Tyrosine phosphorylation of LRP6 by Src and Fer inhibits Wnt/β-catenin signaling

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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.)

Many thanks for your patience while we were waiting to hear back from the reviewers on the suitability of your study for EMBO reports. We have now received the enclosed reports on it and I am happy to tell you that we can offer publication of your study in our journal after minor revision along the lines of the reviewers' suggestions.

Referees 2 and 3 only raise some minor issues that can be addressed in writing. Referee 1 has only one concern that would need to be addressed experimentally, which is testing the effect of short-term Src/Fer inhibition on wnt signaling.

We feel confident that you will be able to address these minor changes in a rather timely fashion so that the publication of the manuscript is not delayed any further. As soon as you have submitted the final version I will make sure that it gets fast-tracked for publication.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.
REFEREE COMMENTS

Referee #1:

This manuscript reports the identification of Src and Fer as the kinases responsible for direct phosphorylation of tyrosine residues in the cytoplasmic tail of LRP6. The study shows that tyrosine phosphorylation inhibits LRP6 ser/thr phosphorylation and Wnt signaling. In the case of Src, the proposed mechanism is inhibition of LRP6 clustering/formation of signalosomes. Altogether, this work is well done, results are clear and interpretations sound. I have only one general criticism, which I will explain below.

But first, I would like to compare this study with the one recently published by Liu et al in JBC, and highlight what I consider important additional contributions from the present manuscript:

Both studies agree on a negative role of tyrosine phosphorylation. In the JBC study, however, this role is only deduced by testing a mutant LRP6 receptor. Here, the analysis is significantly more complete and convincing:

Firstly, it directly implicates Src and Fer, which on its own is an important and surprising discovery. Secondly, the functional analysis includes gain and loss-of-function of the kinases.

Thirdly, a complete characterization of the phosphorylation sites is presented. This includes direct monitoring of LRP6 tyr phosphorylation.

Finally, the authors demonstrate that LRP6 tyrosine phosphorylation is inhibited by Wnt signaling. In terms of the underlying mechanism, the two studies diverge: The JBC paper argues for an effect on routing LRP6 to clathrin/caveolin-mediated endocytosis. The present manuscript proposes that Src inhibits signalosome formation. On this topic, the former model seems based on slightly stronger evidence. The sedimentation experiments of Fig.5 in this manuscript could also potentially be consistent with two types of membrane compartments, rather than different complex sizes. My feeling is that both interpretations may not be mutually exclusive, and could reflect reading different cellular parameters underlying the same molecular events. I would suggest nuancing the interpretation in the discussion.

My only slight concern is about the gain and loss-of-function experiments: I am surprised in particular of the extreme difference between wt and Src -/- MEF cells in terms of response to Wnt. This would imply primordial role of Src on Wnt signaling, which would have been missed by hundreds of studies and innumerable genetic screens. The alternative explanation consists in considering a contribution from indirect effects due to long term Src gain or loss-of-function. Note that I do not doubt that Src and Fer indeed directly phosphorylate LRP6 and negatively regulate its function, as I find the data presented are convincing. Yet I believe that the case would be significantly strengthened by a simple confirmation using short term (e.g. one hour) treatments with soluble Src and Fer inhibitors. This would eliminate the contribution from long term effects, and would provide a straightforward estimate of the actual impact of Src, both in the resting state of the Wnt pathway (is Src required to keep Wnt signaling low) and for the intensity of Wnt signaling. I feel that implicating Src as a negative regulator of the Wnt pathway deserves this small but important additional validation.

Referee #2:

In this manuscript, Chen and co-workers show that the tyrosine kinases Src and Fer negatively regulate Wnt/beta-catenin signaling. They demonstrate that these kinases phosphorylate conserved tyrosine residues in the Wnt co-receptor LRP6 and provide evidence that this inhibits signalosome function. This is a very complete and well thought out study that is suitable for publication in EMBO Reports. I have only a few minor comments:

-It would be helpful if the results of the kinase screen presented in Fig. 1B are included.

-Statistics should be included in figure panels such as 1D (is there a significant difference between wild type and the kinase dead Src mutant and if so, why?); 2C (is the increase in reporter activity observed in the beta-catenin expressing cells significant and if so, why?); 3D (which mutants are statistically different from wild type?).
-Src appears to increase the total protein level of LRP6 (see for example Fig. 2A, but also other figures). The significance of this finding for the proposed mechanism of LRP6 regulation should be discussed.

-Why is the wild type LRP6 band stained with panYP in Fig. 3A of a lower size than the LRP6 band stained with the LRP6 antibody?

-The difference between Src and Fer in their effect on signalosome formation/maintenance should be discussed, especially in light of the mechanism related to the regulation of Fer by CK1gamma.

-To be complete, the authors are advised to include the FOPLASH negative control results in the panels showing TCF reporter data.

-There is an unformatted reference on page 10.

Referee #3:

The authors identified two tyrosine kinase, src and fer to phosphorylate the cytoplasmic domain of LRP6. The kinases block Wnt signalling as shown by overexpression and in knock-out cells, and LRP6 mutants lacking the respective phosphorylation sites are resistant to inhibition by src. These data suggest a new mode of regulation of Wnt signalling at the LRP receptor level which could be involved in negative feedback control as it is also shown that Wnt signalling stimulates LRP6 tyrosine phosphorylation.

Altogether experiments are well performed and controlled and results are clear cut and do not require additional detailed experimental analysis. However, certain aspects should be clarified, as listed below:

1 The authors should clarify how in their screening protocol the phosphorylated LRP6 band was distinguished from all the cellular proteins that become phosphorylated after kinase transfection, in particular as the Western blot in Fig. 1B has no background in the lanes with the other kinases. Probably, this is due the high overexpression of LRP6 but needs to explained.

2 A comment should be made on the apparent stabilisation of LRP6 by src seen in several Western blots (e.g. Fig. 1D, Fig. 2A).

3 The authors show that src blocks signalosome formation but that it apparently does not affect phosphorylation at amino acid 1490 at the PPPsP motif(Fig. 4B, 5A). Does that mean that LRP6 aggregation is not required for this signalling step which was claimed to initiate inhibition of the axin complex? Please comment.

4 The authors state that they could not confirm LRP6 tyrosine phosphorylation to play a role in LRP6 endocytosis which would contradict a previous study (Liu et al., 2914). However as they show no data on endocytosis the basis of their statement is not clear and they should delete this comment or show the data.

5 It is stated that the mutant delta87 lacks four of the five PPPSP motifs but in the scheme in Fig. 3A it appears that only three motifs are missing.

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Referee #1:

This manuscript reports the identification of Src and Fer as the kinases responsible for direct phosphorylation of tyrosine residues in the cytoplasmic tail of LRP6. The study shows that tyrosine phosphorylation inhibits LRP6 ser/thr phosphorylation and Wnt signaling. In the case of Src, the proposed mechanism is inhibition of LRP6 clustering/formation of signalosomes. Altogether, this work is well done, results are clear and interpretations sound.

Thank you for acknowledging the quality of our work.

I have only one general criticism, which I will explain below.

But first, I would like to compare this study with the one recently published by Liu et al in JBC, and highlight what I consider important additional contributions from the present manuscript:

Both studies agree on a negative role of tyrosine phosphorylation. In the JBC study, however, this role is only deduced by testing a mutant LRP6 receptor. Here, the analysis is significantly more complete and convincing:

Firstly, it directly implicates Src and Fer, which on its own is an important and surprising discovery.

Secondly, a complete characterization of the phosphorylation sites is presented. This includes direct monitoring of LRP6 tyr phosphorylation.

Finally, the authors demonstrate that LRP6 tyrosine phosphorylation is inhibited by Wnt signaling. In terms of the underlying mechanism, the two studies diverge: The JBC paper argues for an effect on routing LRP6 to clathrin/caveolin-mediated endocytosis. The present manuscript proposes that Src inhibits signalosome formation. On this topic, the former model seems based on slightly stronger evidence. The sedimentation experiments of Fig.5 in this manuscript could also potentially be consistent with two types of membrane compartments, rather than different complex sizes. My feeling is that both interpretations may not be mutually exclusive, and could reflect reading different cellular parameters underlying the same molecular events. I would suggest nuancing the interpretation in the discussion.

We agree with the referee on this point and, in the discussion, we have further emphasized the likelihood that both LRP6 clustering as well as LRP6 endocytosis are involved in the inhibition mechanism. Indeed, we now have some experimental evidence to support their (Liu et al) findings. Due to the comments from referees 2 and 3 concerning the apparent stabilization of LRP6 by Src, we performed experiments on LRP6 turnover and cell surface levels that actually support the findings of Liu et al. that endocytosis of LRP6 is somehow regulated by Src-mediated phosphorylation. This is now included in the revised manuscript and indeed brings the two studies closer in terms of a mechanistic explanation of how Tyrosine phosphorylation inhibits Wnt/LRP6 signaling. Thus, although our own experimental evidence presented in Fig.5 and Supp. Fig. 5 still points to a greater role for disruption of LRP6 aggregation rather than endocytosis, we come to the conclusion that trafficking of LRP6 is also regulated by Src- and Fer-mediated LRP6 Tyrosine phosphorylation.

My only slight concern is about the gain and loss-of-function experiments: I am surprised in particular of the extreme difference between wt and Src -/- MEF cells in terms of response to Wnt. This would imply primordial role of Src on Wnt signaling, which would have been missed by hundreds of studies and innumerable genetic screens. The alternative explanation consists in considering a contribution from indirect effects due to long term Src gain or loss-of-function. Note that I do not doubt that Src and Fer indeed directly phosphorylate LRP6 and negatively regulate its function, as I find the data presented are convincing. Yet I believe that the case would be significantly strengthened by a simple confirmation using short term (e.g. one hour) treatments with soluble Src and Fer inhibitors. This would eliminate the contribution from long term effects, and would provide a straightforward estimate of the actual impact of Src, both in the resting state of the Wnt pathway (is Src required to keep Wnt signaling low) and for the intensity of Wnt signaling. I feel that implicating Src as a negative regulator of the Wnt pathway deserves this small but important additional validation.

We were also a little surprised to see such sensitization of SYF -/- cells to incoming Wnt signals (hypersensitive to Wnt 3a conditionad media). Indeed this is why we performing the rescue
experiment (co-transfection of Src together with the TOPFLASH reporters in SYF -/- MEF cells), which confirms that Src is responsible for this hypersensitive effect in the KO cells. Whether this is an indirect consequence of long-term Src LoF is unclear but we have performed the suggested short-term experiments and under these more acute Src LoF conditions we do not see such a significant responsiveness of cells to Wnt treatment (Supp. Fig. 4B). The well known pyrazolo[3,4-d]pyrimidine Src inhibitor, PP2, was used. It is worth noting that Src is known to have positive regulatory effects on Wnt signaling and indeed we see that Src usually activates resting Wnt signaling (i.e. cells transfected with TOPFLASH reporters only) when overexpressed. In line with this we see partial inhibition of Wnt signaling by PP2, however this is converted into partial activation when cells are pre-treated with Wnt3a conditioned medium (Supp. Fig. 4B). A similar situation is seen if LRP6 is transfected to stimulate signalling, rather then adding Wnt3a CM (Supp. Fig. 4C). Although we cannot be sure that all endogenous Src activity has been inhibited using PP2, we do agree with the referee that this result potentially suggests that the hyper-responsiveness of the SYF -/- MEF cells could be due to indirect cellular effects as a consequence of prolonged Src LoF. It should nevertheless be mentioned that SYF -/- cells harbour null mutations for all three of the more ubiquitously expressed Src family kinase members, Src, Yes and Fyn. This is likely not comparable to a pharmacological inhibition of Src family kinases by PP2, which also targets other Tyrosine kinases (Bain et al, 2007) and may therefore impinge on other signalling pathways.


Referee #2:

*In this manuscript, Chen and co-workers show that the tyrosine kinases Src and Fer negatively regulate Wnt/beta-catenin signaling. They demonstrate that these kinases phosphorylate conserved tyrosine residues in the Wnt co-receptor LRP6 and provide evidence that this inhibits signalosome function. This is a very complete and well thought out study that is suitable for publication in EMBO Reports. I have only a few minor comments:*

-It would be helpful if the results of the kinase screen presented in Fig. 1B are included.

We have not included all the primary data of this screen because Fer and Src were actually the only significant “hits” that were detected. It should be notes that the screen consisted of about 240 individual kinases, which represents only about 50 % of the kinome and therefore not exhaustive.

-Statistics should be included in figure panels such as 1D (is there a significant difference between wild type and the kinase dead Src mutant and if so, why?); 2C (is the increase in reporter activity observed in the beta-catenin expressing cells significant and if so, why?); 3D (which mutants are statistically different from wild type?).

Statistics have now been included. For Fig.1D the difference between wt and kd Src is actually very significant: wt Src clearly inhibits the LRP6 induced TOPFLASH signal and this corresponds with a clear induction of LRP6 Y-phosphorylation (Pan Yp), whereas the kd mutant of Src neither significantly inhibits TOPFLASH nor induces LRP6 Yp. The reason for this difference is because the kinase dead form of Src is incapable of phosphorylating LRP6 and therefore is a control that Src is not acting in some sort of kinase independent manner (such as through protein complex formation when overexpressed) to inhibit Wnt signaling.

For Fig.2C the slight increase in beta-catenin induced TOPFLASH upon co-expression of Scr is not significant - the error bars actually overlap.

For Fig.3D, although Y1480 and Y1520 single point mutants partially reduce the ability of Src to inhibit signalling, only the Y4F mutant (combination of all 4 Tyrosine to phenylalanine point mutants) is statistically different from the wt.
-Src appears to increase the total protein level of LRP6 (see for example Fig. 2A, but also other figures). The significance of this finding for the proposed mechanism of LRP6 regulation should be discussed.

We indeed observe a dose-dependent upregulation of the total levels of LRP6, as originally mentioned in the results section on the bottom of page 5: “A dose-dependent inhibition of Wnt signaling coincides with increased LRP6 Tyrosine phosphorylation for both Src and Fer and this occurs despite increases in total LRP6 levels (Fig. 2A and Supp. Fig. 1B).” As referee 3 also raised this point, we decided to look in more detail at the half-life of LRP6 to see if reduced LRP6 turnover was responsible. We used enough Src to induce an increase in LRP6 levels (Supp. Fig. 1C, compare lanes 1 and 4) and the results demonstrate that Src increases the turnover of LRP6. This is actually in line with the findings of Liu et al., who showed reduced turnover rates of an LRP6 Tyrosine mutant construct. This finding encouraged us to also perform cell surface LRP6 biotinylation experiments similar to those performed by Liu et al. Our results (Fig. 5A) clearly demonstrate that Src leads to reduced cell surface localization of LRP6, which is in agreement with their model and therefore also suggests there is a regulatory interplay between LRP6 endocytosis and Src mediated LRP6 Tyrosine phosphorylation. We have now added the data and discussed this more in the manuscript.

-Why is the wild type LRP6 band stained with panYp in Fig. 3A of a lower size than the LRP6 band stained with the LRP6 antibody?

We thank the referee for pointing out this discrepancy, which is an error in figure preparation. These are actually two different SDS-PAGE gels and obviously the running times were slightly different, resulting in an apparent difference in size of the full-length LRP6, when in reality they are of the same size. We have now entered the molecular weight size markers for both gels in order to avoid confusion.

-The difference between Src and Fer in their effect on signalosome formation/maintenance should be discussed, especially in light of the mechanism related to the regulation of Fer by CK1gamma.

