Journal of Cell Science

Clathrin regulates Wnt/β-catenin signaling by affecting Golgi to plasma membrane transport of transmembrane proteins

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DOI: 10.1242/jcs.244467

Editor: John Heath

Review timeline
Original submission: 24 January 2020
Editorial decision: 20 February 2020
First revision received: 21 May 2020
Accepted: 4 June 2020

Original submission

First decision letter

MS ID#: JOCES/2020/244467

MS TITLE: Clathrin regulates Wnt/β-catenin signaling by affecting biosynthetic protein transport

AUTHORS: Else Munthe, Camilla Raiborg, Harald Stenmark, and Eva Maria Wenzel
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The role of endocytosis is Wnt signaling has been debated extensively. Munthe et al. contribute to the debate with an interesting set of data demonstrating that depletion of clathrin heavy chain

depletes total cell β-catenin but in a cell type specific manner. The data are consistent with the model that CHC knockdown blocks trafficking from Golgi to PM of cadherins and LRP5/6 (and presumably other PM Wnt regulators such as Frizzled and RNF43/ZNRF3). And that the cell type variability is related to CDH1 abundance. This shifts the debate from endocytosis to exocytosis, a welcome new insight.

Comments for the author

The data appear solid and well-controlled, and the effects are tested in several cell lines. There are several omissions in the data that would help strengthen the model. These are mostly suggestions rather than essential.

First, the authors show that there is a correlation between cellular cadherin expression, and sensitivity to CHC knockdown. But they don't show the data in the main text. This data (Fig S3) should be in the main part of the paper.

The title is a bit vague. What is 'biosynthetic protein transport'? Perhaps a better choice might be "Clathrin regulates Wnt/b-catenin signaling by affecting Golgi-Plasma membrane transport?" Of course, the problem is that the manuscript doesn't really assess β-catenin signaling. Mostly just β-catenin abundance, which is a poor marker for TCF4-dependent nuclear transcription, which is what I think they mean by Wnt/β-catenin signaling

What happens if for example, they over-express CDH1 in CDH1-low MDA-MB-231 or mouse L cells? Does the abundance of β-catenin then become sensitive to CHC knockdown?
Conversely, if they knockdown CDH1 in HeLa cells does this a) decrease membrane β-catenin and b) make cells less responsive to CHC knockdown?

Another limitation of the manuscript is the heavy reliance on SDS lysates to assess β-catenin levels. A key feature of β-catenin is its dual functionality – membrane attachment, and nuclear signaling. It would therefore, be more informative to measure membrane-bound and free β-catenin more often than just in Fig 5A. There are several good assays for this, such as fractionation, or reagents like the ones used here: https://doi.org/10.1083/jcb.200402153

Finally, the manuscript measures total β-catenin but this is a poor surrogate for nuclear Wnt/β-catenin signaling. Why not also look at Wnt-activated TOPFLASH? If the model is correct, CHC knockdown will not have much effect on TOPFLASH in cells with high CDH1, but may have a much larger effect in cells with no CDH1 (where all the effect might be via LRP5/6, FZD, etc.)

Flow cytometry looking at CDH1, LRP5/6 and FZD abundance at the cell surface would markedly enhance their story. Not essential, but a nice alternative way to test their model.

Finally, a cartoon of the proposed model would enhance their message.

A few more specific comments:

Figure 1. Total depletion of CHC with three distinct siRNA leads (slowly) to 50% decrease in total β-catenin.
When β-catenin destruction is blocked with Wnt3a, β-catenin levels go up.

The time course is interesting – the fall in β-catenin is late, suggesting secondary effects. At the early time point, β-catenin actually goes up. This may provide some insight into mechanism, so it's important to know, is this reproducible? There is no indication of how many times this experiment was performed.

Fig 4. The failure to move LRP6-mNG to the membrane after CHC knockdown suggests a more general problem with protein trafficking from Golgi to plasma membrane. The authors confirm this in figure 5 by showing that cadherin levels at the plasma membrane also decrease upon CHC knockdown and this is associated with decrease membrane β-catenin.

Figure six shows a modest change in AXIN2 and MYC mRNA with CHC knockdown, consistent with the decrease in LRP5/6 trafficking to the PM. The problem is that this is basal unstimulated Wnt signaling so it's hard to assess if this is specific. What is the control to tell what robust Wnt inhibition would look like here e.g. β-catenin or WLS or PORCN knockdown?

Minor points

What are the differences between HeLa, HeLaK, and HeLa-CCL2? I am not familiar with these HeLa variants and I suspect most readers will not be either.

