated form of N-cadherin. If the stabilizing effect is not greater is most likely due to the low affinity that b-catenin has for unphosphorylated N-cadherin. In the case of E-cadherin the affinity of b-catenin for the phosphorylated form is 800 times greater than for the unphosphorylated one (McEwen et al., 2014).

2. The authors claim that forced expression of bCat expands the AJC through direct binding to N-cadherin rather than any nuclear signaling activity, based on evidence that a bCat missing its C-terminal "transactivation domain" phenocopies WT bCat. However, it is also known that bCat's N-terminus has transactivation function- via Pygopus and possibly other interactors (ref). So the authors have not really ruled out bCat's capacity as a transcriptional co-activator here.

In Valenta et al 2011, the authors show that the degree of transcriptional activity of b-catenin N-terminus depends on the cell type studied (Valenta et al., 2011). While it may be significant in Drosophila or HEK293T cells, in MEF cultures the b-catΔC construct showed no Wnt dependent transcriptional activity. We used this construct in Herrera et al 2014, there, we demonstrated the lack of transcriptional activity of b-catΔC by using the TOP-Red reporter (Top-Flash but with RFP as reporter). We understand that immunochemistry is a poor quantitative method compared to a luciferase assay, therefore, motivated by the reviewers concern about the lack of transcriptional activity of b-catΔC in neural tube, we have performed luciferase assays to double checked it . We electroporated HH12 neural tubes for 24 h and studied the luciferase activity driven by Top-Flash. In contrast to wild type b-Catenin or Vp16·Tcf3 that increased the transcriptional activity 9.5 and 24.9 times, respectively, b-catΔC did not change it (control 1.00, b-catΔC 1.01). These new data is now the Fig S3E.

3. Dbnl knock-down experiment showing elevation of the Pro-form of N-cadherin and loss of AJs is interesting, and is certainly consistent with the model that Dbnl contributes to N-cadherin/bCat trafficking through the Golgi (especially given known roles of Dbnl in Golgi anterograde trafficking), but clear evidence this model is lacking. Said another way, there are many perturbations that could lead to increased N-cadherin biosynthesis and detection of the Pro-form, or reduced cell-cell adhesion and slowed trafficking. For a paper titled Debrin-like (Dbnl) and bCat promote pro-N-cadherin processing to maintain apico-basal polarity, I think that further analysis of how Dbnl achieves this is expected.

We agree with the reviewer in that the work would benefit from experiments addressing the mechanisms through which DBNL contributes to N-cadherin maturation, especially its involvement on b-catenin induced maturation of N-cadherin. Moreover, the reviewer #1 had a very similar opinion. Therefore, following their advice we performed a new experiment to address this question.

Very recently, by using a dominant negative mutant of DBNL (DBNL-2F), it was demonstrated that DBNL regulates the amount of N-cadherin expressed on the plasma membrane of the cortical neurons (Inoue et al., 2019). Therefore, we decided to use DBNL-2F it to perform the experiment suggested by the reviewer. Using DBNL-2F we have studied the ratio of N-cadherin maturation by western blot using purified Streptactin fractions. In this experiment the effect on N-Cadherin biosynthesis is ruled out since N-cadherin is exogenously expressed. As expected, on the one hand DBNL-2F expression induced Pro-N-cadherin accumulation, and on the other, β-catenin incremented the ratio of mature N-cadherin over pro-N-cadherin. But notably, the effect of β-catenin disappeared when DBNL-2F was also transfected. Therefore, this experiment clearly demonstrates that β-catenin requires DBNL to promote N-cadherin maturation. This new experiment is Fig 7D.

Minor Issues:

1. Stylistic: The first half of the Discussion is thoughtful and well-integrates this group's findings with the studies that have come before. The second section speculating whether bCat tumor suppressor activity may be due to its ability to promote N-cadherin processing in the context of Wnt-driven medulloblastomas seems more appropriate for a review than a central discussion point in this manuscript.

We are sorry to disagree with the reviewer in this regard, in our previous work Herrera et al 2014, we already discussed the importance that the increased adhesion induced by b-catenin could have on the low malignancy demonstrated by Wnt type medulloblastomas. Now we have greatly expanded our knowledge on how b-catenin and DBNL contribute to N-cadherin maturation and on AJs stability. In addition, a very recent report using single cell RNA-seq technology, have demonstrated the presence of rare Wnt-active cells in non-Wnt human MBs. These cells not only retain the impaired tumorigenic potential of Wnt-type MB, but also, are believed to be responsible for the inhibition of tumour growth caused by the ectopic Wnt pathway activation in non-Wnt MB xenografts (Manoranjan et al., 2020). This report has put back in the spotlight whether Wnt pathway activation could be used as a therapy to reduce the malignancy of non-Wnt type medulloblastomas. We are conscious that the work we present here is basic research, but we believe the mechanism we describe here may be of great interest for translational or even clinical investigators, therefore we believe is worth discussing the undeniable relation that our findings have with medulloblastoma growth.

References.

Baum, B., and M. Georgiou. 2011. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *The Journal of cell biology*. 192:907-917.

Grigoryan, T., P. Wend, A. Klaus, and W. Birchmeier. 2008. Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes & development*. 22:2308-2341.

Inoue, S., K. Hayashi, K. Fujita, K. Tagawa, H. Okazawa, K.I. Kubo, and K. Nakajima. 2019. Drebrin-like (Dbnl) Controls Neuronal Migration via Regulating N-Cadherin Expression in the Developing Cerebral Cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 39:678-691.

Klockenbusch, C., and J. Kast. 2010. Optimization of formaldehyde cross-linking for protein interaction analysis of non-tagged integrin beta1. *Journal of biomedicine & biotechnology*. 2010:927585.

