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NgBR is esential for endothelial cell glycosylation and vascular development

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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.)

1st Editorial Decision 22 June 2015

Thank you for the submission of your research manuscript to our editorial office. I would first like to apologize for the delay in getting back to you with a decision on your manuscript, which was due to the high number of submissions we currently receive. I have now had the opportunity to carefully read your study and to discuss its suitability for publication here with my colleagues. I regret to say that the outcome of this process is not a positive one as we all agreed that the manuscript does not seem to be a good fit for EMBO reports.

We acknowledge that you have, for the first time, presented evidence that NgBR is involved in the glycosylation of VEGFR2 and through this regulates vascular development in the early mouse embryo and we agree that these findings will be of interest to researchers in the field. However, if one looks at the study from a broader perspective, and when taking into account that NgBR was known to mediate glycosylation of other proteins and that it regulates vascular development and angiogenesis and that it was also known that VEGFR2 is glycosylated and that this modification is important for its function, we do not think that the manuscript offers the kind of conceptual advance that we or our wide readership of non-specialists are looking for in papers suitable for publication in a general-interest journal such as EMBO reports.

I am providing you with an editorial decision so that you can submit your manuscript elsewhere without delay. Please note that we publish only a small percentage of the many manuscripts submitted to us, and we can therefore only subject to external review those that have a good chance

of faring well with our reviewers and readers. I am sorry to disappoint you on this occasion and hope that this will not prevent you from considering EMBO reports for publication of your work in the future.

Appeal 22 June 2015

I appreciate your insights but when you take the broader perspective argument, you can virtually eliminate any paper from review. For example in the most read paper in EMBO reports online , the authors report that USP30 opposes Parkin dependent ubiquitylation of TOM20 and its depletion enhances depolarization induced cell death. Prior to this paper, it was well described in the published literature since 2008 that USP30 is a ubiquitinase and Parkin dependent ubiquitylation of TOM20 occurs. However, you have published this since the authors have shown the competition between these two pathway and now it is the most widely read paper on your website. I could easily use the "broad perspective argument" here and not reviewed the paper. This is just one example of how the broad perspective argument can eliminate any paper. However, I am a firm believer that the details matter beyond the initial concept. The NgBR pathway is very new and there are a few papers in the field (especially compared to the above pathway used as an example). Yes you are correct in your assertions regarding the published literature, however, this the first description of a conditional KO of NgBR, we have the first data showing that blockade of dolichol synthesis is lethal early (this is not predictable since it was possible that maternal dolichol would rescue), also we directly show that the glycosylation of VEGFR2 is essential for the response in vivo (yes this is known, but we show the enzymatic pathway responsible for this) and show for the first time that dolichol supplementation can rescue these defects. Anyway, I wish you would reconsider sending our paper for full review. Thanks.

2nd Editorial Decision 14 July 2015

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have just now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I would prefer not to repeat the details of them here. What becomes clear is that while all referees in principle agree on the interest of the study, they also point out instances in which they feel the data needs to be strengthened and they all provide feedback and suggestions on how to achieve this. For example, both referees 3 and 2 feel that stronger proof for a causal relationship between the defects in VEGFR glycosylation in the absence of NgBR and the observed (vascular) phenotypes is needed.

Given the potential interest of your findings, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees as listed above and in their reports should be addressed.

Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. Please also note that our scientific reports (as opposed to full articles) contain a combined results and discussion section and I would kindly ask you to modify the text accordingly before submitting the revised version.

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 REPORTS

Referee #1:

NgBR is a subunit of cis-prenyltransferase and acts as receptor of NOG-B, a protein inducing endothelial motility and morphogenesis.
In this paper the authors show that NgBR contributes to the glycosylation of VEGFR2 and this effect explains the in vivo phenotype of mice with endothelial specific NgBR deletion. Actually, cis-prenyltransferase is involved in the biosynthesis of dolichol acid. Dolichol acid is the substrate of a number of enzymes in the biosynthesis of N and O-glycans and the availability of this lipid is one of the rate-limiting factors in the synthesis of these etheropolysaccharides. Based on this information, the authors can rescue VEGFR2 glycosylation by adding dolichol phosphate in the medium of murine EC lacking NgBR.

CRITICISMS
The analysis of vascular embryonic and extra-embryonic defects is clear. I suggest to add a table in supplemental information summarising the number of embryos studied with the shown defects .

