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A genome-wide RNA interference screen uncovers two p24 proteins as regulators of Wingless secretion

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(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 18 April 2011

Thank you for the submission of your manuscript to EMBO reports. Please accept my apologies for the delay in getting back to you, which is due to the fact that we have not yet received the complete set of referee reports. So far we have received reports from two referees, which are copied below, and since both referees are in fair agreement I am making a decision on your manuscript now in order to safe you from any further loss of time. Please note that this is a preliminary decision, and that it is subject to change should the third referee offer very strong and convincing reasons for this. As soon as we receive the third referee report it will be forwarded to you as well.

As you will see, while both referees acknowledge the technical quality of the study, they also both point out that more insight into how the two p24 proteins regulate Wg secretion would need to be provided and that indirect effects of p24 mutants on Wg secretion need to be excluded. Referee 1 indicates that it should be confirmed that Wg lipid modifications occur normally in the absence of p24, and that Wg binds to Eca or Emp24. Referee 2 mentions that it should be examined whether emp24 mutants affect dally secretion and thereby Wg secretion indirectly.

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

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

This manuscript reports a genome-wide RNAi screen for genes involved in the secretion of the Drosophila Wnt protein Wingless (Wg). Wnt proteins are secreted, lipid-modified glycoproteins that play a key role as signaling molecules in animal development and adult tissue homeostasis. Previous studies have revealed that Wg secretion is finely regulated by specific factors that act in endocytosis and late stages of exocytosis. In the RNAi screen, the authors identified, among other genes, Eclair (eca), a gene encoding a transmembrane protein belong to the conserved p24 family. A subsequent analysis of other p24 family members revealed that, in addition to eca, emp24 is also involved in the secretion of Wg. Interestingly, Wg seems to accumulate in the ER whereas other secretory proteins are normally secreted in the absence of Eca or Emp24, suggesting that these two p24 proteins are specifically involved in the ER export of Wnt proteins.

The paper is nicely written. The experiments are well performed and support the author's conclusions for a role of the p24 proteins in the ER exit of Wnt proteins. Understanding of how Wnt protein trafficking is regulated would be of general interest to the readership of the EMBO Reports. However, the study should provide more mechanistic insights regarding the p24 function. The points to be addressed by the authors are raised below:

The specificity of the p24 requirement for Wg secretion leads to the authors to speculate that the p24 proteins could play a direct role in the ER exit of Wnt proteins as cargo receptors. In line with this possibility, the p24 proteins are thought to function as cargo receptors for GPI-anchored proteins in yeast and mammalian cells. This analogy is interesting because GPI-anchored proteins and Wnt proteins are luminally exposed cargoes and they are attached to the membrane by a lipid modification. Thus, as proposed for GPI-anchored proteins, the p24 proteins could link Wg with the cytosolic COPII coat to efficiently incorporate Wg into ER-derived vesicles.

However, to raise the hypothesis that the p24 proteins behave as cargo receptors for Wg, it is necessary to exclude the possibility that the authors are observing an indirect effect in p24 mutants. For instance, lipid modification could be important for the ER exit of Wg. Therefore, the authors should check whether Wg is properly modified by lipids in p24 mutants. Moreover, the cargo receptor hypothesis necessarily implies a specific receptor-ligand interaction. Thus, the authors should address whether Wg specifically binds to Eca or Emp24.

Finally, the ER colocalization experiment shown in Fig. 4A should be quantified.

Referee #2:

In this paper, Port et al briefly describe a genome wide screen for components involved in Wingless secretion. As is usual with cell-based screens, a number of hits are identified. Using secondary assays, they narrow their list of hits to two proteins, snx3 (the subject of a separate paper) and emp24. The rest of the paper is devoted to confirming that emp24 is indeed required for Wingless secretion in vivo and that it does not seem to affect the secretion of two other morphogens, Hh and Dpp. The results are mostly clear-cut and well documented (although some experimental details are only scantily described). However, the material covered is relatively limited and little mechanistic insight is provided.

The main issue I have with this paper is the lack of mechanistic insight. The identification of emp24 is an important first step. As the authors admit themselves, it suggests a number of experiments. Is emp24 required for secretion of GPI-anchored proteins in Drosophila (e.g. dally or dally-like)? Could an effect on these glypicans explain some of the phenotypes seen. If the palmitate on Wg

mediates the interaction with emp4, why is Hh, which is also palmitoylated not affected by emp24? On the whole, a feel that more effort at understanding how emp24 contributes to Wg secretion is needed.

