EMBO reports

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p24 proteins are required for secretion of Wnt ligands

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During development and disease, the exocytosis of signalling molecules, such as Wnt ligands, is essential to orchestrate cellular programs in multicellular organisms. However, it remains a largely unresolved question whether signalling molecules follow specialized transport routes through the exocytic pathway. Here we identify several *Drosophila* p24 proteins that are required for Wnt signalling. We demonstrate that one of these p24 proteins, namely Opossum, shuttles in the early secretory pathway, and that the *Drosophila* Wnt proteins are retained in the absence of p24 proteins. Our results indicate that Wnt secretion relies on a specialized anterograde secretion route with p24 proteins functioning as conserved cargo receptors.

Keywords: Wnt secretion; p24 proteins; protein transporting; signalling

EMBO *reports* (2011) **12**, 1265–1272. doi:10.1038/embor.2011.212

INTRODUCTION

Wnt signalling has been implicated in many developmental processes, such as tissue homeostasis, axis patterning and the maintenance of stem cells, and is misregulated in several types of cancer (van Amerongen *et al*, 2008; Angers & Moon, 2009). Although much progress has been made in dissecting the intracellular Wnt signalling cascade, open questions remain whether and how Wnt ligands follow specialized exocytic pathways. Wnts are secreted as palmitoylated glycoproteins that require the multi-pass transmembrane protein Evenness interrupted (Evi/Wls) for post-Golgi transfer to the plasma membrane (Banziger *et al*, 2006; Bartscherer *et al*, 2006), and Retromer, a multiprotein sorting complex, for plasma membrane-to-Golgi recycling of Evi and the maintenance of Wnt secretion (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Port *et al*, 2008). Although past studies have mainly focused on this recycling pathway, it still remained to be resolved how Wnt proteins are transported through the early secretory pathway to the Golgi apparatus.

Here, we identify members of the p24 protein family, also known as EMP24/GP25L/Erp (endomembrane protein precursor of 24 kD) proteins, as being required for secretion of Wnt proteins, namely Wingless (Wg) and Wnt inhibitor of Dorsal (WntD). p24 proteins have been proposed to be cargo-adaptor proteins implicated in bidirectional transport processes at the endoplasmic reticulum (ER)–Golgi interface (Carney & Bowen, 2004; Strating & Martens, 2009). A specific association of p24 proteins with cargo molecules has also been shown for yeast.

We show that Opossum (Opm), a conserved member of the γ-subfamily of p24 proteins and two other *Drosophila* p24 proteins, CHOp24 and p24-1, are required in Wnt-producing cells for ER export of Wg. Our results indicate that p24 proteins might function as early cargo receptors upstream of Evi in a selective anterograde secretory route of Wnts.

RESULTS

Opm is a conserved regulator of Wnt signalling

To dissect Wnt protein transporting, we analysed the effect of depleting 466 components of the cellular transporting machinery (supplementary Table S1 online) by RNA interference (RNAi) in *Drosophila* cells. Using a new Wg-specific luciferase reporter, we identified CG9053, named Opm, as being required for Wg signalling (Fig 1A). Depletion of Opm using independent double-stranded RNAs (dsRNAs) significantly affected Wg signalling in different cell lines and Wnt-reporter assays (Fig 1B; supplementary Fig S1A–C online). An RNAi-insensitive rescue construct (opm^{resc}) significantly reverted the loss of Wg-reporter activity (Fig 1C; supplementary Fig S1D online).

Opm is a highly conserved type1a transmembrane protein of the conserved γ-subfamily of p24 proteins (supplementary Fig S1E online). Short interfering RNAs (siRNAs) targeting its human homologue, TMED5 (transmembrane emp24 protein transport containing domain 5), also known as CGI-100, significantly reduced Wnt-induced TOPFlash reporter activity in HEK293T cells that were activated by overexpression of mWnt1 (Fig 1D), confirming that Opm is a conserved regulator of canonical Wnt/Wg signalling.