Fig 3. Does NgBR in vitro deletion increase apoptotic rate in ECs?

Fig 4 and EV3 show that: i) VEGFR2 is poorly glycosylated in embryo and in ECs lacking NgBr, ii) dolichol-phosphate treatment of NgBR null ECs rescues VEGFR2 glycosylation, iii) the absence of NgBR induces the unfolded protein response, as inferred by the expression the specific genes Cho and Chac..

In my opinion these results need more experiments to really support the authors' conclusions.
a) The impact of NgBR deletion on glycosylation pathway(s) has to be addressed by measuring the amount of dolichol acid in EC lacking NgBR and by the analysis of enzymatic activity of GlcNAc-1-phosphotransferase, which transfers GlcNAc-P from UDP -GlcNAc to dolichol phosphate. Alternatively the profile of N-glycans should be evaluated by liquid chromatography;
b) Does supplemented dolicholic acid rescue VEGFA/VEGFR2 mediated-activities (i.e proliferation, chemotaxis) in EC lacking NgBR?
c) Based on literature data, the authors discuss that the reduce amount of VEGFR2 detected on the cell membrane is caused by a receptor misfolding. Can they exclude any alteration of receptor trafficking?

The results of paper published on Cell last year (10.1016/j.cell.2014.01.043) has to be discussed

Referee #2:

The study from Bill Sessa's group confirms and extends their previous work on NgBR's crucial role in N-linked glycosylation. NgBR is a subunit in cis-prenyltransferase, which catalyzes the synthesis of dolichol, a membrane anchor for oligosaccharide synthesis. The current manuscript identifies the requirement for NgBR in endothelial cells during development. Mouse strains floxed, constitutively or conditionally, for NgBR show severe defects in vascular development due to decreased endothelial proliferation and increased apoptosis as a consequence of a near complete lack of N-linked glycosylation on essential endothelial proteins such as VEGFR2.

This is a generally interesting and novel work of high quality. My criticisms relate mostly to how the data is presented.

1. The deficiency in glycosylation would not be expected to be specific for VEGFR2 based on the authors earlier work and as confirmed in the blotting for VE-cadherin and CD31 (Fig. 4A and F). Of the three endothelial proteins examined, the VEGFR2 phenotype seems to be established at an earlier time point, perhaps due to a more rapid turnover. The general effect of NgBR deletion is mentioned in passing however, the authors stress that CD31 and VE-cadherin are not affected which I believe is not correct. Please adjust the text.
2. A major cause for the NgBR embryonic phenotype may be first and foremost a very severe

reduction in VEGFR2 cell surface expression (Fig. 4E). Thereby VEGFR2 and other endothelial surface proteins, which appear to be retained in ER due to increased ER stress as shown by the authors, obviously cannot fulfill their function. The main cause of the embryo phenotype is therefore probably not glycosylation per se, but the consequence of the glycosylation defect namely retention in the ER. In the interesting rescue experiments in Fig. 4G, was VEGFR2 cell surface expression restored? If so, the conclusion can be drawn that loss in N-linked glycosylation is inseparable from loss in cell surface expression.

Minor
3. "Glycosylation" should be "N-linked glycosylation".
4. In Fig. 3, please quantify the cleaved caspase 3-postive cells. Correct the part of the Figure 3 legend that reads "Reduced proliferation..." also for panels F and G, which show apoptosis.

Referee #3:

In their MS Park et al describe the effect of endothelial-selective deletion of Nogo-B receptor. Both constitutive and inducible deletions produce embryonically lethal phenotypes characterized by vascular defects that in the case of constitutive deletion are restricted to yolk sac and placenta. The authors also report that these effects are independent of the NgBR ligand Nogo-B, while they depend on impaired glycolsylation of proteins ensuing reduced biosynthesis of dolichol, a glycosyl carrier lipid, which is controlled by NgBR.
The authors identify glycosylation of VEGFR2 as a target of NgBR deletion and conclude that reduced glycosylation of VEGFR2 is responsible for the phenotype observed.

General comment
In general the data reported are interesting and novel. However, the interpretation proposed is not sufficiently supported by conclusive experimental evidence.

The conclusion that inhibition of VEGFR2 glycosylation is central to the phenotype produced by NgBR endothelial-selective deletion is not adequately proved. While the effects of NgBR deletion on VEGFR2 glycosylation are appropriately supported, the proof that such defect is responsible of the phenotype observed is totally lacking.