The authors are to be commended for using dot plots instead of simple plunger plots. I would request that they show all the data points for the controls, though. For example, Figure 2B, the DMSO control does not show the data points, only the mean, normalized to 1. Unless there was only one measurement (which I doubt) they should show all the control data points too, to allow the reader to assess the scatter.

Indirubin 3 oxime is not a very specific GSK3 inhibitor (IC50 = 190 nM), and didn't really have an effect on β-catenin (their figure 3B). It hits multiple other kinases, especially if used at 10 μM. If the authors want to test the role of GSK3 inhibition, compounds like CHIR99021 are more potent (IC50 10 nM, can use at 100-500 nM) and more specific.

Any small molecule used at 100 uM (like Dyngo-4a) are sure to have multiple off-target effects. This makes experiments like that in figure 3B very hard to interpret.

Fig 5C, what does # mean? I assume they are pointing out a non-specific band, but the figure legend ought to make this clear.

Reviewer 2

Advance summary and potential significance to field

The role of endocytosis and Clathrin in Wnt signalling remains unresolved despite numerous papers on the subject including contradictory papers on the role LRP and endocytosis in cells lacking APC. The present study is therefore timely. There is nevertheless scope for improvements, including more complete mechanistic understanding and better-quality controls, which are needed to ensure that the conclusions reached by the authors are solid.

The authors confirm that Clathrin (but not AP2) is needed for for Wnt signalling. Specifically, they show that Clathrin depletion leads to reduction of beta-catenin independently of endocytosis or MVB formation.
They suggest that this conclusion is compatible with previous observations that inhibition of dynamin does prevent Wnt signalling if one considers the role of dynamin in other processes such exocytosis. Their show that Clathrin inhibition reduces the secretion of LRP5/6. They infer that dynamin inhibition has the same effect (but this is not shown). Thus, trafficking of LRP5/6 is essential to maintain sufficient levels of beta-catenin. How this occurs at the molecular level is not addressed. The authors suggest that this phenomenon only partially explains the role of Clathrin in Wnt signalling. They suggest that Clathrin is also needed for trafficking of Cadherin, which in turn would stabilise beta-catenin at the cell surface, and supply signalling competent beta-catenin in the cytoplasm. The latter point is only shown indirectly through a signalling assay.

Comments for the author

Major suggestions
*Role of cadherin-associated beta-catenin; This would be bolstered by directly measuring cytosolic beta catenin upon cadherin KD. The author suggest that this is near the detection limit, but they could focus their measurement on cytosolic protein by IP'ing beta cat from the cytosol. This role of cadherin could also be demonstrated by knocking out LRP5/6 in L cells (no cadherin). This should lead to complete loss of beta-catenin

*No direct validation of clathrin and AP-2 depletion on endocytosis. This is needed to show that endocytosis is equally affected in the two conditions.

*Need to state clearly that the mechanism of beta-catenin stabilisation by LRP5/6 remain unknown, or better still to provide a path towards clarifying this important mechanistic point. There is also scope to clarify the recent controversy between KenyiSaito-Diaz et al, 2018 and Chen & He, 2019)

Additional suggestions.
*The ESCRT depletion experiments do not exclude ESRCT-independent ILV formation, which can occur (Edgar et al. Traffic 2014). A strong experiment would be to express a dominant negative VPS4. Likewise the authors do not consider the possible redundancy by CHC2 (this is why demonstration of endocytosis inhibition is needed).

*In the discussion, the authors suggest that UPR is activated upon Clathrin inhibition. This could be shown with specific markers.

Minor points.

On p7, I assume the authors mean “exogenous LRP6-nMG was present” and not “exogenous LRP6-nMG was absent”

A sentence outlining the nature of the mutations in the various cell lines and specifying if they affect the Wnt pathway is needed for the more lay reader.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
The role of endocytosis is Wnt signaling has been debated extensively. Munthe et al. contribute to the debate with an interesting set of data demonstrating that depletion of clathrin heavy chain depletes total cell β- catenin but in a cell type specific manner. The data are consistent with the model that CHC knockdown blocks trafficking from Golgi to PM of cadherins and LRP5/6 (and presumably other PM Wnt regulators such as Frizzled and RNF43/ZNRF3). And that the cell type variability is related to CDH1 abundance. This shifts the debate from endocytosis to exocytosis, a welcome new insight.

Reviewer 1 Comments for the Author:
The data appear solid and well-controlled, and the effects are tested in several cell lines. There are several omissions in the data that would help strengthen the model. These are mostly suggestions rather than essential.
| We thank Reviewer 1 for nicely summarizing our findings and suggesting experiments to further strengthen our data. We highly appreciated these ideas and think the new data indeed improved our manuscript substantially. You will find the results included in a revised version.

