EMBO reports

Manuscript EMBOR-2011-35238

# p24 proteins are required for secretion of Wnt ligands

Tina Buechling, Varun Chaudhary, Kerstin Spirohn, Matthias Weiss and Michael Boutros

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**Review timeline:**

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| Submission date: | 20 July 2011 |
| Editorial Decision: | 03 August 2011 |
| Revision received: | 29 September 2011 |
| Accepted: | 07 October 2011 |

## 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 03 August 2011

Thank you again for the submission of your research manuscript to EMBO reports. As I mentioned in my previous letter, it was been sent to three referees, and so far we have only received two reports, that I copy below, one of which has already been sent to you. As both referees recommend that you should be given a chance to revise your manuscript, I would like to ask you to continue working your manuscript according to the referees' comments. As soon as we receive the third report, it will be forwarded to you.

Referee #2 is concerned with the relationship between p24 proteins and WntD. In particular, more experimental evidence needs to be provided in order to demonstrate that p24 has a direct effect on WntD secretion. The other two important issues he raised can be addressed directly in the main text of the manuscript. First, he thinks that previous work on TMED5 is not properly acknowledged and, second, he believes that the findings described in your study should be put into context taking into account recent advances concerning p24 proteins and, more specifically, GPI-anchored proteins.

Given these evaluations, I would like to ask you to revise your manuscript, with the understanding that the referee concerns must be fully addressed and their suggestions (as detailed above and in their reports) taken on board.

Browsing through the manuscript myself, I have noticed that statistical analysis is not properly described for all the figures, including supplementary data. Statistical analyses must be described either in the Materials and Methods section or in the legend of the figure to which they apply. Please include a definition of the error bars used and, when necessary, state the statistical significance of the results and the method used to calculate it. I have also noticed that figures do not have scale bars and this needs to be corrected as well.

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

Yours sincerely,

Editor
EMBO reports

REFEREE REPORTS:

Referee #1:

In this paper, Boutros and colleagues show that p24 family members appears to be specifically required for Wnt secretion. These transmembrane proteins have been previously implicated in ER-Golgi transport. From a focused screen, the authors first found that CG9053 (which they name opossum; opm) is required for Wg signalling in cultured S2 cells. They subsequently show that a close homolog (emp24; CG3564) is also required in the same cell-based assay. In accordance with previous work on this family of proteins, the authors find that tagged versions localise in the secretory pathway. They then provide evidence that opm is required for Wg secretion in wing imaginal discs (using mostly RNAi transgenes). Using the same in vivo assay they find that depletion of CG3564 (CHOp24) or CG1967 causes the same phenotype (accumulation of intracellular Wg and loss of signalling). Unfortunately, issue of cross-reactivity of the RNAi tansgene complicate the interpretation and it remains unclear as which p24 family member is directly required for Wg secretion. One exception concerns CG9053 because the authors generated a mutant, which appears to show Wg accumulation in expressing cells (however see caveat about fig S9 below).

In fig S9, Wingless appears not to accumulate in mutant cells that are located near the A\P boundary. I understand the non-autonomous effect on senseless expression but do not see how wild type tissue could 'rescue' the distribution of intracellular Wingless in mutant tissue.

The authors attribute the lack of effect on sens expression in small clones to non-automomy. It seems to me that this could equally be due to perdurance of the opm gene product.

Although the authors downplay the cuticle phenotype of opm M/Z embryos, I find it surprising that it does not mimic better loss of Wg activity during embryogenesis. I guess that this could be attributed to redundant activity from homologs and that somehow this is not as pronounced in imaginal discs

In flybase, the abbreviation of opossum is opo. Perhaps this could be corrected.

Despite the above remarks/concern, this paper makes an important contribution by identifying a new class of proteins that are dedicated to Wnt secretion. If the authors can resolve the issue of non-autonomy, Their story will be of interest to the cell biology community.