NgBR deletion likely affects glycosylation of several different targets, therefore the authors should specifically rescue VEGFR2 glycosylation and observe normalization of the phenotype to conclusively support their interpretation of the results.

The observation that constitutive KO embryo develops normally and VEGFR2 is normally glycosylated, while vascularization of the yolk sac is defective together with impaired VEGFR2 glycosylation, is only correlative evidence.

As show in several figures of this MS other endothelial proteins, namely PECAM and VE-cadherin, are affected by endothelial-selective deletion of NgBR, as their level is strongly reduced in parallel with inhibition of VEGFR2 glycosylation. In particular, reduction of VE-cadherin could contribute to the impaired vascular organization observed.

In addition, the lack of specificity for the effect of NgBR deletion suggests that other critical regulators of endothelial behavior could be targeted and contribute to the phenotype, cooperating or not with VEGFR2.

Specific points

1) The level of dolichol/P-dolichol is never shown, although this mediator would play a central role in deregulating glycosylation. Rescuing of VEGFR2 glycosylation by treating NgBR-KO cultured endothelial cells with Dol-P is suggestive, but not conclusive, evidence that depletion of dolichol is actually taking place.

2) Linked to the previous point, the effect of Dol-P treatment should be shown also on Pecam and VE-cadherin, as these two molecules are strongly reduced in NgBR KO both in vivo and in cultured

endothelial cells. In addition, some functional assay/s should be reported showing that rescuing glycosylation also rescue the functional phenotype.

3) It should be clarified whether NgB KO also affects glycosylation of PECAM and VE-cadherin. There are contradictory statements in the text, stating either no-effect or effect (see p.7 and p.8). Results shown for cell in culture, Fig 4F, in particular for VE-cadherin, could be compatible with altered glycosylation after NgBR KO.

Minor
p.6 line 5: ...by oral gavage of the dam, is lacking

2nd Revision - authors' response 09 October 2015

Response to Referee's Comments:

Referee #1:
We thank the reviewer for the comments and suggestions for improvement of the manuscript. We have incorporated experiments and quantifications to address the reviewer's concern and added text in discussion. We have addressed each point cited by the reviewer as found below.

The analysis of vascular embryonic and extra-embryonic defects is clear. I suggest to add a table in supplemental information summarising the number of embryos studied with the shown defects.

Both the embryonic and extra embryonic phenotypes in $NgBR^{i\Delta EC}$ and $NgBR^{ECKO}$ show almost 100% penetrance. We have added tables for summarizing the number of embryos examined in EV Table 1 and 2.

Fig 3. Does NgBR in vitro deletion increase apoptotic rate in ECs?

NgBR depletion in EC in vivo clearly increases apoptosis as shown in Figure 3. To address if NgBR deletion increase apoptosis in ECs in vitro, we have performed an apoptosis assay by measurement of Caspase 3 activity. We added the data in Figure 3J. In consistent with in vivo data, we detected increased caspase3 activity in NgBR deleted MLECs.

a) The impact of NgBR deletion on glycosylation pathway(s) has to be addressed by measuring the amount of dolichol acid in EC lacking NgBR and by the analysis of enzymatic activity of GlcNAc-1-phosphotransferase, which transfers GlcNAc-P from UDP -GlcNAc to dolichol phosphate. Alternatively the profile of N-glycans should be evaluated by liquid chromatography;

To access reviewer's question about the impact of NgBR deletion on glycosylation pathway, we directly have measured amount of dolichol in EC lacking NgBR instead of accessing GNPAB activity or N-glycan profiling**.** Previous work has shown a reduction in Dol-linked sugars after the knockdown of NgBR (Harrison et al EMBO Journal, May 13;30(12):2490-500, 2011). **The rationale is that NgBR is a subunit required for cisPTase activity and the product of this reaction relevant to glycosylation is dolichol.** We have added the data in Figure EV 3A and B. After deletion of NgBR in MLEC, total amount of dolichol was significantly reduced by >70%. We confirmed the result with HeLa cells in Fig EV 3C and D. HeLa cells were stably infected with lentiviruses expressing a control shRNA (NgBR NS) or a shNgBR (NgBR KD). In addition, NgBR KD cells were transiently transfected with the cDNA for NgBR as a rescue construct. As seen in EC cells, silencing NgBR in HeLa cells reduces dolichol levels and the effects rescued by expression of NgBR. These results clearly show NgBR deletion affects biosynthesis of dolichol which is required for protein glycosylation. Also data in Figure 4 showing Dol-P rescues defects in glycosylation seen in NgBR deleted cells strongly supports the idea that NgBR is essential for glycosylation.

b) Does supplemented dolicholic acid rescue VEGFA/VEGFR2 mediated-activities (i.e proliferation, chemotaxis) in EC lacking NgBR?