First, the authors show that there is a correlation between cellular cadherin expression, and sensitivity to CHC knockdown. But they don’t show the data in the main text. This data (Fig S3) should be in the main part of the paper.
| Following the suggestions from both reviewers, we have now included many new results (2 additional main and 1 additional Supplemental Figures, resulting in 8 main figures). Since JCS allows up to 8 main figures, we decided to keep Fig. S3 in the supplement (now Fig. S4) and chose to rather present the new data in the main part of the paper (see below).

The title is a bit vague. What is ‘biosynthetic protein transport’? Perhaps a better choice might be “Clathrin regulates Wnt/b-catenin signaling by affecting Golgi-Plasma membrane transport?” Of course, the problem is that the manuscript doesn’t really assess ß-catenin signaling. Mostly

just ß-catenin abundance, which is a poor marker for TCF4-dependent nuclear transcription, which is what I think they mean by Wnt/ß-catenin signaling

| We changed the title from "Clathrin regulates Wnt/β-catenin signaling by affecting biosynthetic protein transport" into "Clathrin regulates Wnt/β-catenin signaling by affecting Golgi to plasma membrane transport of transmembrane proteins". We would like to keep "signaling" in the title, since we did measure a reduction in β- catenin target gene expression by qPCR (Fig. 7D) and we also observe a reduction in nuclear β-catenin in three different cell lines (new data, Fig. 7A,B,C).

What happens if for example, they over-express CDH1 in CDH1-low MDA-MB-231 or mouse L cells? Does the abundance of ß-catenin then become sensitive to CHC knockdown?
Conversely, if they knockdown CDH1 in HeLa cells does this a) decrease membrane ß-catenin and b) make cells less responsive to CHC knockdown?

| We highly appreciated these suggestions and therefore decided to do both approaches:

Firstly, we depleted HeLa-CCL2 cells for N-cadherin and saw a stronger decrease in total ß-catenin than with CHC depletion. Indeed, depleting CHC together with N-cadherin did not show an additional effect, making cadherin levels likely the main determinator of ß-catenin stability in this cell line. Further, fractionation experiments show that it is indeed the membrane pool that is mostly affected by clathrin or by CDH2 depletion in HeLa-CCL2 cells (see Fig. R1).

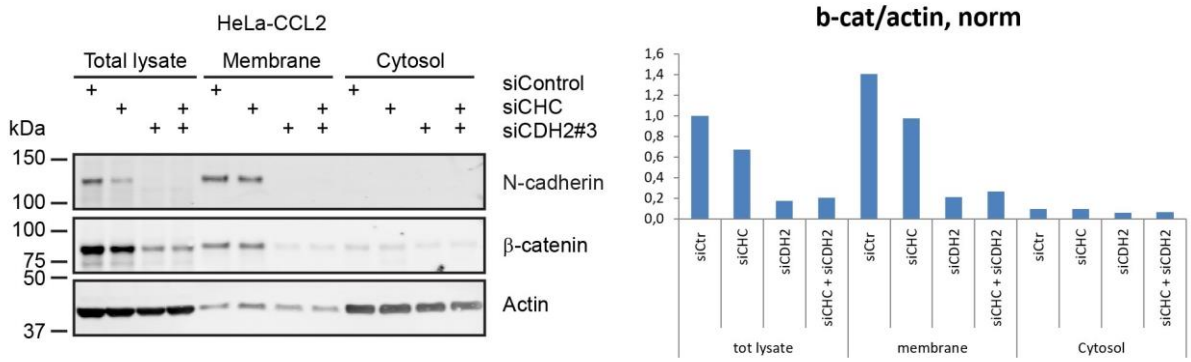


Fig. R1: Fractionation experiment in HeLa-CCL2 cells showing that β-catenin is mostly found in the membrane pool and its protein levels follow the abundance of N-cadherin.

Secondly, we generated stable cell lines expressing cadherins (mouse E-cadherin-GFP, mouse N-cadherin-GFP and human E-cadherin-GFP) in mouse L cells and saw indeed that β-catenin became stabilized (parental L cells have barely detectable β-catenin levels). Importantly, CHC knockdown reduced the levels of β-catenin in the overexpression cell lines. In addition, we used L cells Wnt-3a, which stably express Wnt-3a ligand, leading to an autocrine stimulation and elevated β-catenin levels. Importantly, we could also see a reduction in β-catenin levels upon clathrin depletion in these cells. The L cell experiments thus provided an elegant model to investigate the contributions of the membrane pool and the soluble (cytosolic and nuclear) pools of ß-catenin. We have included these results in the manuscript (new Fig. 6).