Referee #2:

Wnt proteins are secreted, lipid-modified glycoproteins that act as important signaling molecules in animal development and adult tissue homeostasis. Previous studies have revealed that Wnt protein

secretion is finely regulated by specific factors that function in the endocytic and late secretory pathways. However, the mechanisms that drive Wnt protein transport trough the early secretory pathway from the ER to the Golgi remain elusive. In this manuscript, the authors report an RNAi survey for components of the intracellular transport machinery involved in the secretion of the Drosophila Wnt protein Wingless (Wg). This screen uncovered several genes encoding type I transmembrane proteins belong to the conserved p24 family. The requirement of p24 proteins for canonical Wnt/Wg signaling was also observed in human cells, indicating a conserved mechanism. The subsequent functional analysis in Drosophila revealed that Wg seems to accumulate in the ER whereas other secretory proteins are normally secreted in p24 RNAi cells, suggesting that p24 proteins are specifically involved in the ER export of Wnt proteins. Furthermore, a specific physical interaction of Wg with a p24 protein was detected by co-immuoprecipitation. Based on these data, together with much of the existing literature on p24 proteins, the authors propose that p24 proteins can act as ER cargo receptors for Wnt proteins. Overall, these findings are important for the field. However, some points are raised below:

-The major issue of the paper is that p24 proteins might function as cargo receptors for Wg. In addition, the authors claim to show that the Wnt inhibitor of dorsal (WntD), a distant member of the Drosophila Wnt family, can also behaves as p24 cargo since its secretion is affected in p24 RNAi cells. However this effect may be indirect since there is no physical interaction of WntD with p24, and a specific receptor-ligand interaction is an essential requisite for the cargo receptor model. The authors should clarify the role of p24 proteins in WntD secretion. For instance, they can examine whether WntD is also accumulated in the ER like Wg in p24 RNAi cells. WntD is exceptional among Wnts in that it is not lipid modified. Therefore, it is important to address properly whether p24 function does not directly depend on the lipid modification of Wnts proteins by assessing the interaction of Wg with p24 in porc mutant cells.

-The data showing that TMED5 is a component of the early secretory pathway that shuttle between ER and Golgi and it is not required for VSV-G intracellular transport has already been reported (Koegler et al, 2010). Therefore, the authors should clearly mention in the text that they just confirm the previous results obtained by others. The localization of TMED5 in ER and Golgi compartments (Fig. 2) should be shown as supplementary data.

-The text has to be re-written (particularly the Introduction and the Discussion sections) in order to provide more accurate information about the current knowledge of the p24 proteins. Considering the major conclusion of this manuscript, the authors should focus on the p24 receptor role for GPI-anchored proteins that has been now well established and documented in yeast and mammalian cells (Muniz et al, 2000; Takida et al, 2008; Castillon et al, 2009; Bonnon et al, 2010 and very recently Castillon et al, 2011; Fujita et al 2011). The connection of GPI-anchored proteins with Wnt proteins is obvious. Similar to GPI-anchored proteins, Wnt proteins are luminally exposed cargoes and they are attached to the membrane by a lipid modification leading to their incorporation into lipid rafts. Thus, as shown for GPI-anchored proteins (Castillon et al, 2011), the p24 proteins might link Wg with the cytosolic COPII coat to efficiently incorporate Wg into ER-derived vesicles. This possibility has to be considered in the text.

**We would like to thank the reviewers for their constructive criticism concerning our manuscript and have addressed their concerns as outlined below. We have also significantly shortened the revised manuscript to adhere to the EMBO reports format requirements.**

**Referee #1:**

*"In this paper, Boutros and colleagues show that p24 family members appears to be specifically required for Wnt secretion. These transmembrane proteins have been previously implicated in ER-Golgi transport. From a focused screen, the authors first found that CG9053 (which they name opossum; opm) is required for Wg signalling in cultured S2 cells. They subsequently show that a close homolog (emp24; CG3564) is also required in the same cell-based assay. In accordance with previous work on this family of proteins, the authors find that tagged versions localise in the secretory pathway. They then provide evidence that opm is required for Wg secretion in wing imaginal discs (using mostly RNAi transgenes). Using the same in vivo assay they find that depletion of CG3564 (CHOp24) or CG1967 causes the same phenotype (accumulation of intracellular Wg and loss of signalling). Unfortunately, issue of cross-reactivity of the RNAi tansgene complicate the interpretation and it remains unclear as which p24 family member is directly required for Wg secretion. One exception concerns CG9053 because the authors generated a mutant, which appears to show Wg accumulation in expressing cells (however see caveat about fig S9 below)."*