Kubben, N., J.W. Voncken, J. Demmers, C. Calis, G. van Almen, Y. Pinto, and T. Misteli. 2010. Identification of differential protein interactors of lamin A and progerin. *Nucleus*. 1:513-525.

Manoranjan, B., C. Venugopal, D. Bakhshinyan, A.A. Adile, L. Richards, M.M. Kameda-Smith, O. Whitley, A. Dvorkin-Gheva, M. Subapanditha, N. Savage, N. Tatari, D. McKenna, B. Bassey-Archibong, N. Winegarden, R. Hallett, J.P. Provias, B. Yarascavitch, O. Ajani, A. Fleming, G.D. Bader, T.J. Pugh, B.W. Doble, and S.K. Singh. 2020. Wnt activation as a therapeutic strategy in medulloblastoma. *Nature communications*. 11:4323.

McEwen, A.E., M.T. Maher, R. Mo, and C.J. Gottardi. 2014. E-cadherin phosphorylation occurs during its biosynthesis to promote its cell surface stability and adhesion. *Molecular biology of the cell*. 25:2365-2374.

Nelson, W.J., and R. Nusse. 2004. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science*. 303:1483-1487.

Rogers, C.D., L.K. Sorrells, and M.E. Bronner. 2018. A catenin-dependent balance between N-cadherin and E-cadherin controls neuroectodermal cell fate choices. *Mechanisms of development*. 152:44-56.

Valenta, T., M. Gay, S. Steiner, K. Draganova, M. Zemke, R. Hoffmans, P. Cinelli, M. Aguet, L. Sommer, and K. Basler. 2011. Probing transcription-specific outputs of beta-catenin in vivo. *Genes & development*. 25:2631-2643.

March 3, 2021

RE: JCB Manuscript #202007055R

Dr. Sebastián Pons
MOLECULAR BIOLOGY INSTITUTE OF BARCELONA
Department of Cell Biology
C/ Baldiri Reixac, 10-12
Edificio Cluster, Parc Cientific de Barcelona
Barcelona 08028
Spain

Dear Dr. Pons,

The re-review of your manuscript "Debrin-like (Dbnl) and β-catenin promote pro-N-cadherin processing to maintain apico-basal polarity" is now complete and we are pleased to say that the reviewers feel you have addressed their concerns. The revised manuscript nicely demonstrates in vivo interactions that link cadherin proteins to intracellular developmental processes. Congratulations and thank you for sending this interesting paper to the Journal of Cell Biology. We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Articles may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. You currently do not have scale bars in Figures 1B'/C', 3E, 4Q/R/S, 7A'/B, 8A'/A''/C'/C'', S1K/L, and S5'. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis. You currently do not have a marker in Figure S3D.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both

in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

5) Abstract and title: The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership. We suggest shortening your title to "Dbnl and β-catenin promote pro-N-cadherin processing to maintain apico-basal polarity" and moving the "Debrin-like (Dbnl)" definition to the abstract.
The words 'catenin' and 'cadherin' are capitalized in the last sentence of the abstract but not in the rest of the abstract, please use a consistent style. Also in the abstract, it seems that the word 'this' is missing from "although the mechanism through which occurs remained unclear."

6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

8) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. Imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures and 10 videos. You currently exceed this limit but, in this case, we will be able to give you the extra space but please try not to add to the current total. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

12) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

13) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

14) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution. Please also provide a brief description.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Marianne Bronner, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have adequately addressed all my previous concerns.

Reviewer #2 (Comments to the Authors (Required)):

This revised manuscript identifies that N-cadherin interacts with β-catenin in its phosphorylated full-length form (pro-N-cadherin) in the Golgi prior to being processed by Furin and inserted into the apical membrane. Interaction with Dbnl is necessary for the maturation of N-cadherin via Furin and the formation of adherens junctions in the posterior neural tube (early spinal cord). Using high resolution imaging and multiple gain and loss of function experiments, and IP Mass Spectrometry, the authors describe novel information about the molecular mechanisms involved in in vivo processing of N-cadherin and that excess pro-N-cadherin leads to a loss of the apical adherens junctions causing abnormal neural tube morphology and integrity. Overall, this paper provides strong mechanistic information about the intracellular post-translational processing and function of adherens junction proteins during early trunk neural tube development. The experiments and results in the manuscript are clearly a significant amount of work, and I still believe that the paper will move the cell and developmental biology fields forward with a intricate understanding of cell-cell adhesion proteins, differential processing and function, identification of new protein-protein interactions in vivo, and how those molecular events affect the development of epithelial tissues.

Overall, this is a very elegant paper that demonstrates in vivo interactions and provides rationale for intracellular molecular mechanisms that link cadherin proteins to intracellular developmental processes, and will likely have a strong impact on work in other cell types and organisms moving forward. The authors have addressed all of my concerns by either updating figures, using new experiments, or providing additional information to rationalize their choice of experimental methods. They have improved the flow of the figures and it is significantly easier to navigate them, and their additional experiments, added figures, and new clarity of labeling is very helpful. I appreciate their attention to detail with response to the original comments, their willingness to head back to the lab and perform updated experiments during a global pandemic, and that they provided in depth information and responses for why they chose not to perform some of the requested experiments. I support publication of this paper in JCB.

Reviewer #3 (Comments to the Authors (Required)):

The DBLN-2F dominant inhibitor strategy is an improvement/shows that DBLN works with b-catenin to promote N-cadherin. Although it seems like a previous publication also showed this basic effect (Inoue et al., 2019), which clips novelty of this study a bit, the overall conclusions are now better supported by the data.