Another limitation of the manuscript is the heavy reliance on SDS lysates to assess ß-catenin levels. A key feature of ß-catenin is its dual functionality – membrane attachment, and nuclear signaling. It would, therefore, be more informative to measure membrane-bound and free ß-catenin more often than just in Fig 5A. There are several good assays for this, such as fractionation, or reagents like the ones used here: https://doi.org/10.1083/jcb.200402153

| Initially, we analysed total protein levels of ß-catenin by making whole cell lysates with an SDS-lysis buffer. At a later time point and while looking for the mechanism, we started with fractionation assays (Fig. 5A), which indeed helped us to unravel the mechanism. We have now included more fractionation experiments in the revised version of the manuscript (Fig. 6G, Fig. 7A, B, C) and investigated the effect of clathrin on the different ß-catenin pools much more

thoroughly (Fig. 6 and Fig. 7).

Finally, the manuscript measures total β-catenin but this is a poor surrogate for nuclear Wnt/ß-catenin signaling. Why not also look at Wnt-activated TOPFLASH? If the model is correct, CHC knockdown will not have much effect on TOPFLASH in cells with high CDH1, but may have a much larger effect in cells with no CDH1 (where all the effect might be via LRP5/6, FZD, etc.)
| We have tried to perform TOP/FOP reporter assays to address the transcriptional activity of ß-catenin and how it is affected by clathrin depletion. However, clathrin depletion severely impaired the transfection efficiency of the reporter plasmids (surely due to impaired endocytic uptake of the DNA/liposome mixture) and in addition changed cell morphology and viability heavily. We did not succeed to obtain reasonable luciferase measurements and therefore chose to perform qPCR to measure ß-catenin target gene expression instead (Fig. 7D). In the revised version of the manuscript, we have now also added nuclear fractionation experiments, which show a reduction in nuclear ß-catenin upon CHC- or CDH-depletion in three different cell lines (HeLaK, HeLa-CCL2 and L cells), under various stimulation conditions (new data, Fig. 7A-C). We think that the combination of qPCR and nuclear fractionations can justify our conclusion that clathrin depletion also affects the transcriptional activity of ß-catenin.

Flow cytometry looking at CDH1, LRP5/6 and FZD abundance at the cell surface would markedly enhance their story. Not essential, but a nice alternative way to test their model.
| Flow cytometry is a powerful method to quantify surface expression of plasma membrane proteins. Our IF stainings showed a marked decrease not only in surface localization (see Fig. 4B), but actually reduced total LRP6 levels, which we confirmed by WB analysis. We do not think that flow cytometry would add to these findings and on the contrary, it might be difficult to interpret surface fluorescence intensity measurements, since clathrin depleted cells become multinucleated and thus larger due to clathrin's function at the mitotic spindle (Royle, 2012), rendering surface fluorescence measurements hard to interpret.

Finally, a cartoon of the proposed model would enhance their message.
| We gladly added a summarizing cartoon (new Fig. 8).

A few more specific comments:

Figure 1. Total depletion of CHC with three distinct siRNA leads (slowly) to 50% decrease in total ß-catenin. When ß-catenin destruction is blocked with Wnt3a, ß-catenin levels go up. The time course is interesting – the fall in ß-catenin is late, suggesting secondary effects. At the early time point, ß-catenin actually goes up. This may provide some insight into mechanism, so it's important to know, is this reproducible? There is no indication of how many times this experiment was performed.
| We think that the apparently "slow" reduction in ß-catenin protein levels following clathrin depletion is due to the necessity of a *complete* depletion of clathrin, since small amounts of clathrin may suffice to pinch off vesicles: CME requires merely 45-80 sec (Kirchhausen et al., 2014). This indicates that a reduced number of clathrin molecules may still be enough to maintain essential cellular functions, such as CME or generation of Golgi-derived secretory vesicles, although less efficiently. Even more importantly, some time for the turnover of transmembrane molecules will be required, meaning newly synthesized molecules have to enter the secretory pathway, which might be another factor explaining the slow effect. The initial increase in ß-catenin at 48 h post transfection was indeed reproducible. We have now added quantifications from three independent experiments to this figure (Fig. 1C). We could imagine that it might reflect an initial queuing effect of transmembrane proteins at the Golgi in cells that have already good enough depletion. This transient accumulation of proteins seems to be resolved at a later stage, when the cell has managed to upregulate Endoplasmic-reticulum-associated protein degradation (ERAD) and/or downregulated biosynthesis (possibly via eIF2a) as a response to the impaired Golgi trafficking and an induction of ER stress and the unfolded protein response. We have indeed observed an accumulation of cadherins and ß-catenin in the trans-Golgi network of clathrin-depleted cells and included these data now in the manuscript (new data, Fig. 6C, D).