Additional comments.
-No information is given on the Luc-Wg fusion protein.
-As part of the justification for in vivo assay, the authors suggest (rightly) that it is important to rescreen gene function in the context of cells that naturally express Wg but then proceed to assay suppression of a misexpression phenotype in the eye. There is nothing wrong with this assay but the logic must be streamlined.
-The reference to (Bartoszewski 2004) on p 6 is misleading because it implies that this paper studied Wg secretion.
-As mentioned above, the evidence that emp24 is required for Wg secretion is convincing. It is also shown that Hh secretion does not seem to require emp24. The situation with Dpp is somewhat confusing in light of the earlier report that Dpp signalling is affected in emp4 mutant embryos. This should be addressed more thoroughly. In addition, secretion of Dpp could be addressed with the published dpp-gfp fusion constructs.
-'resistent' instead of resistant on p8.
-Colocalisation can not be objectively assessed at the magnification shown in figure 4A.

Correspondence 26 April 2011

As mentioned in the decision letter, we are forwarding the comments of our 3rd referee to you, which have just been received. Please see the comments below. Many thanks for your patience.

Referee #3:

Drosophila Wg acts as a morpohgen to pattern tissues during development. Wg secretion from its producing cells is critical for its gradient formation, but the mechanism(s) of Wg secretion is still not fully understood. Basler lab has made important contributions in the Wg secretion pathway by identifying Wls and retromer as key molecules involved in this pathway. In the current manuscript, Basler and his colleagues have performed a genome-wide RNAi screen aiming at identification of other molecules involved in the Wg secretion pathway. Three new genes including Snx3, Emp24 and Eca have been uncovered as essential molecules required for proper Wg secretion. The current manuscript has characterized the functions of Emp24 and Eca, which have been proposed to be involved in ER to Golgi trafficking. Based on their data, they propose that Emp24 and Eca act as specific cargo receptors for Wg to concentrate it in forming vesicles at sites of ER export. Overall, this is an important piece of work in the field. I have following specific comments:

1. The Wg secretion defects associated with loss-of-function of Emp24 and Eca (Fig. 3) are weak compared with those from Wls mutant cells. As shown in Fig3. (3B-B' and 3D-D'), reductions of extracellular Wg levels in P compartment are marginal. One possibility is that P24 family members are functionally redundant. However, based on their data, Eca are P24 are not functionally redundant as simultaneously knock-down of both of them does not generate any stronger Wg secretion defects. I would like to know authors' interpretations on these data.

2. It would be important to determine whether Emp24 and Eca are required for Wg secretion in other developmental contacts such as embryos or in leg discs.

3. Both Wls and Porc are essential for Wg secretion. Reduced Wg secretion in cells lacking Emp24 and Eca activity could be due to defects in Wls trafficking or Porc activities. This can be done by examining the levels and subcellular localization of Wls in clones expressing Emp24 and Eca. I would like to know whether Emp24 and Eca are required for Wls trafficking from ER to Golgi or there is other mechanism(s) controlling Wg exit from ER to Golgi. The authors should also discuss possibility of Emp24 and Eca in regulating Porc activity, which could subsequently affect Wg lipid

modifications.

1st Revision - authors' response 12 July 2011

Please find below a detailed point-by-point response to all of the reviewers comments.

Referee #1:

This manuscript reports a genome-wide RNAi screen for genes involved in the secretion of the Drosophila Wnt protein Wingless (Wg). Wnt proteins are secreted, lipid-modified glycoproteins that play a key role as signaling molecules in animal development and adult tissue homeostasis. Previous studies have revealed that Wg secretion is finely regulated by specific factors that act in endocytosis and late stages of exocytosis. In the RNAi screen, the authors identified, among other genes, Eclair (eca), a gene encoding a transmembrane protein belong to the conserved p24 family. A subsequent analysis of other p24 family members revealed that, in addition to eca, emp24 is also involved in the secretion of Wg. Interestingly, Wg seems to accumulate in the ER whereas other secretory proteins are normally secreted in the absence of Eca or Emp24, suggesting that these two p24 proteins are specifically involved in the ER export of Wnt proteins.

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However, to raise the hypothesis that the p24 proteins behave as cargo receptors for Wg, it is necessary to exclude the possibility that the authors are observing an indirect effect in p24 mutants.

For instance, lipid modification could be important for the ER exit of Wg. Therefore, the authors should check whether Wg is properly modified by lipids in p24 mutants.