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Received 20 July 2011; revised 7 October 2011; accepted 7 October 2011; published online 18 November 2011

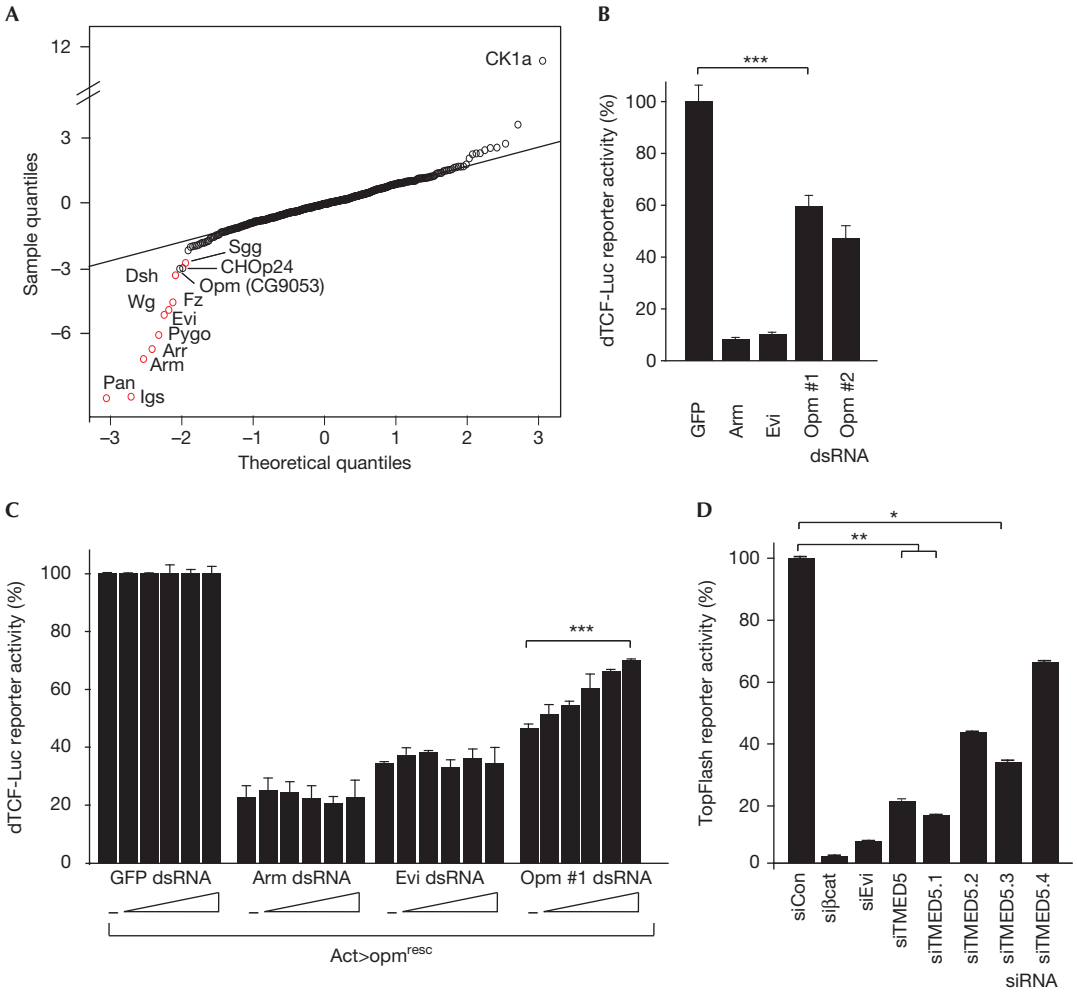


Fig 1 | Opm is a conserved positive regulator of Wg signalling. (**A**) Graphical representation of screening results as Q–Q plot of normally distributed quantiles against screening result quantiles. A perfect fit to a normal distribution is represented by a line. Values represent *z*-scores. Known positive regulators are highlighted in red. (**B**) dTCF-Luc reporter assay in S2R+ cells stimulated by overexpression of Wingless (Wg), in the presence of the indicated double-stranded RNAs (dsRNAs; ***$P<0.0001$, paired Student's *t*-test). (**C**) dTCF-Luc reporter system in Kc_{167} cells in the presence of increasing amounts of opm^{resc} (***$P<0.0001$, analysis of variance test). (**D**) TOPFlash reporter assay in Hek293T cells activated by overexpression of mWnt1, in the presence of the indicated short interfering RNAs (siRNAs; **$P<0.005$, *$P<0.05$, paired Student's *t*-test). Data are shown as mean ± s.d. of at least three independent experiments. Evi, Evenness interrupted; GFP, green fluorescent protein; opm, opossum.

Opm shuttles in the early secretory pathway