We have examined cell proliferation and apoptosis with dolichol-P (Dol-P) supplement to MLEC after Ad-Cre or Ad-GFP infection. We have included this result in Figure 4 H and I. Proliferation was increased and apoptosis was decreased in NgBR depleted cells with Dol-P supplement compare to without dolichol-P.

c) Based on literature data, the authors discuss that the reduce amount of VEGFR2 detected on the cell membrane is caused by a receptor misfolding. Can they exclude any alteration of receptor trafficking?

We think the reduced amount of the receptor on the cell surface is because VEGFR2 is not glycosylated. The lack of glycosylation may cause receptor to not traffic to cell surface due misfolding in the ER and possibly degradation. As shown in the cell surface biotinylation experiments (Figure 4G), NgBR deletion reduced cell surface VEGFR2 levels, an effect rescued by Dol-P suggesting that glycosylation of VEGFR2 is required for localization on the plasma membrane. This result is consistent with a previous study (PMID: 9160888) showing that fully glycosylated form of VEGFR 2 is expressed on the cell surface. Protein N-glycosylation is an important post-translational modification and involved in many cellular processes, including protein folding, intracellular trafficking, secretion and enzyme activity. Our results support previous studies showing N-glycosylation is required for cell surface expression of VEGFR2. However, further investigation is required to understand how N-glycosylation regulates VEGFR2 transit from the ER and localization to the cell surface.

The results of paper published on Cell last year (10.1016/j.cell.2014.01.043) has to be discussed
This is now included in our revised discussion.

Referee #2:
We thank the reviewer for noting that our study is interesting and novel work and high quality and for the comments and insights regarding improvement of the manuscript. We agree to reviewer's criticisms relate to how the data is presented. We agree the phenotype in EC in vivo and in vitro are not likely an exclusive VEGFR2 specific defect. However, the timing and extent of the defects observed in EC NgBR deleted embryos compelled us to look at VEGFR2 considering that it is glycosylated and very important for vascular development. Indeed, in the revised MS, we clearly see defects in protein glycosylation of other important endothelial proteins including CD31 and VE-cad. We have modified the text in an attempt to make the logic of the paper more easily accessible and performed the suggested experiments to support the conclusion. We have addressed each point cited by the reviewer as found below.

1. The deficiency in glycosylation would not be expected to be specific for VEGFR2 based on the authors earlier work and as confirmed in the blotting for VE-cadherin and CD31 (Fig. 4A and F). Of the three endothelial proteins examined, the VEGFR2 phenotype seems to be established at an earlier time point, perhaps due to a more rapid turnover. The general effect of NgBR deletion is mentioned in passing however, the authors stress that CD31 and VE-cadherin are not affected which I believe is not correct. Please adjust the text.

We agree that other endothelial proteins including CD31 and VE-cadherin are affected in NgBR deleted EC. We have adjusted the text to reflect the reviewer's concern.

2. A major cause for the NgBR embryonic phenotype may be first and foremost a very severe reduction in VEGFR2 cell surface expression (Fig. 4E). Thereby VEGFR2 and other endothelial surface proteins, which appear to be retained in ER due to increased ER stress as shown by the authors, obviously cannot fulfill their function. The main cause of the embryo phenotype is therefore probably not glycosylation per se, but the consequence of the glycosylation defect namely retention in the ER. In the interesting rescue experiments in Fig. 4G, was VEGFR2 cell surface expression restored? If so, the conclusion can be drawn that loss in N-linked glycosylation is inseparable from loss in cell surface expression.

We now have included the data for VEGFR2 surface expression after dolichol-P supplement in revised Fig 4G. .We clearly show that dolichol-P supplement to NgBR deleted cells rescued both

glycosylation defects and cell surface expression of VEGFR2 suggesting VEGFR2 glycosylation affects its ultimate trafficking to the cell surface.

Minor

3. "Glycosylation" should be "N-linked glycosylation".
We prefer to be consistent throughout the manuscript with use of the term “glycosylation” since dolichol has roles for several different types of glycosylation reactions including N-linked glycosylation, C-mannosylation and GPI anchor formation.

4. In Fig. 3, please quantify the cleaved caspase 3-postive cells. Correct the part of the Figure 3 legend that reads "Reduced proliferation..." also for panels F and G, which show apoptosis.

We have included the quantification of the cleaved caspase-3 positive cells in Figure 3 G and I. We have changed the figure legend for panels F and G (in original MS, Figure F-I in revised MS) in accord with the reviewer’s comment.

Referee #3:
General comments by the reviewer:
In general the data reported are interesting and novel. However, the interpretation proposed is not sufficiently supported by conclusive experimental evidence.

The conclusion that inhibition of VEGFR2 glycosylation is central to the phenotype produced by NgBR endothelial-selective deletion is not adequately proved. While the effects of NgBR deletion on VEGFR2 glycosylation are appropriately supported, the proof that such defect is responsible of the phenotype observed is totally lacking.

NgBR deletion likely affects glycosylation of several different targets, therefore the authors should specifically rescue VEGFR2 glycosylation and observe normalization of the phenotype to conclusively support their interpretation of the results. The observation that constitutive KO embryo develops normally and VEGFR2 is normally glycosylated, while vascularization of the yolk sac is defective together with impaired VEGFR2 glycosylation, is only correlative evidence.

As show in several figures of this MS other endothelial proteins, namely PECAM and VE-cadherin, are affected by endothelial-selective deletion of NgBR, as their level is strongly reduced in parallel with inhibition of VEGFR2 glycosylation. In particular, reduction of VE-cadherin could contribute to the impaired vascular organization observed. In addition, the lack of specificity for the effect of NgBR deletion suggests that other critical regulators of endothelial behavior could be targeted and contribute to the phenotype, cooperating or not with VEGFR2.

We appreciate the reviewer’s insightful comments and suggestions and have changed the title not to highlight VEGFR2 exclusively. As pointed out, we detected glycosylation defects on critical endothelial proteins including CD31, VE-cad and VEGFR2 in NgBR KO EC and glycosylation defects in those proteins can be rescued by dolichol-P supplementation (new Fig 4F). Our study is the first report clearly demonstration the essential role of glycosylation in endothelium in vivo and in vitro. In addition, we provide the first evidence that dolichol-p can be internalized by EC and utilized as a glycan carrier for protein N-glycosylation.

Specific points
1) The level of dolichol/P-dolichol is never shown, although this mediator would play a central role in deregulating glycosylation. Rescuing of VEGFR2 glycosylation by treating NgBR-KO cultured endothelial cells with Dol-P is suggestive, but not conclusive, evidence that depletion of dolichol is actually taking place.

We have measured amount of dolichol in EC lacking NgBR using mass spectrometry and these results are in EV Figures 3A and B. After deletion of NgBR in MLEC, total amount of dolichol was significantly reduced by >70%. Dolichol-P was not detectable in both cells even with MS since in

our experience dolichol-P is difficult to detect in mammalian cultured cells because it is an intermediate used for the synthesis dol-linked sugars and in a previous paper we have shown that the loss of NgBR reduces the abundance of dol-linked sugars (Harrison et al EMBO Journal, May 13;30(12):2490-500, 2011). In addition the reduction in dolichol in EC, we confirmed these results in HeLa cells using lentiviruses expressing a control shRNA (NgBR NS) or a shNgBR (NgBR KD). In addition in this cell line, NgBR KD cells were transiently transfected with the cDNA for NgBR as a rescue construct. As seen in EC cells, silencing NgBR in HeLa cells reduces dolichol levels and these effects rescued by expression of NgBR. These results clearly show NgBR deletion affect biosynthesis of dolichol which are required for protein glycosylation in two distinct cell types.

2) Linked to the previous point, the effect of Dol-P treatment should be shown also on Pecam and VE-cadherin, as these two molecules are strongly reduced in NgBR KO both in vivo and in cultured endothelial cells. In addition, some functional assay/s should be reported showing that rescuing glycosylation also rescue the functional phenotype.

We have repeated the experiments to examine rescue of protein glycosylation defects on CD31 and VE-cadherin both in vivo and in vitro. As seen in Fig 4F, NgBR depleted MLEC and control cells were incubated with dolichol-P or DMSO for 72 hrs and glycosylation of key proteins were examined by western blotting. As with VEGFR2, we detected rescue of glycosylation on CD31 and VE-cadherin.. We also examined proliferation and apoptosis rate of NgBR KO EC with dol-P or DMSO supplement. Both proliferation and apoptosis were rescued by dol-P treatment. This result presented in Figure 4H and I.

3) It should be clarified whether NgB KO also affects glycosylation of PECAM and VE-cadherin. There are contradictory statements in the text, stating either no-effect or effect (see p.7 and p.8). Results shown for cell in culture, Fig 4F, in particular for VE-cadherin, could be compatible with altered glycosylation after NgBR KO.

I apologize for the confusion. We have changed the text in p.7. As shown in Figure 4A and 4B, we detected reduced N-linked glycosylation defect on CD31, VE-cadherin as well as VEGFR2.

Minor
p.6 line 5: ...by oral gavage of the dam, is lacking

We have added words to clearly explain that tamoxifen was given to pregnant females.

3rd Editorial Decision 10 November 2015

Many thanks for the submission of your revised study to EMBO reports. The manuscript was sent back to the original referees and while reviewers 1 and 2 now support publication of the study more or less in its current form, referee 3 still raises some concerns about the completeness and conclusiveness of some parts of the data set. Upon further discussion of these issues with the other two referees we came to the conclusion that these concerns should be addressed before acceptance.

I would thus like to give you the opportunity to revise the study a second time along the lines indicated in the new set of referee reports. Formally, papers in EMBO reports have to be accepted within 6 months of the initial decision, which in your case would be January, 14, 2016. We are, of course, still interested in publishing your study after this time-point, but we would need to take the novelty into account if your study can only be accepted after this date. Given that the final version of the manuscript might still need to be evaluated by one of the reviewers, please also outline briefly in a point-by-point manner how you have addressed the remaining referee concerns.

I look forward to seeing a revised form of your manuscript when it is ready.

REFEREE REPORTS

Referee #1:

The authors properly addressed my concerns

Referee #2:

The authors have performed an ambitious revision which has further improved the quality of their very impressive study. I spotted a minor issue which should be corrected before acceptance namely the missing description of the ER stress data and reference to Fig. 4D in the Results section.

Referee #3:

The authors have moderated the emphasis given in their previous version of the manuscript to VEGFR2 glycosylation as a crucial effector of NgBR depletion on vascular development. Experiments have also been performed to answer the referees concerns.

Nevertheless, the manuscript still contains inconsistencies, over-interpretations and conceptual weaknesses. These relate both to the novelty of the observations reported and to the mechanistic interpretation of the results obtained.

The novel aspect of the manuscript is the requirement of NgBR for protein glycosylation in mammalian endothelial cells and for organization of the vascular network in vivo. However, the effect of NgBR on angiogenesis has already been reported in Z.fish (see ref 21). In addition, the same authors have already described that NgBR controls glycosylation in various models. While it is not surprising that protein glycosylation plays a critical role in vascular development, the mechanism of such effect is not unambiguously defined.

For example, the potential involvement of cholesterol depletion, which is also caused by NgBR abrogation (ref2, by the same authors), is not tested.

Specific issues
1) Discussion, p.12, states partial rescue of hypo-glycosylation by Dol-P. However, the blots in figure 4F show complete rescue of glycosylation, while the functional rescuing is not adequately documented. See following point 2.

2) Fig.4H- The difference in proliferation and apoptosis between DMSO and Dol-P treatments is small (although significant? the asterisk is not defined in the legend) in comparison to the strong rescuing effect of Dol-P on glycosylation of VEGFR2, VE-cadherin and CD31 shown in Fig.4F. Therefore, figure 4H should report also the proliferation and apoptosis of wild type control cells as in Figure 4F. This would allow proper evaluation of the Dol-P effect on the functions of NgBRdeltaEC MLEC.

3) Introduction p.3 3rd paragraph. The statement "The in vivo function of NgBR has not been demonstrated" is not correct considering ref 21. It also contradicts the following sentence in the manuscript "Several studies..." and needs to be cancelled.

4) Fig. 4A and B- In yolk sac and embryo extracts NgBR knockout appears to affect the total amount of VE-cadherin and CD31 more than the motility of respective bands. Could the authors comment on this? This also, in the light of the clear effect of NgBR depletion on the migration pattern of both VE-cadherin and CD31 in cultured endothelial cells (see Fig. 4E and F).

5) The role of cholesterol depletion in the phenotype induced by NgBR KO is not tested. Could cholesterol supply rescue the NgBR KO phenotype? If not, this would reinforce the specific role of glycosylation in the phenotype described.

Typos

p.2 7th line, glycisyaltion is glycosylation
p.9 3th line, 2nd paragraph intersomatic is intersomitic
p.19 bottom line, J is (J)

4th Revision - authors' response 24 November 2015

Response to Referee's Comments:

Referee #1:

The authors properly addressed my concnerns
We are pleased that the reviewer found our revision satisfactory. We thank the reviewer for his/her support.

Referee #2:

The authors have performed an ambitious revision which has further improved the quality of their very impressive study. I spotted a minor issue which should be corrected before acceptance namely the missing description of the ER stress data and reference to Fig. 4D in the Results section.

We thank the reviewer for the helpful suggestions for improvement of the manuscript.
We have added description of ER stress data in the result section (page 8).

Referee #3:

The authors have moderated the emphasis given in their previous version of the manuscript to VEGFR2 glycosylation as a crucial effector of NgBR depletion on vascular development. Experiments have also been performed to answer the referees concerns.

Nevertheless, the manuscript still contains inconsistencies, over-interpretations and conceptual weaknesses. These relate both to the novelty of the observations reported and to the mechanistic interpretation of the results obtained.

The novel aspect of the manuscript is the requirement of NgBR for protein glycosylation in mammalian endothelial cells and for organization of the vascular network in vivo. However, the effect of NgBR on angiogenesis has already been reported in Z.fish (see ref 21). In addition, the same authors have already described that NgBR controls glycosylation in various models. While it is not surprising that protein glycosylation plays a critical role in vascular development, the mechanism of such effect is not unambiguously defined. For example, the potential involvement of cholesterol depletion, which is also caused by NgBR abrogation (ref2, by the same authors), is not tested.

We respectfully disagree with the reviewer's point about novelty of our study. In this study, we report the first characterization of endothelial cell specific deletion of NgBR in mice. We clearly show that defective glycosylation of endothelial proteins due to the loss of NgBR in vivo and in vitro. This is first in vivo model that shows clear protein glycosylation defects due to reduced dolichol. Moreover, to date, no studies shown Dolichol-P rescues protein glycosylation defects in cells. The published data in zebrafish was performed only with morpholinos which target NgBR in the entire fish and are notorious for off target effects and can be phenotypically different from knockout approaches in fish or mice (Kok et al., Dev Cell. 2015, 12;32(1):97-108; Didier et al., 2015, Dev Cell. 12;32(1):7-8). Moreover, in that paper, the authors suggested that NgBR function in endothelial cells during vascular development is Nogo-B dependent, whereas our data indicate NgBR regulates dolichol synthesis and protein glycosylation in endothelial cells, independent of Nogo-B. We would like to respectfully point out that the mechanisms and phenotypes due to NgBR ECKO that we describe (glycosylation defects) are very different than the published work.

Finally, the loss of NgBR increases cellular free cholesterol, not cholesterol depletion. This is explained below and cannot account for the main phenotypes reported in this paper (see point 5).

Specific issues

1) Discussion, p.12, states partial rescue of hypo-glycosylation by Dol-P. However, the blots in figure 4F show complete rescue of glycosylation, while the functional rescuing is not adequately documented. See following point 2.

Even though Dol-P treatment of NgBR KO endothelial cells improved VEGFR2, VE-cad and CD31 glycosylation it is not fully rescued since we can still detect hyoglycosylated VEGFR2 and CD31. Thus, we have tempered our interpretation of the data in Fig 4F and restructured the statements to avoid the confusion.

2) Fig.4H- The difference in proliferation and apoptosis between DMSO and Dol-P treatments is small (although significant? the asterisk is not defined in the legend) in comparison to the strong rescuing effect of Dol-P on glycosylation of VEGFR2, VE-cadherin and CD31 shown in Fig.4F. Therefore, figure 4H should report also the proliferation and apoptosis of wild type control cells as in Figure 4F. This would allow proper evaluation of the Dol-P effect on the functions of NgBRdeltaEC MLEC.

Even though the ability of Dol-P to rescue growth and apoptosis is small, the experiments show a clear statistical difference that was reproducible (asterisk defines P value =0.005). We have reported proliferation and apoptosis of WT and NgBR KO EC in Fig 3E and J and no effect of Dol-P supplement on glycosylation in WT cells (Fig.4F). Thus, present Fig4 H and I show better representation of our point than comparison with WT data.

More importantly, we did not expect to see a strong rescue effect of Dol-P on apoptosis and proliferation relative to the glycosylation status of a few EC proteins due to technical challenges of this experiment. To determine the ability of Dol-P to rescue, primary $NgBR^{f/f}$ mouse lung endothelial cells were first infected with Ad-GFP or Ad-CRE to induce NgBR gene deletion. After 48hrs, we then supplied Dol-P to the infected cells for additional 72hrs. As we mentioned in the discussion section, of the paper, little is known about the uptake and utilization of exogenous Dol-P and additional experiments are clearly warranted to elucidate the mechanisms involved. Based on existing knowledge, exogenous Dol-P (Dolichyl monophosphate) needs to be transferred to the ER, converted to GlcNAc-P-P-dol by dolichyl-phosphate (UDP-N-acetylglucosamine) N-acetylglucosamine phosphotransferase 1 and processed to form lipid-linked oligosaccharide (LLO) for protein glycosylation to occur. Clearly there must be a kinetic lag between correction of protein glycosylation and complete rescue of complex integrated cellular functions such as growth and apoptosis. This has now been added to the discussion.

3) Introduction p.3 3rd paragraph. The statement "The in vivo function of NgBR has not been demonstrated" is not correct considering ref 21. It also contradicts the following sentence in the manuscript "Several studies..." and needs to be cancelled.

We have modified this paragraph.

4) Fig. 4A and B- In yolk sac and embryo extracts NgBR knockout appears to affect the total amount of VE-cadherin and CD31 more than the motility of respective bands. Could the authors comment on this? This also, in the light of the clear effect of NgBR depletion on the migration pattern of both VE-cadherin and CD31 in cultured endothelial cells (see Fig. 4E and F).

It is well known that protein glycosylation plays a pivotal role in protein folding and quality control, and misfolded proteins undergo ubiquitin- and proteasome-dependent degradation. Thus, it is not surprising to see reduced amount of protein with the protein glycosylation defects and the reduced expression of a glycoprotein is used as an indicator for glycosylation defects in many studies. For example, reduced protein expression of ICAM1 was detected in Congenital Disorder of Disorder (CDG)-Ia, CDG-Ib, and CDG-Ik fibroblasts from patients and used as a marker for glycosylation defect (HE et al, J Bio Chem. 2012 25; 287(22): 18210–18217). Detection of non- or hypo-glycoform of a protein is affected by many factors including protein half-life and its clearance via

degradation pathways. Thus, different patterns of VE-cad and CD31 between in vivo and in vitro study can be explained by these factors. These fundamental concepts are well accepted in the literature.

5) The role of cholesterol depletion in the phenotype induced by NgBR KO is not tested. Could cholesterol supply rescue the NgBR KO phenotype? If not, this would reinforce the specific role of glycosylation in the phenotype described.

NgBR is critical for regulating intracellular cholesterol trafficking by stabilizing NPC2 protein and loss of NgBR induces free cholesterol accumulation in cells (Harrison et al Cell Metabolism, 2009). Mutations in NPC2 cause abnormal accumulation of unesterified cholesterol in lysosomes and defective sterol sensing resulting in the elevated uptake and accumulation of LDL derived cholesterol. **We want to respectively point out that the loss of NgBR does not result in cholesterol depletion, but free cholesterol enrichment.**

More importantly, an increase in free cholesterol in NgBR KO EC cannot explain the in vivo or in vitro phenotypes in the present paper. NPC2 deficient mice shows marked accumulation of free cholesterol in cells but no embryonic lethality and mice grow with normal body weight until ~55 days (Sleat et al, PNAS, 2004 20;101(16):5886-91) and NPC1 knockout also increases cellular free cholesterol and does not result in embryonic lethality or abnormal embryogenesis. The global loss of NgBR in early embryonic lethality before E6.5 (Park et al Cell Metabolism, 2014). As shown in this study, endothelial specific NgBR knockout also show early embryonic vascular ethality with severe phenotypes. Thus, developmental phenotypes shown in NgBR ECKO embryos are unlikely related to NPC2 functions.

Typos
p.2 7th line, glycisyaltion is glycosylation

p.9 3th line, 2nd paragraph intersomatic is intersomitic

We apologize for the misspelling and we have now corrected these typos.

p.19 bottom line, J is (J)

To be consistent with other figure legends, this should be J, not (J)

4th Editorial Decision 27 November 2015

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.