We have tried to discuss more why only Src and not Fer mediated LRP6 Tyrosine phosphorylation results in disruption of LRP6 aggregation. It is perhaps worth noting that Src is membrane tethered due to myristoylation and has been reported to be localized to lipid raft regions of the membrane (detergent soluble fractions), thus it is possible that Src mediates its effect on LRP6 due to this physical localization.

-To be complete, the authors are advised to include the FOPLASH negative control results in the panels showing TCF reporter data.

We apologize for not including this control, which we will introduce in future experiments.

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Thank you, this has been corrected.

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Altogether experiments are well performed and controlled and results are clear cut and do not require additional detailed experimental analysis.
Thank you for the positive comments

However, certain aspects should be clarified, as listed below:

1 The authors should clarify how in their screening protocol the phosphorylated LRP6 band was distinguished form all the cellular proteins that become phosphorylated after kinase transfection, in particular as the Western blot in Fig. 1B has no background in the lanes with the other kinases. Probably, this is due the high overexpression of LRP6 but needs to explained.

As the referee correctly points out, the use of the Pan-phospho-Tyrosine Antibody (panYp) on cells overexpressing LRP6 specifically allows LRP6 phosphorylation to be detected at a sensitivity significantly above that required to detect any Tyrosine phosphorylation of endogenous cellular proteins. This explanation has been added to the materials and methods section.

2 A comment should be made on the apparent stabilisation of LRP6 by src seen in several Western blots (e.g. Fig. 1D, Fig. 2A).

We indeed observe a dose-dependent upregulation of the total levels of LRP6, as originally mentioned in the results section on the bottom of page 5: “A dose-dependent inhibition of Wnt signaling coincides with increased LRP6 Tyrosine phosphorylation for both Src and Fer and this occurs despite increases in total LRP6 levels (Fig.2A and Supp. Fig. 1B).” As referee 2 also raised this point, we decided to look in more detail at the half-life of LRP6 to see if reduced LRP6 turnover was responsible. We used enough Src to induce an increase in LRP6 levels (Supp. Fig. 1C, compare lanes 1 and 4) and the results demonstrate that Src increases the turnover of LRP6. This is actually in line with the findings of Liu et al., who showed reduced turnover rates of an LRP6 Tyrosine mutant construct. This finding encouraged us to also perform cell surface LRP6 biotinylation experiments similar to those performed by Liu et al. Our results (Fig. 5A) clearly demonstrate that Src leads to reduced cell surface localization of LRP6, which is in agreement with their model and therefore also suggests there is a regulatory interplay between LRP6 endocytosis and Src mediated LRP6 Tyrosine phosphorylation. We have now added the data and discussed this more in the manuscript.

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It is difficult to give a definitive answer to this question. Although LRP6 aggregation has been suggested to be required for the phosphorylation of LRP6 by CK1g (Bilic et al. 2007), it is not clear whether aggregation is a prerequisite for PPSP site phosphorylation of LRP6. Our data appear to suggest that this is not the case, because 1) disruption of LRP6 aggregation by Src clearly takes place and 2) Src-induced LRP6 Tyrosine phosphorylation does not significantly affect PPSP (Sp1490) phosphorylation. We would therefore propose that PPSP site phosphorylation (at least S1490) and LRP6 clustering are relatively independent events.

4 The authors state that they could not confirm LRP6 tyrosine phosphorylation to play a role in LRP6 endocytosis which would contradict a previous study (Liu et al., 2914). However as they show no data on endocytosis the basis of their statement is not clear and they should delete this comment or show the data.

We have added one Figure (Supp. Fig. 5B) demonstrating that siRNA mediated silencing of either Clathrin or Caveolin does not affect the ability of Src to inhibit LRP6-induced Wnt/β-catenin signalling.

5 It is stated that the mutant delta87 lacks four of the five PPPSP motifs but in the scheme in Fig. 3A it appears that only three motifs are missing.

Thank you for pointing out this mistake, we have now corrected the figure to more clearly indicate
that four (4) of the five (5) PPSP motifs are missing in the delta-87-LRP6 mutant.

6 Several references lack pages, vol etc.
This has now been corrected.

2nd Editorial Decision 17 October 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.