**We agree with the reviewer on the potential cross-reactivity of the RNAi transgenes, but we indeed believe that not a single, but multiple p24 proteins are involved in Wg secretion. p24 proteins were described to form multimeric complexes with different subfamily members and oligomerization seems to be required for many known interactions (Carney and Bowen, 2004; Bethune et al., 2006).**

**We believe that p24 proteins act partially redundant. We have attempted to address this issue by analyzing CHOp24; opm double mutant embryos. Hemizygous CHOp24 mutant embryos (P-element insertion PBac{RB}CHOp24$^{e04526}$) survive until first instar larval stage and, similar to opm mutants, do not exhibit a clear Wg loss of function phenotype. We have generated CHOp24; opm$^{9.3}$ double mutants by recombination and analysed their cuticular phenotype. Here, we observe a much stronger phenotype in hemizygous double mutant males than observed in either CHOp24 or opm single mutants, indicating that they can act in a partially redundant manner. We have added an additional panel to supplementary Fig S4C. It is likely that additional p24 proteins contribute to a full loss-of-function phenotype in a tissue specific manner which would be consistent with the report by Port et al. (2011).**

*"In fig S9, Wingless appears not to accumulate in mutant cells that are located near the A\P boundary. I understand the non-autonomous effect on senseless expression but do not see how wild type tissue could 'rescue' the distribution of intracellular Wingless in mutant tissue."*

**We do not believe that there is a non-autonomous effect on intracellular Wg levels and rather think that this was an imaging artifact. We have repeated the experiment and have replaced the original image with a new one in figure S4D. Another example is shown below where no non-autonomous rescue of intracellular Wg accumulation can be observed.**

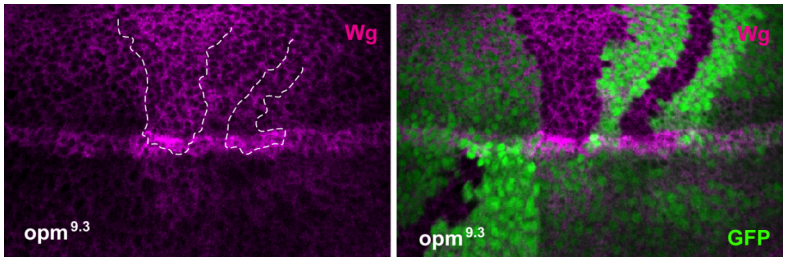


**Figure R1. Opm mutant clones stained for total Wg protein.** Opm mutant clones marked by the absence of GFP show accumulation of total Wg inside the clones, clonal boundaries are indicated.

*"The authors attribute the lack of effect on sens expression in small clones to non-automomy. It seems to me that this could equally be due to perdurance of the opm gene product. "*

**We do agree and have commented on the possibility in the main text on page 8.**

*"Although the authors downplay the cuticle phenotype of opm M/Z embryos, I find it surprising that it does not mimic better loss of Wg activity during embryogenesis. I guess that this could be attributed to redundant activity from homologs and that somehow this is not as pronounced in imaginal discs."*

**We believe that the lack of an embryonic phenotype is due to redundancies among p24 protein family members and have commented on this in the main text on page 8.**

*"In flybase, the abbreviation of opossum is opo. Perhaps this could be corrected."*

**This will be corrected in Flybase.**

*"Despite the above remarks/concern, this paper makes an important contribution by identifying a new class of proteins that are dedicated to Wnt secretion. If the authors can resolve the issue of non-autonomy, Their story will be of interest to the cell biology community."*