p24 proteins have been proposed to be recycling cargo adaptor proteins, as they are abundantly present in membranes of the early secretory pathway (Carney & Bowen, 2004; Strating and Martens, 2009). Therefore, we first examined the subcellular localization of an Opm–enhanced green fluorescent protein fusion protein, which colocalized with the ER marker KDEL in the *Drosophila* follicular epithelium. Similarly, TMED5–enhanced green fluorescent protein colocalized to the ER, the ER–Golgi intermediate compartment (ERGIC) and partially to the *cis*-Golgi network in HeLa cells. In accordance with a previous report (Koegler *et al*, 2010), TMED5 colocalized to ERGIC53-positive vesicles in cells treated with nocodazole and Brefeldin A, indicating that it shuttles in the early secretory pathway (supplementary Fig S2A,B online).

Loss of Opm does not affect general protein secretion

To test whether TMED5 is required for general protein transporting, we performed a vesicular stomatitis virus G-protein secretion assay. In accordance with a recent study (Koegler *et al*, 2010), vesicular stomatitis virus G-protein–GFP was detected at the plasma membrane in TMED5-depleted cells (supplementary Fig S2C online). Similar results were obtained with a secreted version of firefly luciferase (Fig 2A). In addition, both a JAK/signal transducers and activators of transcription (STAT)- and Hedgehog (Hh)-specific reporter assay were unaffected by *opm* depletion (Fig 2B,C), indicating that Opm is not required for global protein secretion.

Epistatically, Opm function was mapped to the Wnt/Wg-secreting cells as cell-mixing experiments revealed that Opm function is not required for signal transduction in the receiving cell (Fig 2D–E).

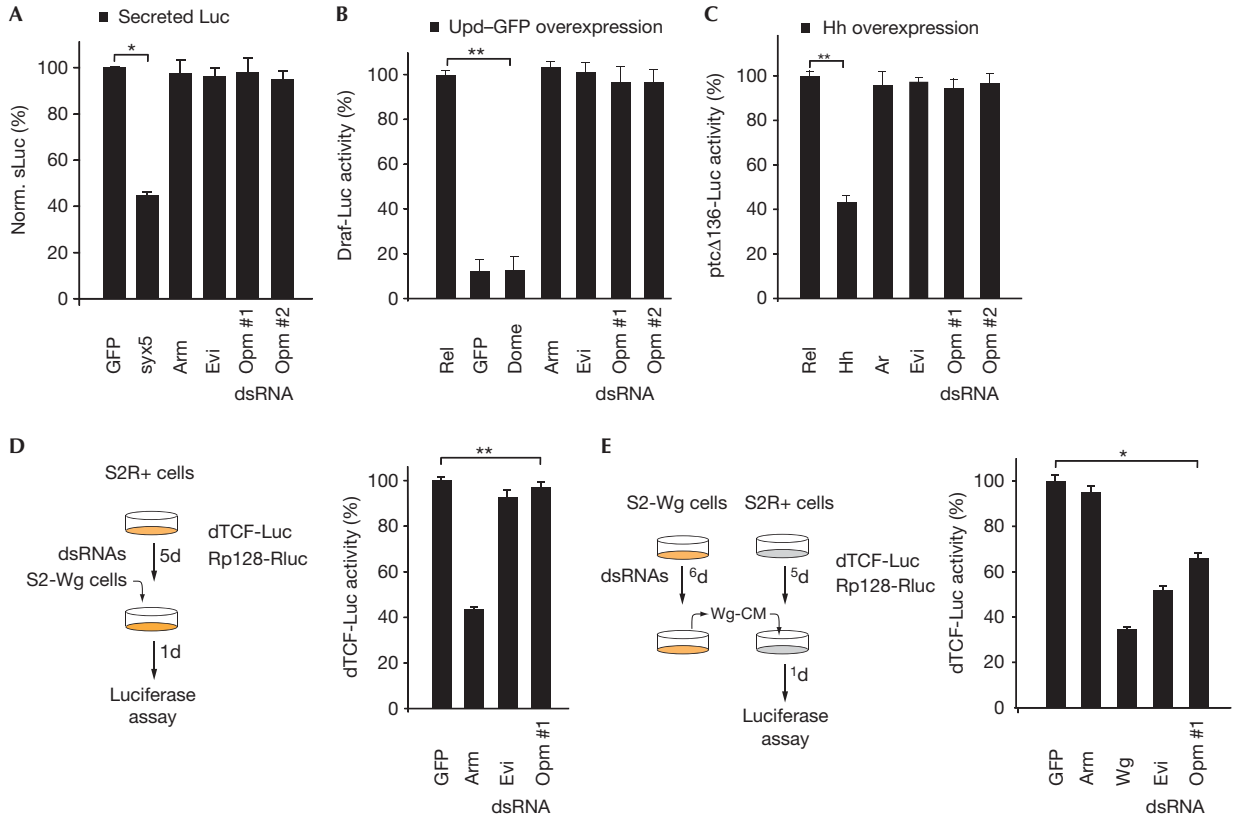


Fig 2 | Opm functions in the Wg-secreting cell. (**A**) sLuc secretion assay in the presence of the indicated double-stranded RNAs (dsRNAs). Syntaxin 5 (syx5) serves as control. (**B,C**) JAK/signal transducers and activators of transcription- and Hedgehog (Hh)-specific reporter assays in the presence of the indicated dsRNAs. (**D**) S2R+ cells treated with the indicated dsRNAs (highlighted in yellow) and transfected with the dTCF-Luc reporter system were mixed with S2-Wg cells. Time of treatment in days (d) is indicated. (**E**) S2R+ cells activated with Wingless (Wg)-conditioned medium collected from S2-Wg cells (Wg-CM) pretreated with the indicated dsRNAs (highlighted in yellow). Data are shown as mean ± s.d. of at least three independent experiments (*$P<0.001$, **$P<0.0001$, paired Student's *t*-test). Evi, Evenness interrupted; GFP, green fluorescent protein; opm, opossum; sLuc, secreted version of firefly luciferase; Upd, unpaired.

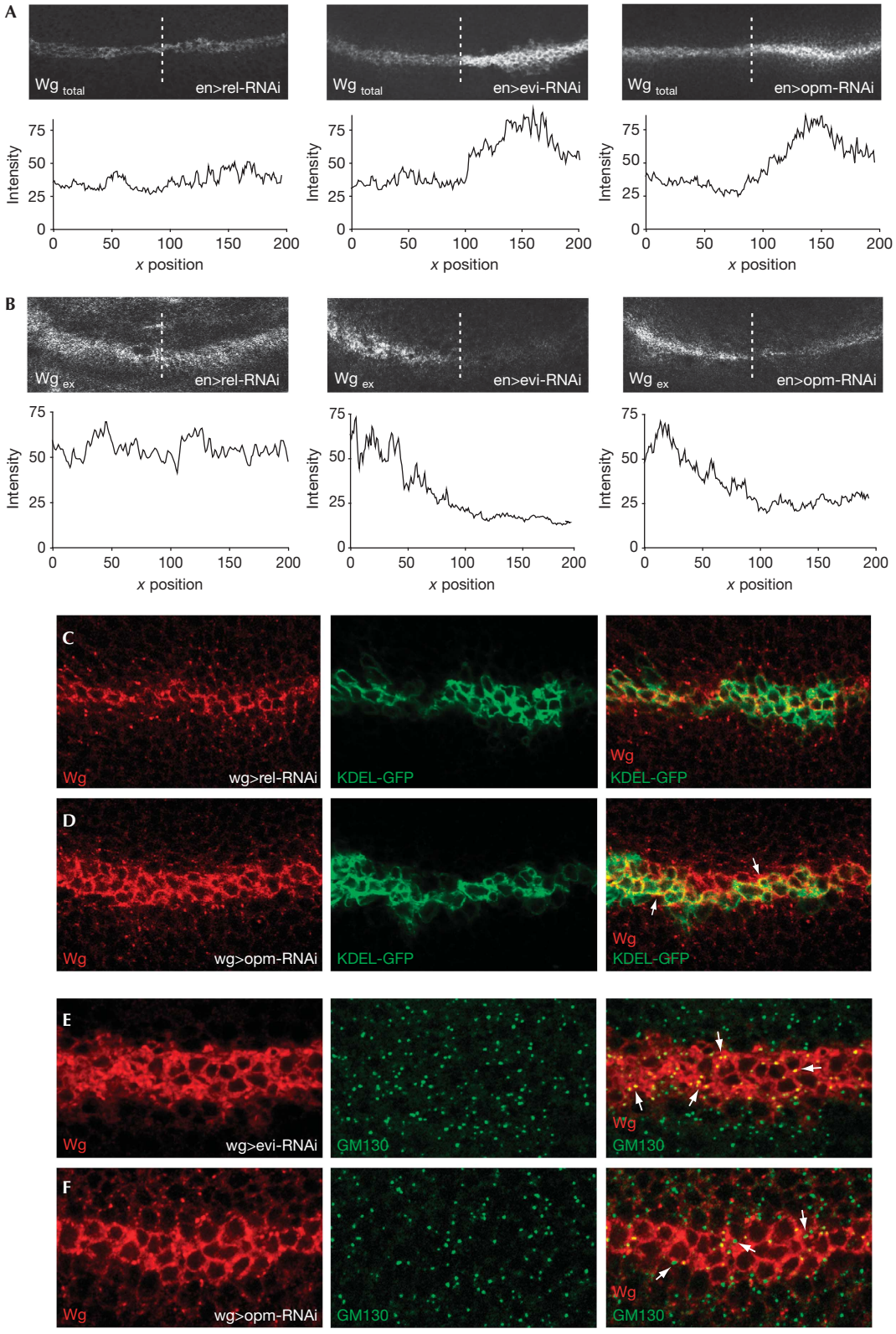
Opm is required for Wg function *in vivo*

To determine the requirement of Opm for Wg signalling *in vivo*, we depleted *opm* by tissue-specific RNAi in the developing *Drosophila* wing disc. Total Wg protein accumulated in Wg-expressing cells of the *opm*-depleted tissue, whereas the extracellular Wg gradient was significantly reduced (Fig 3A,B). Furthermore, Wg colocalized to a higher extent with the ER marker KDEL, but not the Golgi marker GM130, as observed for *evi*-deficient cells (Fig 3C–F; supplementary Fig S3A online). These experiments suggest that Wg does not reach the Golgi apparatus in the absence of Opm.

We next examined the Wg short-range target Senseless (Sens), which was reduced in *opm*-depleted cells leading to adult wing margin defects that phenocopy a loss of Wg function (Fig 4A,B). However, *opm* depletion seems not to block Wg secretion completely as the observed phenotypes are milder compared with *evi* knockdown, indicating that *opm* might function in part redundantly with other factors.

To test for the specificity of the phenotype, we first examined the Hh target gene Patched in *opm*-depleted wing discs, which was unaffected, confirming that Opm is not required for Hh signalling (supplementary Fig S3B online). Second, we rescued the effect on Wg protein by expression of an RNAi-insensitive opm^{resc} construct in the *opm*-RNAi background, which restores total Wg protein to almost wild-type amounts and partially rescues the RNAi-induced wing margin defects (Fig 4C,D; supplementary Fig S3C,D online).

As Opm is a member of the p24 protein family consisting of nine predicted members in *Drosophila*, we systematically analysed RNAi-induced phenotypes of other p24 proteins *in vivo* (supplementary Table S2 online). Knockdown of CHOp24 (CG3564 or p24/p24β1) and p24-1 (CG1967 or p26/p24γ4) caused wing margin defects mimicking *opm* depletion and an accumulation of total Wg protein (Fig 4E,F). However, as we were not able to completely rescue the observed wing margin defects of opm-RNAi animals, we analysed the expression of other p24 transcripts. Indeed, we observed both reduction and upregulation of several p24 transcripts on knockdown of a single p24 protein (supplementary Fig S3E online), indicating that several p24 proteins might be affected and suggesting a cross-regulatory compensation by several p24 proteins on knockdown of a single family member. In addition, we cannot exclude changes in protein expression of



p24s causing a combined RNAi phenotype. Taken together, our results strongly indicate that several p24 proteins contribute to Wnt secretion and might have partially redundant functions.

Opm is required for embryonic development

To further analyse the role of *opm* during development, we generated a loss-of-function allele by homologous recombination

◀ **Fig 3** | Opm is required for Wg secretion *in vivo*. (**A,B**) Total and extracellular Wg staining of larval wing discs of the indicated genotypes ($n > 5$ for all genotypes). (**C,D**) Total Wg protein staining of larval wing discs colabelled with KDEL-GFP. Quantification of the degree of colocalization for $n = 3$ was done through the JACoP plugin in ImageJ, resulting in an average Pearson's coefficient of $P = 0.56$ and $P = 0.68$ for rel-RNAi and opm-RNAi, respectively, which supports an enhanced colocalization in the ER on opm-RNAi. To complement the analysis, Mander's correlation coefficients were calculated ($M = 0.47$ for rel-RNAi versus $M = 0.59$ for opm-RNAi). (**E,F**) Total Wg protein staining of third-instar larval wing discs co-stained with GM130 ($n = 5$ for all genotypes). ER, endoplasmic reticulum; Evi, Evenness interrupted; GFP, green fluorescent protein; opm, opossum; RNAi, RNA interference; Wg, Wingless.