Fig 4. The failure to move LRP6-mNG to the membrane after CHC knockdown suggests a more general problem with protein trafficking from Golgi to plasma membrane. The authors confirm this in figure 5 by showing that cadherin levels at the plasma membrane also decrease upon CHC

knockdown and this is associated with decrease membrane ß-catenin.
| Indeed, many proteins trafficking from the Golgi to the plasma membrane might be affected by clathrin depletion. We assume that the extend of clathrin sensitivity may depend on the turnover of these molecules: endogenous LRP6 appeared only modestly affected, while exogenous (overexpressed) LRP6-mNG was more sensitive to clathrin depletion (Fig. 4A). A possible explanation could be that the clathrin sensitivity for certain transmembrane molecules reflects the amount of synthesized protein, which needs to be trafficked.

Figure six shows a modest change in AXIN2 and MYC mRNA with CHC knockdown, consistent with the decrease in LRP5/6 trafficking to the PM. The problem is that this is basal unstimulated Wnt signaling so it's hard to assess if this is specific. What is the control to tell what robust Wnt inhibition would look like here, e.g. ß-catenin or WLS or PORCN knockdown?
| We agree that the reduction in *AXIN2* and *cMyc* mRNA is modest and likely close to baseline. We regret that we did not include a positive control in these qPCR experiments. As an alternative, we have now included fractionation data, showing a reduction in nuclear ß-catenin in three different cell lines (HeLaK, HeLa-CCL2 and L cells) under conditions of cadherin overexpression or Wnt-3a stimulation. In all conditions nuclear ß-catenin was reduced upon clathrin depletion. We have included these data now in Fig. 7A,B,C.

Minor points

What are the differences between HeLa, HeLaK, and HeLa-CCL2? I am not familiar with these HeLa variants and I suspect most readers will not be either.
| HeLa cells were established in 1951 and have been widely distributed over laboratories around the world. Due to their genomic instability, they have diverged into different variants. Two well characterized variants are HeLa-CCL2, which are thought to be close to the original HeLa cell line, and a variant called HeLa K ("Kyoto"), which is more homogenous in morphology, a bit less motile and therefore particularly well suited for (live-cell) imaging studies. HeLa variants have been subject to multi-omics studies and were shown to be surprisingly distinct (Liu et al., 2019). This was fortunate for us, since HeLa-CCL2 appear to solely express N-cadherin, while HeLaK seem to express a different, unknown cadherin family member as judged by a Pan-cadherin WB (Fig. S4). We have now included a short explanation on the two HeLa variants in the results section of our manuscript.

The authors are to be commended for using dot plots instead of simple plunger plots. I would request that they show all the data points for the controls, though. For example, Figure 2B, the DMSO control does not show the data points, only the mean, normalized to 1. Unless there was only one measurement (which I doubt) they should show all the control data points too, to allow the reader to assess the scatter.
| The data were normalized to the control (set to 1) in every experiment, therefore there is no variation in the control. To avoid confusion, we have now included the correct number of dots in the control bars.

Indirubin 3 oxime is not a very specific GSK3 inhibitor (IC50 = 190 nM), and didn't really have an effect on ß- catenin (their figure 3B). It hits multiple other kinases, especially if used at 10 µM. If the authors want to test the role of GSK3 inhibition, compounds like CHIR99021 are more potent (IC50 10 nM, can use at 100-500 nM) and more specific.

Any small molecule used at 100 uM (like Dyngo-4a) are sure to have multiple off-target effects. This makes experiments like that in figure 3B very hard to interpret.
| We thank the reviewer for making us aware of a better GSK3 inhibitor! For the current manuscript, we think that the indirubin-3-oxime data are valid, since it is backed up by either LiCl or siAPC when used as a means to manipulate the destruction complex (Fig. 2). Likewise it shows the same tendency as Wnt-3a stimulation when used as a means to activate Wnt signaling (Fig. 3). We completely agree with the reviewer that Dyngo-4a may have multiple off-target effects, especially at the high concentration used by us and others (Gagliardi et al., 2014). A strength in our study is however, that we not only rely on inhibitors, but also employ knock downs and KO systems, helping us to corroborate our findings. In addition, we now point out the possibility of off-target effects in the discussion.

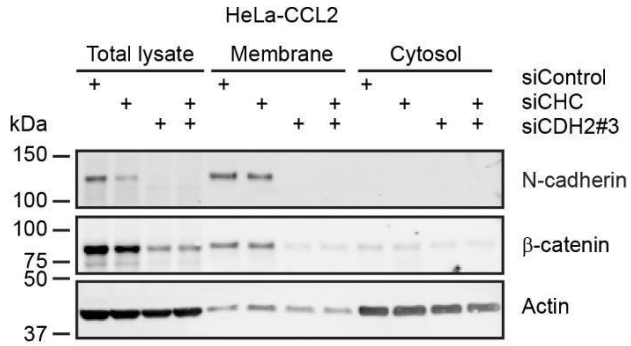
Fig 5C, what does # mean? I assume they are pointing out a non-specific band, but the figure legend ought to make this clear.
| Yes, indeed we are pointing out non-specific bands and have now added this information to the figure legend of Fig. 5C.

Reviewer 2 Advance Summary and Potential Significance to Field:
The role of endocytosis and Clathrin in Wnt signalling remains unresolved despite numerous papers on the subject including contradictory papers on the role LRP and endocytosis in cells lacking APC. The present study is therefore timely. There is nevertheless scope for improvements, including more complete mechanistic understanding and better-quality controls, which are needed to ensure that the conclusions reached by the authors are solid.

The authors confirm that Clathrin (but not AP2) is needed for for Wnt signalling. Specifically, they show that Clathrin depletion leads to reduction of beta-catenin independently of endocytosis or MVB formation. They suggest that this conclusion is compatible with previous observations that inhibition of dynamin does prevent Wnt signalling if one considers the role of dynamin in other processes such exocytosis. Their show that Clathrin inhibition reduces the secretion of LRP5/6. They infer that dynamin inhibition has the same effect (but this is not shown). Thus, trafficking of LRP5/6 is essential to maintain sufficient levels of beta- catenin. How this occurs at the molecular level is not addressed. The authors suggest that this phenomenon only partially explains the role of Clathrin in Wnt signalling. They suggest that Clathrin is also needed for trafficking of Cadherin, which in turn would stabilise beta-catenin at the cell surface, and supply signalling competent beta-catenin in the cytoplasm. The latter point is only shown indirectly through a signalling assay.

Reviewer 2 Comments for the Author: Major suggestions
*Role of cadherin-associated beta-catenin; This would be bolstered by directly measuring cytosolic beta catenin upon cadherin KD. The author suggest that this is near the detection limit, but they could focus their measurement on cytosolic protein by IP'ing beta cat from the cytosol. This role of cadherin could also be demonstrated by knocking out LRP5/6 in L cells (no cadherin). This should lead to complete loss of beta-catenin
| We highly appreciated the suggestions from the reviewer and have now focused a lot more on the role of the different ß-catenin pools, both by including more fractionation experiments besides Fig. 5A (new Fig. 6G, new Fig. 7A, B, C) and by manipulating the abundance of cadherins by two different means: Firstly, by depleting N-cadherin in HeLa-CCL2 cells and secondly, by overexpressing cadherins in mouse L cells which do not express cadherins.

Firstly, we depleted HeLa-CCL2 cells for N-cadherin and saw a stronger decrease in total ß-catenin than with CHC depletion. Indeed, there was no additional effect in cells co-depleted for CHC and N-cadherin, making cadherin levels likely the main determinator of ß-catenin stability in this cell line. Further, fractionation experiments show that it is indeed the membrane pool that is mostly affected by clathrin or by CDH2 depletion in HeLa-CCL2 cells (see Fig. R1).



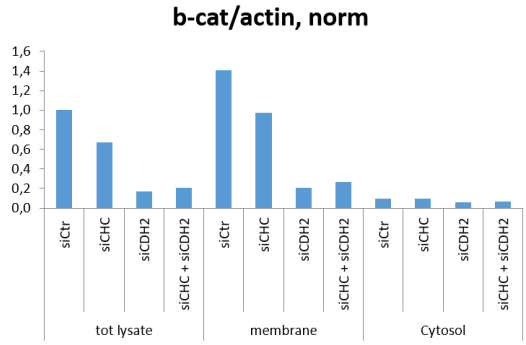


Fig. R1: Fractionation experiment in HeLa-CCL2 cells showing that ß-catenin is mostly found in the membrane pool and its protein levels follow the abundance of N-cadherin.

Secondly, we used mouse L cells to address the role of the different ß-catenin pools. Mouse L cells have only marginal levels of ß-catenin (new data, Fig. 6B, C), making it difficult to detect a further reduction by knocking out or knocking down LRP5/6. Instead, we attempted to increase the membrane pool of ß-catenin by stably expressing cadherins (mouse N-cadherin-GFP, mouse E-cadherin-GFP, human E-cadherin-GFP) in these cells. Indeed, expressing any of those cadherin family members increased total ß-catenin levels and rendered ß-catenin sensitive to CHC depletion (new data, Fig. 6B). In addition, we employed L cells which stably express Wnt-3a, leading to an autocrine stimulation loop in these cells. L cells Wnt-3a have higher ß-catenin levels, which could be reduced upon clathrin depletion (new data, Fig. 6C, E, F). We interpret these findings in L cells as strengthening our model that clathrin depletion affects ß-catenin levels either via affecting the cadherin-associated pool of ß-catenin or by affecting the LRP5/6 mediated signaling competent pool of ß-catenin. Importantly, the consequence is in both cases a reduction in nuclear ß-catenin as determined by fractionation experiments in HeLaK, HeLa-CCL2 and L cells. These results are now included in the manuscript (new data, Fig. 7A, B, C).

*No direct validation of clathrin and AP-2 depletion on endocytosis. This is needed to show that endocytosis is equally affected in the two conditions.
| We agree that it is an important control experiment to show that the depletion of CHC and AP-2 inhibits endocytosis to a similar extend. We have now included a transferrin uptake experiment, which proofs a complete inhibition of endocytosis upon KD of CHC and the AP-2µ2 subunit on a single cell resolution. Depletion of the AP-2a subunit was marginally less efficient compared to AP-2µ2 and CHC depletion. These data are now included in the manuscript (new data, Fig. S3).

*Need to state clearly that the mechanism of beta-catenin stabilisation by LRP5/6 remain unknown, or better still to provide a path towards clarifying this important mechanistic point. There is also scope to clarify the recent controversy between KenyiSaito-Diaz et al, 2018 and Chen & He, 2019)
| The question of how ß-catenin is stabilized following ligand binding is one of the central questions in the Wnt field. A plethora of studies exists addressing this fundamental question: Suggestions range from an inhibition of GSK3 by competitive binding to the phosphorylated intracellular tail of LRP6 (Hernandez et al., 2012; Piao et al., 2008; Wu et al., 2009) or through its internalization into multivesicular endosomes (Taelman et al., 2010), to a disassembly of the destruction complex (Lee et al., 2003; Liu et al., 2005; Luo et al., 2007) or a recruitment of Axin to LRP5/6 (MacDonald et al., 2009; Tamai et al., 2004) to impaired ß-catenin binding (Jho et al., 1999; Kim et al., 2013) or ubiquitination (Li et al., 2012). Likely, a combination of these events is important for efficient Wnt activation, but it could also be cell-type dependent which mechanism dominates. We state now in the figure legend of our model (new Fig. 8) that the mechanism how LRP5/6 leads to the stabilization of ß-catenin is still unknown.

The second fundamental question is about a recently suggested second role of APC in Wnt signaling. Saito-Diaz et al. 2018 provide data that APC may not solely function as a critical member of the destruction complex, but in addition protects the cell against ligand-independent Wnt signaling activation by preventing clathrin-dependent signalosome formation in the absence

of ligand. The implications of these findings are important, since they challenge the current prevailing view in the field: When APC as a supposedly downstream component of the Wnt pathway is mutated, an inhibition of upstream events (such as blocking Wnt receptors) is therapeutically not promising. Saito-Diaz showed that receptor inhibition or downregulation greatly reduces Wnt/ß-catenin signaling despite a mutation or depletion of APC. The group of Xi He could not reproduce that LRP5/6 depletion or knock out reduces Wnt signaling activity in APC mutant cells, putting these new findings into question.
Given the clinical importance of whether or not the Wnt receptor status plays a role in APC mutated cells, we do not feel that we are in a position to answer this conundrum along the way. We believe that the complexity of the contrasting findings cannot be resolved by a single experiment and be presented as a side finding within our current manuscript, which does not focus on endocytosis/Wnt initiation, but rather on exocytosis.

Additional suggestions.

*The ESCRT depletion experiments do not exclude ESRCT-independent ILV formation, which can occur (Edgar et al. Traffic 2014). A strong experiment would be to express a dominant negative VPS4. Likewise, the authors do not consider the possible redundancy by CHC2 (this is why demonstration of endocytosis inhibition is needed).
| To elucidate the mechanism of how clathrin affects ß-catenin stability we interrogated various known cellular functions of clathrin. While the functional importance of clathrin was clearly shown for ESCRT-dependent ILV formation (Raiborg et al., 2006; Wenzel et al., 2018), we are not aware that the CD63-mediated ESCRT-independent ILV formation (Edgar et al., 2014) may involve clathrin. Dominant-negative VPS4 would inhibit specifically ESCRT- dependent ILV formation and would in that sense serve as an alternative approach to HRS depletion. We chose HRS knockdown as a means to abrogate ESCRT function, since HRS is the direct recruiter of clathrin to the endosome (Raiborg et al., 2001; Wenzel et al., 2018). We have now slightly reformulated the paragraph about ILV formation in the results part to make our point clearer.

*In the discussion, the authors suggest that UPR is activated upon Clathrin inhibition. This could be shown with specific markers.
| We have tried to measure ER stress and the activation of an unfolded protein response (UPR) by detecting phosphorylated PERK. We chose to investigate the induction of the UPR at various time points after transfection, since we suspect ER stress/UPR to be an early reaction on clathrin depletion, which could be later resolved through an inhibition of protein synthesis (via eIF1a) and/or through upregulation of ERAD-mediated protein degradation. Our results with PERK were not very clear: We might observe a higher molecular weight smear particularly at 48 and 72 h post siRNA transfection, which could indicate a small fraction of phospho-PERK in clathrin depleted cells (Fig. R2).

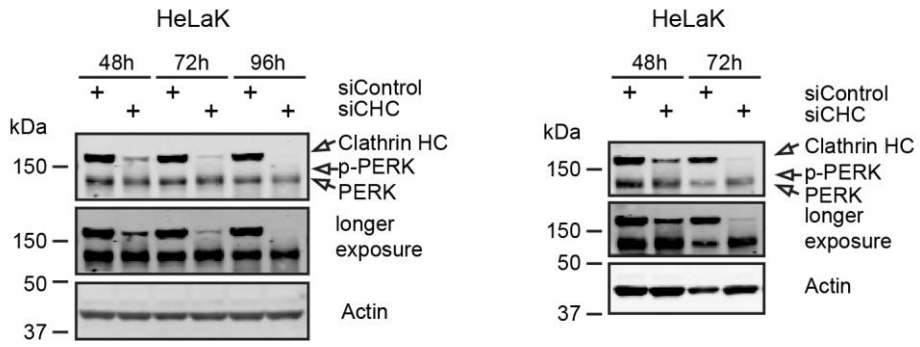


Fig. R2: Phosphorylated PERK might be weakly detected at 48 and 72 h post siRNA against clathrin HC transfection in HeLaK cells, indicating a mild induction of ER stress and the activation of an unfolded protein response.

However we think these data are too weak to be included in our manuscript. A reason for the weak signal could be that the clathrin depletion leads to a chronic, but mild activation of ER

stress/UPR over several days, which is harder to detect than acute ER stress induction by for example a three hour treatment with Thapsigargin, a typical positive control for the UPR response. We are formulating the possible mechanism on how clathrin downregulates LRP5/6 and cadherin protein abundance now a bit more carefully in the discussion. In addition, we have included data on accumulated cadherins together with ß-catenin in the trans-Golgi-network of clathrin-depleted cells (new data, Fig. 6C,D), which supports our mechanistic explanation.

Minor points.

On p7, I assume the authors mean “exogenous LRP6-nMG was present” and not “exogenous LRP6-nMG was absent”
| We have now clarified this sentence.

A sentence outlining the nature of the mutations in the various cell lines and specifying if they affect the Wnt pathway is needed for the more lay reader.
| We have now included this information in Fig. S4.

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Second decision letter

MS ID#: JOCES/2020/244467

MS TITLE: Clathrin regulates Wnt/β-catenin signaling by affecting Golgi to plasma membrane transport of transmembrane proteins

AUTHORS: Else Munthe, Camilla Raiborg, Harald Stenmark, and Eva Maria Wenzel
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The authors make a substantial addition to the discussion of the role of endocytosis and exocytosis on Wnt signaling.

Comments for the author

The authors have nicely addressed the issues raised in the initial reviews. This is a well written clear and straightforward paper that moves the field forward by introducing robust evidence for the role of exocytosis in control of Wnt/β-catenin signaling.

Reviewer 2

Advance summary and potential significance to field

See previous review

Comments for the author

The manuscript flows better and the title change also makes it stronger as the data fits it better. I recommend accept as is.