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*member of the Drosophila Wnt family, can also behaves as p24 cargo since its secretion is affected in p24 RNAi cells. However this effect may be indirect since there is no physical interaction of WntD with p24, and a specific receptor-ligand interaction is an essential requisite for the cargo receptor model. The authors should clarify the role of p24 proteins in WntD secretion. For instance, they can examine whether WntD is also accumulated in the ER like Wg in p24 RNAi cells. WntD is exceptional among Wnts in that it is not lipid modified. Therefore, it is important to address properly whether p24 function does not directly depend on the lipid modification of Wnts proteins by assessing the interaction of Wg with p24 in porc mutant cells."*

**We cannot exclude that the effect on WntD might be indirect, however we have performed additional experiments to support our model that also WntD is dependent on p24 proteins for export.**

**To address the role of p24 proteins on WntD secretion, we performed a cell mixing experiment of opm and control RNAi treated cells expressing a tagged version of WntD. We then analyzed whether opm-treated cells retain more WntD than control cells. One cell population was stably marked by Histon2B-RFP. As shown below, we do see increased levels of WntD in opm-depleted cells compared to control treated cells, suggesting that Opm is indeed required for WntD secretion on a cellular level.**

**Data of a similar experiment where both RNAi populations have been marked (H2B-CFP cells treated with rel dsRNAs mixed with H2B-RFP cells treated with opm dsRNAs) has also been added to the manuscript (see supplementary Fig. S5G).**

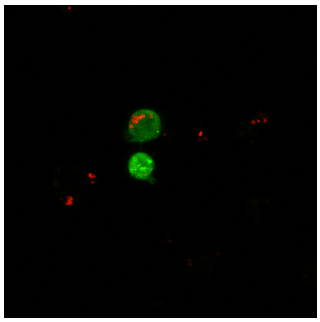

**Figure R2. WntD accumulated in opm RNAi treated cells.**
Example of His-2B-RFP cells treated with rel-RNAi (red) and unmarked cells treated with opm-RNAi (marked by the absence of RFP), higher levels of WntD-HA can be seen in unmarked cells suggesting defects in secretion.

**WntD is not expressed in imaging discs which prevented us to perform a secretion analysis similarly to the experiments done for Wg. We could not detect WntD neither on protein nor on mRNA level (which is in accordance with Gordon et al., 2005).**

**We have attempted to look at Wg-p24 interaction in porc RNAi treated cells however were not able to produce a sufficient knockdown (of <50%) with multiple independent RNAi constructs for porc. We further attempted to perform immunoprecipitation experiments in S2 cells overexpressing Wg mutant proteins (Wg-C93A and Wg-S239). However, these experiments were unfortunately inconclusive, most likely because these mutant proteins have different stabilities and remain stuck in different subcellular compartments.**

*"The data showing that TMED5 is a component of the early secretory pathway that shuttle between ER and Golgi and it is not required for VSV-G intracellular transport has already been reported (Koegler et al, 2010). Therefore, the authors should clearly mention in the text that they just confirm the previous results obtained by others. The localization of TMED5 in ER and Golgi compartments (Fig. 2) should be shown as supplementary data."*

**We have moved parts of Fig 2 (an experiment similar to Koegler et al, 2010**

**immunofluorescence in HeLa cells) to the supplement as suggested. The Koegler et al study is now referenced as follows: 'In accordance with a previous report (Koegler et al, 2010), TMED5-EGFP colocalized to ERGIC53-positive vesicles' and 'confirmed findings by a recent report (Koegler et al, 2010) that VSVG is secreted normally in TMED5-depleted cells'.**

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**We thank the reviewer for the suggestion and have added an explanatory sentence to the discussion of the manuscript. However, we would like to point out that we do not have biochemical data to support this hypothesis and therefore prefer not to re-focus our story on GPI-anchored proteins and the COP-coat.**

3rd Editorial Decision 07 October 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 EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports