Wnt directs the endosomal flux of LDL-derived cholesterol and lipid droplet homeostasis

Cameron C. Scott, Stefania Vossio, Fabrizio Vacca, Berend Snijder, Jorge Larios, Olivier Schaad, Nicolas Guex, Dimitry Kuznetsov, Olivier Martin, Marc Chambon, Gerardo Turcatti, Lucas Pelkmans and Jean Gruenberg

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Review timeline:

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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.)

Transfer Note:

Please note that this manuscript was originally submitted to the journal Molecular Systems Biology, where it was peer-reviewed. It was then transferred to EMBO reports with the original decision letter and the authors' responses to the referees attached. (Please see below)

Editor: Barbara Pauly

Original decision letter and referees' comments - MSB

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees raise substantial concerns on your work, which, I am afraid to say, preclude its publication in Molecular Systems Biology.

While referee #1 is positive on your work, the two other reviewers would not recommend publication. The reviewers appreciate your findings related to the role Wnt in regulating cholesterol metabolism. They note however that the underlying molecular mechanisms remain to be elucidated. With regard to the high-content screens presented in the study, reviewers #2 and #3 raise serious issues and indicate that in view of the lack of overlap between the various screens and fundamental issues with their interpretation, the value of these datasets as a community resource will be limited.
Under these circumstances, while the interesting data on Wnt-regulated cholesterol metabolism might be suitable for publication in a more classical cell and molecular biology journal, I am afraid that for Molecular Systems Biology the level of support remains too limited and I see no other choice than to return the manuscript with the message that we cannot offer to publish it.

We have nevertheless taken the liberty to discuss your study with our colleagues from EMBO reports next door. They indicated that the would be interested to consider the study provided the following points would be addressed (comments from Barbara Pauly, Senior Editor, EMBO reports):
- the details and results of the initial screen would be removed altogether and the study would focus on the characterization of the Wnt3a-mediated effects on cholesterol and lipid droplet homeostasis
- the minor concerns raised by referees 2 and 3 would be clarified
- while the full elucidation of the molecular mechanism by which Wnt3a regulates intracellular transport and lipid metabolism is beyond the scope of this manuscript, all three referees feel that some further insights in this direction would strengthen the study for example by following up some potential leads, as indicated by referee 2.

If you are interested in the possibility to have your manuscript considered by EMBO reports, you can transfer the full manuscript and the reviewers' reports.

In any case, thank you for the opportunity to examine your work and I hope that the points raised in the reports will prove useful to you.

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REFEREE REPORTS

Reviewer #1:

This paper reports the results of an image based, siRNA screen for modulators of endolysosomal cholesterol levels and bis-monoacylglycerol (LBPA) levels in cultured cells. The screen confirms a link between these two lipids; the experiments confirm that LDL-receptor function is important for LBPA levels (likely because it influences cholesterol levels). Of the various pathways that altered lysosomal lipid staining upon siRNA treatment, the Wnt signaling pathway was chosen for further study. Others have shown that loss of beta catenin in mice leads to steatosis and cholesterol accumulation in liver, consistent with the present findings. The authors go on to evaluate the effect of Wnt3a addition on cholesterol content, trafficking and lipid droplets. Workers in the Wnt signaling field will find the story very interesting. In general, the work represents a solid contribution that appears to be carried out at a high level. A great deal of work is summarized.

Specific suggestions: The authors should compare their Wnt3a-responsive genes with previous transcriptional profiling studies and explain how their results compare.

Minor points: Page 3 and 4 have numerous language and syntax errors; last line of p4, what is the previously unknown cellular program that the authors refer to? Fig. 2B, can the authors add error bars? The authors call this "highly significant" thus should bolster the statement by providing a P value. Figure 3, should read: principle component; also the 3C figure is not fully labeled. Figure 4A. How were these data normalized? How long were LPDS treatments? Please add more information to the figure legends.

Reviewer #2:

This manuscript by Scott et al. reports the results of a RNAi screen using staining by filipin and an antibody against LBPA as a readout. Based on this, the authors propose that Wnt-signaling regulates cholesterol metabolism and storage.

This paper has previously been reviewed by others. As I feel it would be unfair to raise too many experimental criticisms at this point, I will mainly provide an evaluation on what is there, rather than
suggest how to improve the paper.

In my mind, this is a difficult work to assess; it's a case of "the good, the bad and the ugly" and it depends on how one values the relative strengths and weaknesses:

The "Good": the finding that Wnt regulates cholesterol metabolism, and predominantly CE storage is likely true and exciting.

The "Bad": it remains unclear what the mechanism is. Maybe this is too much to ask for in one paper, but the authors have interesting leads (specifically the apparent up-regulation of SOAT1).

Would have been nice to see some follow-up. An alternative hypothesis to the authors' argument that Wnt regulates directly lipid metabolism would be that it regulates cell division and that indirectly this impacts lipid metabolism. Would have been nice to see more time course type of experiments.

The "Ugly": it is really unclear what the quality of the screen is. There is very little confirmation of any hits (i.e. pick the top 50 and repeat screening with independent sets of reagents), there has been minimal overlap between screens, which would be expected to yield similar results. The authors argue that the method of combining the top hits is common. I really cannot follow this argument for the screen provided here. As the authors argue, the identification of the Wnt pathway and confirmation by other means shows that one can find something interesting (peculiarly though figure 3A shows that Wnt pathway genes are all over the place). However, the authors report the whole screen and in my opinion this has VERY limited value without some quality control (also, on a more subtle point, figures like 2E are pointless in my view).

Another major contingency for the interpretation of the screen result concerning the correlation between LBPA and cholesterol signals in the membrane is the nature of the assay. It is very possible that the amount of lipids such as LBPA changes the accessibility of filipin to cholesterol. There is ample evidence for this effect in the literature and would explain the correlation independent of biology.

In summary, I find the identification of Wnt signaling as a regulator of cholesterol metabolism potentially very interesting, but the context in which it is presented makes it hard for me to support publication of this paper.

A number of more specific points that would need to be addressed are:

1) Concerning the data on orlistat. This needs to be interpreted much more carefully. Orlistat is a general lipase inhibitor, not a LIPA inhibitor (as the authors repeatedly claim).

2) The methods do not show how cholesterol is measured (e.g. Fig 4A), as a consequence its impossible to evaluate these data (What are the axes? Relative to what?)

3) the writing of the paper is convoluted, often unclear. A major reason is that the authors continuously present "mini-conclusions" in the results section (often unwarranted). For example: "WNT pathway genes were highly enriched in our screens, confirming WNT signaling as a likely regulator of cellular cholesterol". This is not really true. The enrichment says (if true which is hard to assess given the weaknesses in the screen data, but likely based on what follows) that Wnt is required for normal cholesterol levels. It also is a regulator in other contexts. So, the INTERPRETATION is that it is a regulator of cholesterol. Also "confirming ....as a likely candidate" is a bit of an empty statement: was it not a candidate before? What is the difference between a candidate and a validated hit?

While this seems like a small point, there are many instances of this logic lapses in the paper, which I find very off-putting when reading it.

4) Similar some of the subheadings use suggestive titles rather than reporting the results: example: LDLR unexpectedly regulates LBPA. Well, I guess whether it is expected or not is in the eye of the beholder.

Reviewer #3:

Using siRNA-based screening and image analysis, the authors identify multiple cellular pathways (mostly metabolic) as regulators of cellular cholesterol homeostasis. They then focus on the Wnt signaling pathway as a regulator of cholesterol endocytosis and transport in the endocytic pathway. This study confirms and extends previous siRNA-based studies identifying a role for Wnt in
endocytosis (Collinet et al., 2010). Additional experiments indicate that Wnt3-conditioned medium reduces fluid phase uptake and the flux of cholesterol through early and late endosomes. The work is of interest for a large audience. However, it does not clarify how specific the effect of Wnt3-conditioned medium is, and how intracellular transport and lipid metabolism are regulated by Wnt signaling. There are several points that need to be addressed.

Major points

1. Screen validation. Two cell lines have been used in the study, each treated with two siRNA libraries (druggable genome from Qiagen, 3 siRNAs per gene, and whole genome from Dharmacon, pooled siRNAs), in duplicates. For all these conditions, LBPA (lyso bis phosphatidylic acid) and cholesterol mean cellular fluorescence levels were measured. The auxiliary dataset presents the results for each gene. However, it is not clear how many of the siRNAs targeting the same gene caused the phenotypes. It is also not clear how the gene Z-scores shown in the Dataset was calculated (average value of the duplicates/siRNAs/how many siRNAs show the phenotype?). For pathway analysis (pg. 8) the authors use hits detected in one screen only, due to the low overlap between the screens (see Dataset). The siRNA-based screens use in general multiple siRNAs (6-7) in order to exclude off-target effects. In this study phenotypes are based however, at least in some cases, on a single siRNA.

2. To confirm the role of Wnt in lipid metabolism, Wnt3a conditioned medium was used as a source of ligand. Does this conditioned medium activate other signaling pathways, especially since a high number of metabolic genes are modified after this treatment (Table S1)? The cell line used for these experiments is different than those used for screening. Does the knockdown of the hit genes affects lipid metabolism in the same way in this cell line? Treatment with U18666A modifies a similar (to Wnt3) set of genes, however it affects differently cholesterol-related genes (Table S2). What is the significance of this finding? The conclusions on pg.12 do not appear to be experimentally supported.

Minor points

2. Parameter values were corrected for the influence of population context. Even though the reference article is mentioned, it would be useful for the reader if this method were shortly described (pg. 20). This analysis is presented in Fig. 1E, however without a color legend. Some other figure legends are incomplete, and no statistical information (number of experiments, variation, significance) or color legends are shown. In addition, it is difficult to find the information about mass spectrometry mentioned in the legend of Fig. 2A.

3. Based on the analysis of the siRNA screens, Wnt-pathway is selected as an interesting candidate. The implication of many genes such as CSNK2B, CSNK2A2, MYC etc. (Fig. 3B), which likely play a role in multiple pathways other than Wnt, could be discussed.

4. Fig. 3C: the effect of Wnt3a-conditioned medium stimulation on beta-catenin protein levels (also Fig. S3) is shown, however a loading control is missing in both conditions.

5. The reduction in cholesterol (filipin) levels in Wnt3-treated cells is very small (Fig. S3B, C).

6. Wnt3a-medium treatment and APC knockdown also increased lipid droplet formation. Incubation with lipoprotein-depleted serum reduced lipid droplet formation and cholesterol-esterification (Fig. 5). Wnt3-dependent lipid droplet formation was also reduced by overexpression of Rab5Q79L and of anti-LBPA, which blocks cholesterol export form late endosomes. How significant is a reduction from 0.6 to 0.3 lipid droplets per cell (Fig. 5E)? How was the amount of Rab5Q79L quantified and what are the thresholds for the groups shown as distinct bars (Fig. 5D)?

6. Wnt3-conditioned media decreased the amount of LDLR and NPC2 (Fig. 6) and initial fluid-phase uptake (Fig. S7). No change was observed in transferrin receptor, EEA1, Rab7 or LAMP1 protein levels or in EGFR phosphorylation. Uptake experiments measuring the uptake of the Transferrins and EGF could indicate how Wnt3 stimulation affects transport in these endocytic pathways.
Authors’ responses to:

Your editorial comments:

The Chief Editor of MSB, informed us that he took the liberty to discuss our paper with you, and that you indicated that you would be interested to consider our study provided the following points would be addressed:

- the details and results of the initial screen would be removed altogether and the study would focus on the characterization of the Wnt3a-mediated effects on cholesterol and lipid droplet homeostasis

- As you suggested, we have deleted the details and results of the initial screens, and the manuscript now focuses on the characterization of the Wnt3a-mediated effects on cholesterol and lipid droplet homeostasis

- the minor concerns raised by referees 2 and 3 would be clarified

- The minor concerns of the referees have been clarified.

- while the full elucidation of the molecular mechanism by which Wnt3a regulates intracellular transport and lipid metabolism is beyond the scope of this manuscript, all three referees feel that some further insights in this direction would strengthen the study for example by following up some potential leads, as indicated by referee 2.

- As you requested, we have added new experiments that provide further insight into the mechanisms by which Wnt regulates intracellular transport and lipid metabolism. In particular, our new data indicate that Wnt controls lipid storage and transport via the SREBP-dependent pathway (see below and new Fig 5H and I).

Reviewer #1:

This paper reports the results of an image based, siRNA screen for modulators of endolysosomal cholesterol levels and bis-monoacyl glycerol (LBPA) levels in cultured cells. The screen confirms a link between these two lipids; the experiments confirm that LDL-receptor function is important for LBPA levels (likely because it influences cholesterol levels). Of the various pathways that altered lysosomal lipid staining upon siRNA treatment, the Wnt signaling pathway was chosen for further study. Others have shown that loss of beta catenin in mice leads to steatosis and cholesterol accumulation in liver, consistent with the present findings. The authors go on to evaluate the effect of Wnt3a addition on cholesterol content, trafficking and lipid droplets. Workers in the Wnt signaling field will find the story very interesting. In general, the work represents a solid contribution that appears to be carried out at a high level. A great deal of work is summarized.

Specific suggestions: The authors should compare their Wnt3a-responsive genes with previous transcriptional profiling studies and explain how their results compare.

-As requested, we have carried out this analysis and added the findings as a new Figure S5A.

Minor points: Page 3 and 4 have numerous language and syntax errors; last line of p4, ...

-We have reworded these sections to more precisely describe our findings.

… what is the previously unknown cellular program that the authors refer to?
We have clarified this sentence. We now state that our data reveal “the existence of an unforeseen program controlling lipid storage and endosome transport through Wnt signaling”, rather than “…a previously unknown cellular program controlling lipid storage and endosome transport under the control of Wnt signaling”.

Fig. 2B, can the authors add error bars? The authors call this "highly significant" thus should bolster the statement by providing a P value.

Following your suggestions, this whole section of the manuscript, including this figure, has been removed.

Figure 3, should read: principle component; also the 3C figure is not fully labeled.

These have been corrected.

Figure 4A. How were these data normalized?

The captions of the Y-axis in Fig 4A were wrong. This was a mistake and we thank the reviewer for this comment. We had normalized the data to simplify comparison with the other panels, but we forgot to change the Y-axis in Fig 4A. This has been corrected.

How long were LPDS treatments? Please add more information to the figure legends.

The requested information has been added to the Legends. The cells were treated with LPDS for 24h for the same time as with the conditioned media. Information on GC-MS was originally cited as a reference, and we have now added a detailed description to the methods.

Reviewer #2:

This manuscript by Scott et al. reports the results of a RNAi screen using staining by filipin and an antibody against LBPA as a readout. Based on this, the authors propose that Wnt-signaling regulates cholesterol metabolism and storage. This paper has previously been reviewed by others. As I feel it would be unfair to raise too many experimental criticisms at this point, I will mainly provide an evaluation on what is there, rather than suggest how to improve the paper.

The reviewer was mistaken. The manuscript was not reviewed previously.

In my mind, this is a difficult work to assess; it’s a case of "the good, the bad and the ugly" and it depends on how one values the relative strengths and weaknesses: The "Good": the finding that Wnt regulates cholesterol metabolism, and predominantly CE storage is likely true and exciting. The "Bad": it remains unclear what the mechanism is. Maybe this is too much to ask for in one paper, but the authors have interesting leads (specifically the apparent up-regulation of SOAT1). Would have been nice to see some follow-up.

It is unclear to us what the reviewer is requesting regarding "following-up the lead" with SOAT1. It would be a euphemism to state that we strongly feel that we have followed this lead – in fact this point is central to the story. We show that Wnt affects SOAT1 expression (mRNA levels) and activity (as assessed by drug treatment and direct biochemical quantification of esterification). We take this as strong evidence that Wnt signaling results in the upregulation of SOAT1, which results in the esterification of cholesterol for storage in lipid droplets.

We also wish to mention that the SOAT1 inhibitor 58-035 does not block lipid droplet formation – if anything the inhibitor potentiates droplet formation. After addition of radiolabeled oleic acid as
tracer, however, the bulk of the radioactivity appears in TAGs, not CEs (~10-fold, Fig. 4), indicating that the bulk of incoming oleate is incorporated by DGATs into the TAGs of the droplets. It is very likely that similar or increased amounts of TAGs are synthesized by DGATs in response to Wnt in the presence of the SOAT1 inhibitor. Synthesis of TAGs is likely to depend on a feedback loop that senses CEs in the lipid droplets. Although we left these data out of the manuscript, because the control of the TAG-to-CE ratio in forming lipid droplets is much beyond the scope of this paper, we briefly discuss this issue after Wnt treatment in the supplementary materials (“Wnt-dependent regulation of LD formation: CEs and TAGs”).

An alternative hypothesis to the authors’ argument that Wnt regulates directly lipid metabolism would be that it regulates cell division and that indirectly this impacts lipid metabolism. Would have been nice to see more time course type of experiments.

-We have really no evidence that Wnt regulates the cell division cycle in our unsynchronized cultures. Having said that, we can carry out time-course experiments or directly test the effects of Wnt on cell division.

The "Ugly": it is really unclear what the quality of the screen is. There is very little confirmation of any hits (i.e. pick the top 50 and repeat screening with independent sets of reagents), there has been minimal overlap between screens, which would be expected to yield similar results. The authors argue that the method of combining the top hits is common. I really cannot follow this argument for the screen provided here.

-Following your suggestions, these parts have been deleted in the revised version of the manuscript. However, we still wish to reply to the arguments raised by this reviewer:

-Perhaps it is “not common” (which we never claimed), but extracting data by combining data from multiple screens has been done before (as we have cited). We treated our data with the same principles and presented it as one solution to the inherent problems of siRNA screening today. We maintain that this work describes a novel approach for dealing with the large number of false positive, false negative, and off-target effect of existing libraries. In fact, we feel that this is one of the strengths of the work. The paper was structured as a presentation of the screens, including a figure (ex-1F) documenting the difference between screens and a detailed description of how we dealt with the large inherent variation between the libraries. The remainder of manuscript shows that this was fruitful by validating the Wnt pathway resulting from this analysis.

-We may need to test more systematically other pathways (not just hits) also found by this strategy in order to further validate our approach, and to be able to conclusively state that a combinatorial pathway analysis is better than the analysis of each screen independently. This is of course ongoing work, which extends far beyond the present study and will require significant additional effort.

As the authors argue, the identification of the Wnt pathway and confirmation by other means shows that one can find something interesting (peculiarly though figure 3A shows that Wnt pathway genes are all over the place). However, the authors report the whole screen and in my opinion this has VERY limited value without some quality control (also, on a more subtle point, figures like 2E are pointless in my view).