Previous studies have suggested that the correct lipid modification of Wg is necessary for protein secretion. However, we believe it is very unlikely that the Wg secretion defects seen in p24 mutants are caused by changes in lipid modification. This is suggested by our finding that secretion of Hedgehog protein, which is dually lipid modified by palmitate and cholesterol, is not altered in these mutants, indicating that overall the post-translational modification of proteins with lipids in not affected. Furthermore, the large body of literature on p24 proteins unambiguously points to a role in regulating ER-to-Golgi trafficking. Lipid modification of Wg is thought to be mediated by the acetyltransferase Porcupine (Porc), an ER resident protein that is not expected to be affected by changes in ER-to-Golgi trafficking. Additionally, deciphering the role of the lipid modifications in

Wg secretion and activity is not trivial – not least because of the conflicting reports about the relative requirement of the two lipid modifications. Therefore properly resolving this, aside from being technically extremely challenging, is beyond the scope of our manuscript. However, we now mention the possibility of an effect on Porc in the text and further discuss our view on the role of lipid modification in the context of p24 mediated ER export.

Moreover, the cargo receptor hypothesis necessarily implies a specific receptor-ligand interaction. Thus, the authors should address whether Wg specifically binds to Eca or Emp24.

In the revised Figure 5 we now show that Emp24 specifically interacts with Wg. We failed to detect binding of Eca to Wg. However, in several experiments the expression level of Eca was consistently low, which might have lead to this negative result. Alternatively, Eca and Emp24 might act in a complex, as would be expected from previous work, and the Wg-binding interface of the complex might entirely reside on Emp24. Endogenous Emp24 levels are not sufficient for this complex to be detectable. This is the interpretation we favour. In any case, our demonstration of a physical interaction of Emp24 and Wg provides direct evidence for our cargo receptor hypothesis.

Finally, the ER colocalization experiment shown in Fig. 4A should be quantified.

We now show higher magnification images of Wg colocalization with YFP-ER in both genotypes and compare it to a staining of a wildtype disc. Moreover, we now present more direct evidence that in p24 mutants Wg accumulates in the ER: Whereas elevating Wg levels in the entire secretory pathway leads to an accumulation of Wls in the Golgi, high Wg levels in p24 mutants lead to a reduction of Wls in this compartment. This demonstrates that less Wg protein reaches the Golgi in Eca or Emp24 mutant cells and that the excessive protein accumulates in an earlier compartment.

Referee #2:

In this paper, Port et al briefly describe a genome wide screen for components involved in Wingless secretion. As is usual with cell-based screens, a number of hits are identified. Using secondary assays, they narrow their list of hits to two proteins, snx3 (the subject of a separate paper) and emp24. The rest of the paper is devoted to confirming that emp24 is indeed required for Wingless secretion in vivo and that it does not seem to affect the secretion of two other morphogens, Hh and Dpp. The results are mostly clear-cut and well documented (although some experimental details are only scantily described). However, the material covered is relatively limited and little mechanistic insight is provided.

The main issue I have with this paper is the lack of mechanistic insight. The identification of emp24 is an important first step. As the authors admit themselves, it suggests a number of experiments. Is emp24 required for secretion of GPI-anchored proteins in Drosophila (e.g. dally or dally-like)? Could an effect on these glypicans explain some of the phenotypes seen.

We now show that knock-down of eca or emp24 does not have any major effect on Dlp levels or localization. Occasionally Dlp levels appeared to be slightly elevated in Emp24 RNAi clones, but this phenotype was not detected in all discs and was not seen in Eca RNAi clones. Most importantly, Dlp levels were not higher in Wg producing cells in either Eca or Emp24 RNAi.
Unfortunately, currently no antibody against Dally is available, preventing a similar analysis. To test a role of Dally we would have to cross the existing Dally-GFP transgene into our mutant background. The necessary crosses would consume more time than appropriate for a revision. In addition, our other results, in particular the physical interaction of Wg with Emp24, make a purely indirect mode of action through Dally unlikely.

If the palmitate on Wg mediates the interaction with emp24, why is Hh, which is also palmitoylated not affected by emp24?

It is now demonstrated that Emp24 binds Wg, but we have not tested whether this interaction involves the lipid modifications on Wg. The fact that Hh does not rely on Emp24, suggests that palmitoylation is at least not sufficient to render a protein an Emp24 target.

On the whole, a feel that more effort at understanding how emp24 contributes to Wg secretion is needed.

Additional comments.
-No information is given on the Luc-Wg fusion protein.

The WgRluc was first described in Katanaev et al 2008 (PMID: 18219274) More specific details of the site into which the Wg was clones are described in Zecca et al 1996 (PMID: 8945511). We have appended the Methods to include this information: The WgRluc construct was cloned by inserting the Renilla luciferase coding sequence between codons R32 and S36 of the Wg coding sequence (see Zecca et al, 1996).

-As part of the justification for in vivo assay, the authors suggest (rightly) that it is important to rescreen gene function in the context of cells that naturally express Wg but then proceed to assay suppression of a misexpression phenotype in the eye. There is nothing wrong with this assay but the logic must be streamlined.

The reviewer is correct. We have modified the text to alert the reader that our screen in the eye is based on overexpressed protein.
We now write:
"We set out to rescreen selected candidate genes from our tissue culture screen in living flies using a sensitized system in the eye, as well as a system where endogenous Wg signaling in the wing is affected."

-The reference to (Bartoszewski 2004) on p 6 is misleading because it implies that this paper studied Wg secretion.

We removed the reference.

-As mentioned above, the evidence that emp24 is required for Wg secretion is convincing. It is also shown that Hh secretion does not seem to require emp24. The situation with Dpp is somewhat confusing in light of the earlier report that Dpp signalling is affected in emp24 mutant embryos. This should be addressed more thoroughly. In addition, secretion of Dpp could be addressed with the published dpp-gfp fusion constructs.

In fact the earlier paper by Bartoszewski et al reports that whereas Dpp signaling in the embryo is severely disrupted in eca and baiser mutants, Dpp signaling in many other developmental contexts, including wing development, is not. Our results are entirely consistent with this conclusion. Our Dpp data intends to further strengthen our conclusion that Eca and Emp24 are not required for secretion in general, which so far we mainly inferred from the analysis of endogenous Hh. In the new version of the manuscript we now show that two other endogenous proteins, Wls and Dlp, are not affected in Eca and Emp24 mutants. We therefore moved the Dpp data into the supplementary material.

-'resistent' instead of resistant on p8.

The text is now modified.

-Colocalisation can not be objectively assessed at the magnification shown in figure 4A.

Please refer to comment to Referee 1.

Referee #3:

Drosophila Wg acts as a morpohgen to pattern tissues during development. Wg secretion from its producing cells is critical for its gradient formation, but the mechanism(s) of Wg secretion is still not fully understood. Basler lab has made important contributions in the Wg secretion pathway by identifying Wls and retromer as key molecules involved in this pathway. In the current manuscript, Basler and his colleagues have performed a genome-wide RNAi screen aiming at identification of other molecules involved in the Wg secretion pathway. Three new genes including Snx3, Emp24 and Eca have been uncovered as essential molecules required for proper Wg secretion. The current manuscript has characterized the functions of Emp24 and Eca, which have been proposed to be involved in ER to Golgi trafficking. Based on their data, they propose that Emp24 and Eca act as specific cargo receptors for Wg to concentrate it in forming vesicles at sites of ER export. Overall, this is an important piece of work in the field. I have following specific comments:

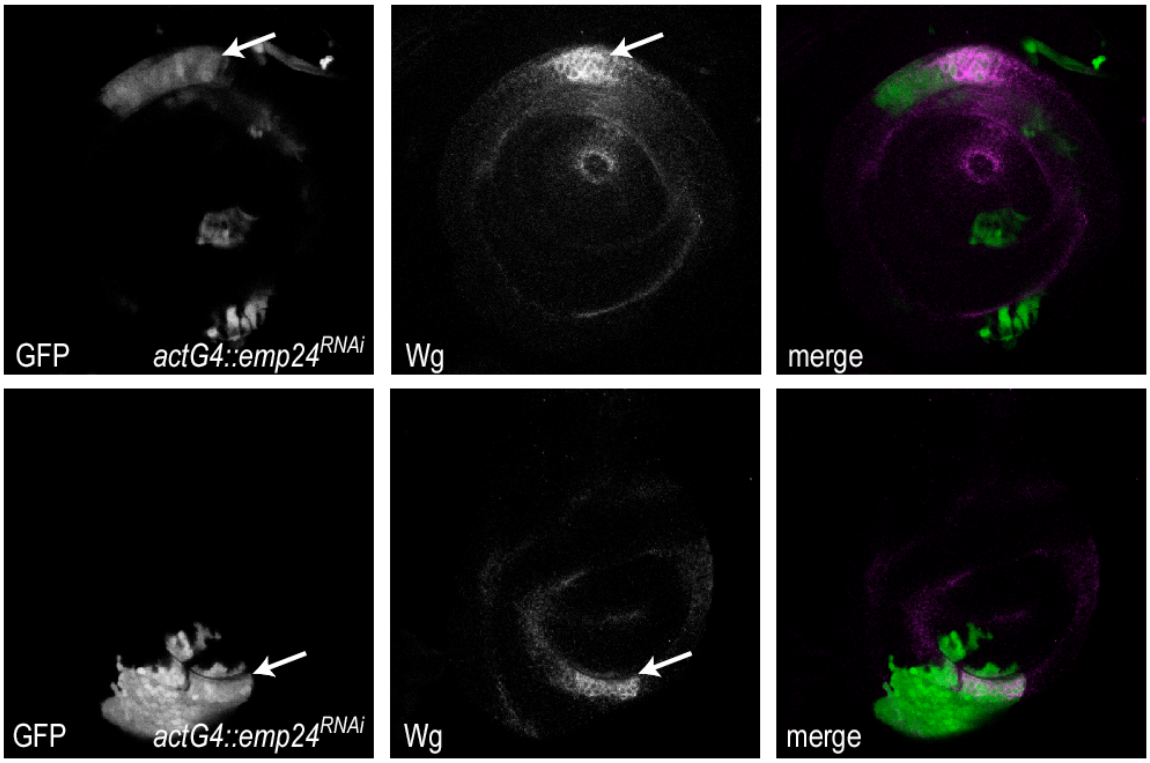
1. The Wg secretion defects associated with loss-of-function of Emp24 and Eca (Fig. 3) are weak compared with those from Wls mutant cells. As shown in Fig3. (3B-B' and 3D-D'), reductions of extracellular Wg levels in P compartment are marginal. One possibility is that P24 family members are functionally redundant. However, based on their data, Eca are P24 are not functionally redundant as simultaneous knock-down of both of them does not generate any stronger Wg secretion defects. I would like to know authors' interpretations on these data.

Indeed the Wg secretion phenotype of the p24 mutants is weaker compared to Wls mutants. Whereas the loss of Wls leads to a near complete block of post-Golgi trafficking of Wg, in the absence of Eca or Emp24 a significant amount of Wg is still secreted. This is entirely consistent with many previous studies that have shown for other p24 cargoes that loss of p24s leads to a mere reduction or delay in trafficking. One possible explanation is that in the absence of p24s a fraction of cargo protein escapes the ER by bulk flow. Alternatively there might exist other transmembrane proteins that could function in a partially redundant way to the p24 proteins. Why such mechanisms are unable to partially compensate for a loss of Wls remains one of the exciting puzzles in the Wnt secretion field. We now have incorporated these thoughts into the manuscript.

2. It would be important to determine whether Emp24 and Eca are required for Wg secretion in other developmental contacts such as embryos or in leg discs.

We have now analysed whether knock-down of eca or emp24 also leads to Wg accumulation in other imaginal discs. Indeed we do find evidence that depletion of either Eca or Emp24 does lead to Wg accumulation in leg and haltere imaginal discs, as well as the notum region of the wing imaginal disc. These phenotypes were generally weaker and more variable than in the pouch region of the wing disc. It remains to be determined whether other p24 proteins play a partially redundant role in these tissues. Moreover, future studies need to test whether p24 proteins are also required for secretion of Wnt proteins in other organisms.

Because space restrains do not allow incorporation of these results into our manuscript, we provided here the results for the reviewer.



Reviewer Figure 1: Clones of cells expressing *emp24 RNAi* constructs under the control of *actin-Gal4* were generated under conditions that lead to Wg accumulation in wing imaginal discs. Staining against endogenous Wg in a leg imagin disc (to panel) and haltere imaginal disc (bottom panel) is shown.

3. Both Wls and Porc are essential for Wg secretion. Reduced Wg secretion in cells lacking Emp24 and Eca activity could be due to defects in Wls trafficking or Porc activities. This can be addressed by examining the levels and subcellular localization of Wls in clones expressing Emp24 and Eca. I would like to know whether Emp24 and Eca are required for Wls trafficking from ER to Golgi or there is other mechanism(s) controlling Wg exit from ER to Golgi. The authors should also discuss the possibility of Emp24 and Eca in regulating Porc activity, which could subsequently affect Wg lipid modifications.

In our revised manuscript we describe the analysis of Wls protein in cells expressing eca or emp24 RNAi. ER-to-Golgi trafficking of Wls appears unaffected. Furthermore, these experiments provide further evidence that Wg accumulation occurs in the ER (see comments to reviewer 1). We now also raise the possibility that p24 proteins affect Porc and explain why we regard it as unlikely.

2nd Editorial Decision 22 July 2011

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.

Yours sincerely,

Editor
EMBO Reports