-We are pursing a direct validation of a larger number of hits of these screens in the course of a related project. In the current version of the manuscript, Figures 2A-D show examples of the validation of a limited number of hits (although granted they were chosen because of their obvious biological relevance).

-However, we are not surprised at all with the scattered nature of the hits all over the Wnt pathway — particularly given the complexity and in-built redundancies of this pathway. By nature, siRNA screens can only reveal knockdowns causing sufficient decrease of the protein level to become limiting — assuming that the activity is limiting — but without
deleterious effects on viability.

- Figure 2E was not included as validation of the screen, but rather as an unbiased description of the pathway analysis illustrating how the Wnt pathway was identified (the subject of the rest of this paper), and the relative ranking of this pathway amongst the other top hits.

Another major contingency for the interpretation of the screen result concerning the correlation between LBPA and cholesterol signals in the membrane is the nature of the assay. It is very possible that the amount of lipids such as LBPA changes the accessibility of filipin to cholesterol. There is ample evidence for this effect in the literature and would explain the correlation independent of biology.

-We take issue with this assertion. In contrast to the contention of the reviewer, we are not aware of any study showing that the amount of lipids such as LBPA changes the accessibility of filipin to cholesterol. In fact, filipin has proven to be a highly reliable cholesterol tracer in cells from patients with the cholesterol-storage disorder Niemann-Pick type C, which is accompanied by increased levels of LBPA. Last but not least, cholesterol remains the most consistent and robust cholesterol indicator in many experimental contexts, including in screens (see for example the studies of Fred Maxfield or Heiko Runz). Finally, from a biochemical viewpoint, it is not clear what the reviewer means with “lipids such as LBPA”. Does he/she mean glycerophospholipids? Or negatively charged lipids? Or lipids believed to have a negative molecular spontaneous curvature?

-Regardless of this, we went to great lengths to confirm our lipid observations with direct determinations using mass spectrometry and thin-layer chromatography.

In summary, I find the identification of Wnt signaling as a regulator of cholesterol metabolism potentially very interesting, but the context in which it is presented makes it hard for me to support publication of this paper.

A number of more specific points that would need to be addressed are:

1) Concerning the data on orlistat. This needs to be interpreted much more carefully. Orlistat is a general lipase inhibitor, not a LIPA inhibitor (as the authors repeatedly claim.

-Perhaps this is just semantics but we never claimed Orlistat was a specific inhibitor of LIPA in the text, just an inhibitor of LIPA (which it is). We do not share the reviewers concerns as in our hands, we see that Orlistat completely recapitulates the effect of the silencing of LIPA by siRNA (some of this data are shown in Figure 2), suggesting that both treatments target the same acidic lipase activity relevant for cholesterol processing in endosomes. However, we have modified the text to make it clear that Orlistat is a general inhibitor of lipases, and therefore could have effects on cellular lipid homestasis beyond those mediated LIPA in endosomes.

2) The methods do not show how cholesterol is measured (e.g. Fig 4A), as a consequence its impossible to evaluate these data (What are the axes? Relative to what?)

- In the previous version of the manuscript, these methods were referenced (Ding, 2013), which is standard for well-established and commonly used methods. We now describe these methods in detail.

3) the writing of the paper is convoluted, often unclear. A major reason is that the authors continuously present "mini-conclusions" in the results section (often unwarranted) . For example: "WNT pathway genes were highly enriched in our
screens, confirming WNT signaling as a likely regulator of cellular cholesterol". This is not really true. The enrichment says (if true which is hard to assess given the weaknesses in the screen data, but likely based on what follows) that Wnt is required for normal cholesterol levels. It also is a regulator in other contexts. So, the interpretation is that it is a regulator of cholesterol. Also "confirming ...as a likely candidate" is a bit of an empty statement: was it not a candidate before? What is the difference between a candidate and a validated hit? While this seems like a small point, there are many instances of this logic lapse in the paper, which I find very off-putting when reading it.

-Wording may not have been optimal in this (and perhaps other) instance. However, beyond hairsplitting arguments, we see nothing wrong with the statement that our analysis confirms that Wnt is a likely candidate. A likely candidate means exactly what it said. The comment about the empty statement is also strange: Wnt comes up earlier in the text, and thus the assertion is correct.

4) Similar some of the subheadings use suggestive titles rather than reporting the results: example: LDLR unexpectedly regulates LBPA. Well, I guess whether it is expected or not is in the eye of the beholder.

-We appreciate the reviewer’s opinion and have done our best to amend the text.

Reviewer #3:

Using siRNA-based screening and image analysis, the authors identify multiple cellular pathways (mostly metabolic) as regulators of cellular cholesterol homeostasis. They then focus on the Wnt signaling pathway as a regulator of cholesterol endocytosis and transport in the endocytic pathway. This study confirms and extends previous siRNA-based studies identifying a role for Wnt in endocytosis (Collinet et al., 2010). Additional experiments indicate that Wnt3-conditioned medium reduces fluid phase uptake and the flux of cholesterol through early and late endosomes. The work is of interest for a large audience. However, it does not clarify how specific the effect of Wnt3-conditioned medium is, and how intracellular transport and lipid metabolism are regulated by Wnt signaling.

There are several points that need to be addressed.

Major points

1. Screen validation. Two cell lines have been used in the study, each treated with two siRNA libraries (druggable genome from Qiagen, 3 siRNAs per gene, and whole genome from Dharmacon, pooled siRNAs), in duplicates. For all these conditions, LBPA (lyso bis phosphatidic acid) and cholesterol mean cellular fluorescence levels were measured. The auxiliary data set presents the results for each gene. However, it is not clear how many of the siRNAs targeting the same gene caused the phenotypes. It is also not clear how the gene Z-scores shown in the Dataset was calculated (average value of the duplicates/siRNAs/how many siRNAs show the phenotype?).

- The methods have been expanded to make these points clearer. Indeed the duplicates of each of the separate siRNAs of the QIAGEN library were averaged, after exclusion of toxic (low number of cells) wells, while for the pooled library (Dharmacon), the two duplicates were averaged.

For pathway analysis (pg. 8) the authors use hits detected in one screen only, due to the low overlap between the screens (see Dataset).

- This is incorrect. As stated in the text, we used the combined three screens for pathway analysis.
The siRNA-based screens use in general multiple siRNAs (6-7) in order to exclude off-target effects. In this study phenotypes are based however, at least in some cases, on a single siRNA.

-This is also incorrect. All “hits” were identified by three siRNAs, in a pool (3 siRNAs per pool, Dharmacon library) or separate (3 separate siRNAs, QIAGEN library). We have modified the text to make this clearer.

2. To confirm the role of Wnt in lipid metabolism, Wnt3a conditioned medium was used as a source of ligand. Does this conditioned medium activate other signaling pathways, especially since a high number of metabolic genes are modified after this treatment (Table S1)?

-For all experiments, conditioned medium was prepared in parallel from sister L-cells that do not secrete WNT3A and was used as a control medium. Therefore, we expect all differences to result solely from the presence of WNT3A in the media.

The cell line used for these experiments is different than those used for screening. Does the knockdown of the hit genes affects lipid metabolism in the same way in this cell line?

-The screen was done using HeLa-MZ cells and the conditioned medium prepared from L-cells that secrete Wnt. Thus, we preferred to use a homologous system and used L-cells to study the effects of Wnt. However, we have included in the manuscript typical examples showing that the effects of Wnt are recapitulated in the HeLa-MZ cells (Fig. S3B, S4, S6B) used in the screen.

-Further, we have now confirmed the same effects in a human hepatocyte cell (HepaRG cells, see new Fig 3).

Treatment with U18666A modifies a similar (to Wnt3) set of genes, however it affects differently cholesterol-related genes (Table S2). What is the significance of this finding?

-This suggests that the treatments differentially affect the cholesterol sensing mechanism, which is expected (and is now discussed in the text). We are currently investigating the role of SREBP response in Wnt signaling (see new Fig 5H and I).

The conclusions on pg.12 do not appear to be experimentally supported.

-This is a very puzzling comment. Did this reviewer miss the point, or was our wording so unclear? The conclusions are that, in response to Wnt, the cholesterol esters arriving in lipid droplets transit through the LDLR/SOAT pathway. These conclusions are supported by multiple experiments presented in Figure 5. We have reworded the conclusions to avoid any ambiguity.

3. Knockdown of APC causes a small reduction (20%) in the amount of membrane cholesterol. Could this be also due to non-specific effects of APC knockdown, e.g. apoptosis, transcriptional activation?

-These comments are also very puzzling. So-called free cholesterol is a key component of membranes: it regulates the functions of many proteins but also the membrane physical properties. As discussed in the paper, membrane cholesterol can only be depleted to a limited extend in living cells, without destroying the membrane barrier properties and thus killing cells. Acute depletion of cholesterol can be achieved, e.g. with cyclodextrins, but only over a short time-period. We were actually amazed that cells tolerated a 20% reduction in membrane cholesterol, which is highly significant – to the best of my
knowledge a greater decrease is not seen by any other treatment, including in our screen (see below).

- The APC knockdown presumably reduces cholesterol by the same mechanism as the WNT treatment, which indeed results in transcription changes, as discussed in the paper (see Fig. S5). We did not detect any difference between the Wnt treatment and the APC siRNA that would suggest apoptosis or another process is differentially regulated between the two treatments.

Minor points

2. Parameter values were corrected for the influence of population context. Even though the reference article is mentioned, it would be useful for the reader if this method were shortly described (pg. 20). This analysis is presented in Fig. 1E, however without a color legend. Some other figure legends are incomplete, and no statistical information (number of experiments, variation, significance) or color legends are shown. In addition, it is difficult to find the information about mass spectrometry mentioned in the legend of Fig. 2A.

- As requested, we have now added a description of the method used for population context analysis.
- Thank you for pointing out these oversights, they have been corrected or the figures removed from the current manuscript.
- Since the GC-MS methods are standard biochemical methods, they were just referenced. We now added a description of the methods in this new version.

3. Based on the analysis of the siRNA screens, Wnt-pathway is selected as an interesting candidate. The implication of many genes such as CSNK2B, CSNK2A2, MYC etc. (Fig. 3B), which likely play a role in multiple pathways other than Wnt, could be discussed.

- Indeed, we do not know what other pathways may be involved and what cross-talk may exist with the Wnt pathway – this is a topic that we are pursuing in the lab. In any case, the siRNA screen has been removed in this new version of the paper, and this issue is no longer raised in the paper.

4. Fig. 3C: the effect of Wnt3a-conditioned medium stimulation on beta-catenin protein levels (also Fig. S3) is shown, however a loading control is missing in both conditions.

- We thank the reviewer for mentioning this omission. The loading controls have now been added.

5. The reduction in cholesterol (filipin) levels in Wnt3-treated cells is very small (Fig. S3B, C).

- This point is discussed above. As mentioned (above and in the text), a 20% reduction in membrane cholesterol is very significant. During the whole screening process, we did not identify siRNAs, which could reduce cholesterol below this, because lower levels of cholesterol are incompatible with intact biological membranes and therefore cell viability.

6. Wnt3a-medium treatment and APC knockdown also increased lipid droplet formation. Incubation with lipoprotein-depleted serum reduced lipid droplet formation and cholesterol-esterification (Fig. 5). Wnt3-dependent lipid droplet formation was also reduced by overexpression of Rab5Q79L and of anti-LBPA, which blocks cholesterol export form late endosomes. How significant is a reduction from 0.6 to 0.3 lipid droplets per cell (Fig. 5E)?

- These experiments were performed in a large number of cells by automated microscopy and image analysis. There for the exact settings used to count lipid droplets were optimized for specificity, and thus are restricted to the brighter/larger droplets to ensure proper identification, and therefore significantly underestimate the total number of lipid droplets, however these measurements are consistent between conditions. The absolute
number of lipid droplets here is less than elsewhere in the paper as it was necessary to
dilute the Wnt3a conditioned media with the hybridoma supernatant containing the anti-
LBPA antibodies. In any case, a two-fold difference is highly significant – as also obvious
by eye.

-Rab5Q79L was tagged with mCherry and quantified by measuring fluorescence intensity.
The methods have been expanded to include more detail on this experiment.

7. Wnt3-conditioned media decreased the amount of LDLR and NPC2 (Fig. 6) and
initial fluid-phase uptake (Fig. S7). No change was observed in transferrin receptor,
EEA1, Rab7 or LAMP1 protein levels or in EGFR phosphorylation. Uptake
experiments measuring the uptake of the Transferrin and EGF could indicate how
Wnt3 stimulation affects transport in these endocytic pathways.

-Again we are a bit puzzled by this comment. We quantified EGFR degradation (Fig. S7)
and the data clearly shows that no detectable difference can be seen in the kinetics and
extent of EGFR degradation between the Wnt and Control conditioned media. These
observations rule out the possibility that Wnt had a significant effect on EGFR endocytosis.

-We did measure transferrin uptake and found a slight, but significant reduction in uptake
over the short times we tested. However, given the known reduction in endocytosis caused
by partial cholesterol depletion (including clathrin-mediated endocytosis, e.g. Kirsten
Sandvig and colleagues), this small reduction in Tf uptake may be a consequence of
reduced cholesterol levels after Wnt treatment.

Thank you for your patience while we have reviewed your revised manuscript. As you will see from
the reports below, the referee who also saw the original version of it at Molecular Systems Biology
appreciates the efforts in improving and re-focusing of the study.

I am therefore happy to accept your manuscript for publication in EMBO reports provided a few
minor issues will be addressed:

1. At the moment there are no materials & methods described in the main body of the text. Please
move parts (or the entire section) of what is now placed in the supplementary section to the main
manuscript. Please do not worry about length restrictions. I much rather enable our readers to see the
M&M in the main body of the paper.

2. Please indicate clearly throughout the study which statistical tests have been performed. Please
also note that in general, standard errors should not be shown if the number of biological replicates
is only 2 (n=2; e.g. as it appears to be the case in Fig 3C). In cases in which the experiment has been
repeated twice (i.e. two biological replicates) but in each case 30 cells have been counted
(representing technical replicates, e.g. in Fig 2I-J) I would recommend showing individual data
points for the 30 cells and state that the experiment has been repeated twice. Please go through the
main and supplementary figures and adjust them accordingly.
If all remaining corrections have been attended to, you will then receive an official decision letter
from the journal accepting your manuscript for publication in the next available issue of EMBO
reports. This letter will also include details of the further steps you need to take for the prompt
inclusion of your manuscript in our next available issue.

REFEREE REPORT:
Referee #1:

This is a revision of a previous paper submitted to a different journal, where I had reviewed it.
At the heart of the story, the authors identify Wnt3a as a modulator of lipid metabolism and Lipid
Droplets. Consistent with the mission of EMBO reports, this is a novel and interesting finding
justifying the publication of this paper. Despite the authors rebuttal, I maintain that the screen is largely un-interpretable without more rigorous confirmation of candidates (not sure about the point of figure 1) and that there are numerous experimental problems (e.g., based on the images provided, I cannot see how the authors could possibly count LDs per cell), as well as problems of over-interpretation throughout the text and figures. Nonetheless, the paper summarizes a very substantial amount of work with an important finding that could be important for the field of lipid metabolism research. It should thus be published.

1st Revision - authors’ response 05 March 2015

Thank you very much for your very positive comments and for accepting in principle our paper by Scott et al. entitled: “Wnt directs the endosomal flux of LDL-derived cholesterol and lipid droplet homeostasis”, in EMBO reports.

We have addressed all the points that you have raised in your editorial letter, and you will find below our detailed responses.

1. At the moment there are no materials & methods described in the main body of the text. Please move parts (or the entire section) of what is now placed in the supplementary section to the main manuscript. Please do not worry about length restrictions. I much rather enable our readers to see the M&M in the main body of the paper.

The entire Materials and Methods were indeed present in the last version, although they were entitled “Experimental Procedures”. They have been renamed “Methods” as you have requested, and are part of the main body of the text.

2. Please indicate clearly throughout the study which statistical tests have been performed. Please also note that in general, standard errors should not be shown if the number of biological replicates is only 2 (n=2; e.g. as it appears to be the case in Fig 3C). In cases in which the experiment has been repeated twice (i.e. two biological replicates) but in each case 30 cells have been counted (representing technical replicates, e.g. in Fig 2I-J) I would recommend showing individual data points for the 30 cells and state that the experiment has been repeated twice. Please go through the main and supplementary figures and adjust them accordingly.

We have added to the Methods a paragraph detailing the statistical methods, which we have used. We now provide the requested details on the number of replicates, and explain what type of data is presented in the Figure Legends. In summary, we have added or precisely specified p-values to panels 2A, 2D-E, 2H-J, 3B, 4A-C, 4E, 5C-G, S6D, removed a time point from 2A, and changed panels 2B-C, 2G-J, 3C, 4B, 4D, 4F-G, 5B, 5G-I, S3E, S6C-H to conform with your recommendation above.

Referee #1 comment:
This is a revision of a previous paper submitted to a different journal, where I had reviewed it. At the heart of the story, the authors identify Wnt3a as a modulator of lipid metabolism and Lipid Droplets. Consistent with the mission of EMBO reports, this is a novel and interesting finding justifying the publication of this paper. Despite the authors rebuttal, I maintain that the screen is largely un-interpretable without more rigorous confirmation of candidates (not sure about the point of figure 1) and that there are numerous experimental problems (e.g., based on the images provided, I cannot see how the authors could possibly count LDs per cell), as well as problems of over-interpretation throughout the text and figures. Nonetheless, the paper summarizes a very substantial amount of work with an important finding that could be important for the field of lipid metabolism research. It should thus be published.
Authors reply:
We do strongly disagree with the reviewer’s assertion that there are any significant experimental problems with the study. As the reviewer failed to provide specifics (as in the initial review) we cannot provide a detailed rebuttal to this statement, however as to the quantification of LDs by software, we are confident that our imaging pipeline can robustly identify and quantify LDs in cells. These images were acquired in large numbers by an automated microscope using a 60X objective. Since LDs are of a distinct, regular shape, it is quite easy to identify them in cells. In fact, LDs are easily quantified in a robust fashion in these experiments, since they cannot be mistaken for another organelle and since they are essentially absent in untreated cells.

We wish to thank you very warmly for your insightful comments, which we feel, have contributed to significantly improve the paper.

2nd Editorial Decision 06 March 2015

Many thanks for submitting the final version of your manuscript to EMBO reports. I am happy to formally accept it for publication in the next available issue.