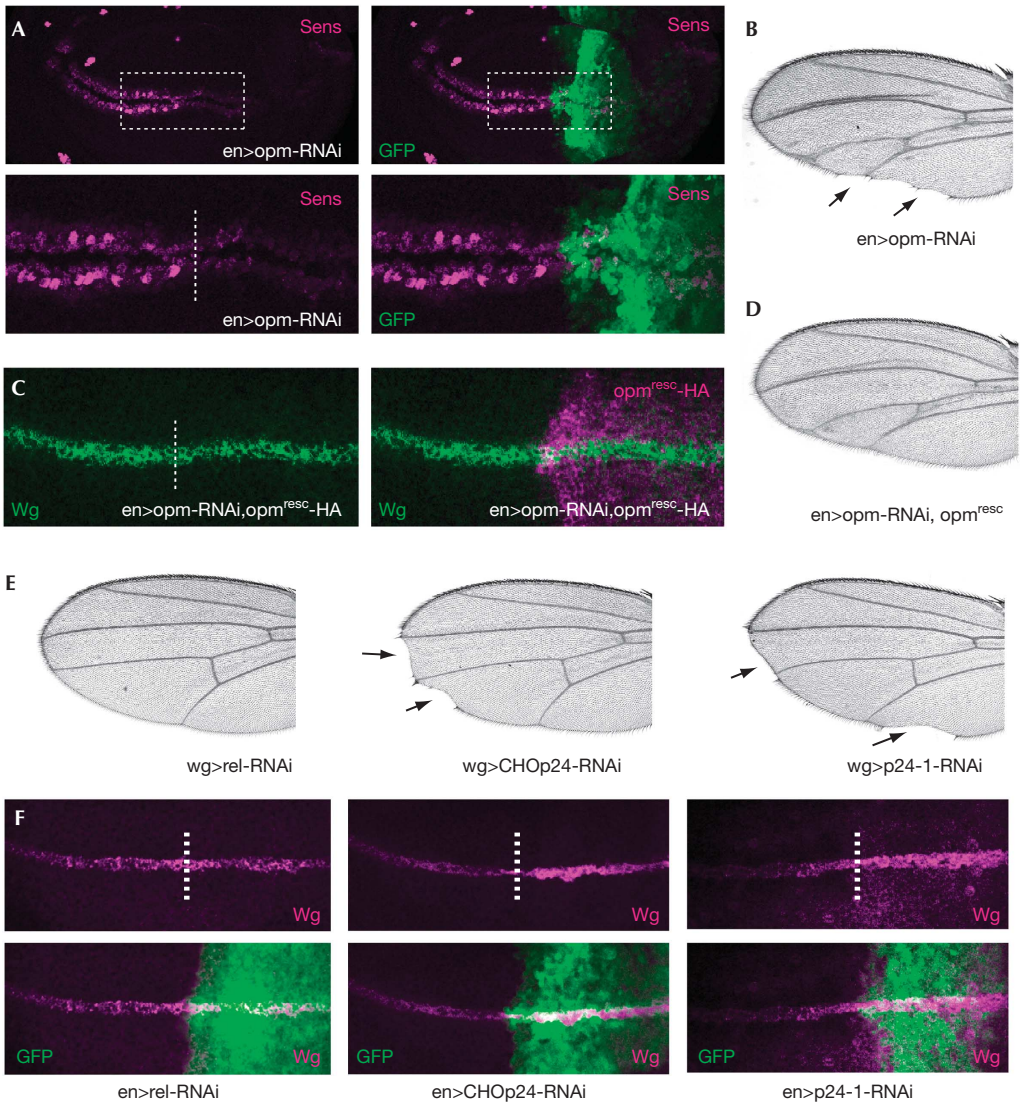


Fig 4 | p24 proteins are required for Wg secretion. (**A**) Sens staining of wing discs of the genotype enGal4, UAS-GFP/UAS-opm-RNAi ($n = 5$ analysed). GFP marks the RNAi expression domain. The dashed line indicates the anterior/posterior (A/P) boundary. (**B**) Adult wing of the genotype enGal4/UAS-opm-RNAi ($n > 50$ analysed). Note the characteristic wing margin defects. (**C**) Total Wg staining of third-instar larvae of the genotype: enGal4/UAS-opm-RNAi;UAS-opm^{resc}-HA/+. The dashed line indicates the A/P boundary. (**D**) Adult wing of the genotype enGal4/UAS-opm-RNAi;UAS-opm^{resc}/+ ($n > 50$ analysed). (**E**) Adult wings of the genotypes wgGal4/UAS-relRNAi, wgGal4/UAS-CHOp24-RNAi and wgGal4/+; UAS-p24-1-RNAi/+ ($n > 100$ analysed). Note the wing margin defects and the loss of sensory bristles (arrows). (**F**) Total Wingless (Wg) staining of wing discs of the indicated genotypes, anterior to the left: enGal4,UAS-GFP/UAS-relRNAi, enGal4,UAS-GFP/UAS-CHOp24-RNAi, enGal4,UAS-GFP/UAS-p24-1-RNAi ($n = 4$ analysed). GFP marks the RNAi expression domain; the dashed line indicates the A/P boundary. GFP, green fluorescent protein; HA, haemagglutinin; opm, opossum; RNAi, RNA interference; Sens, Senseless.

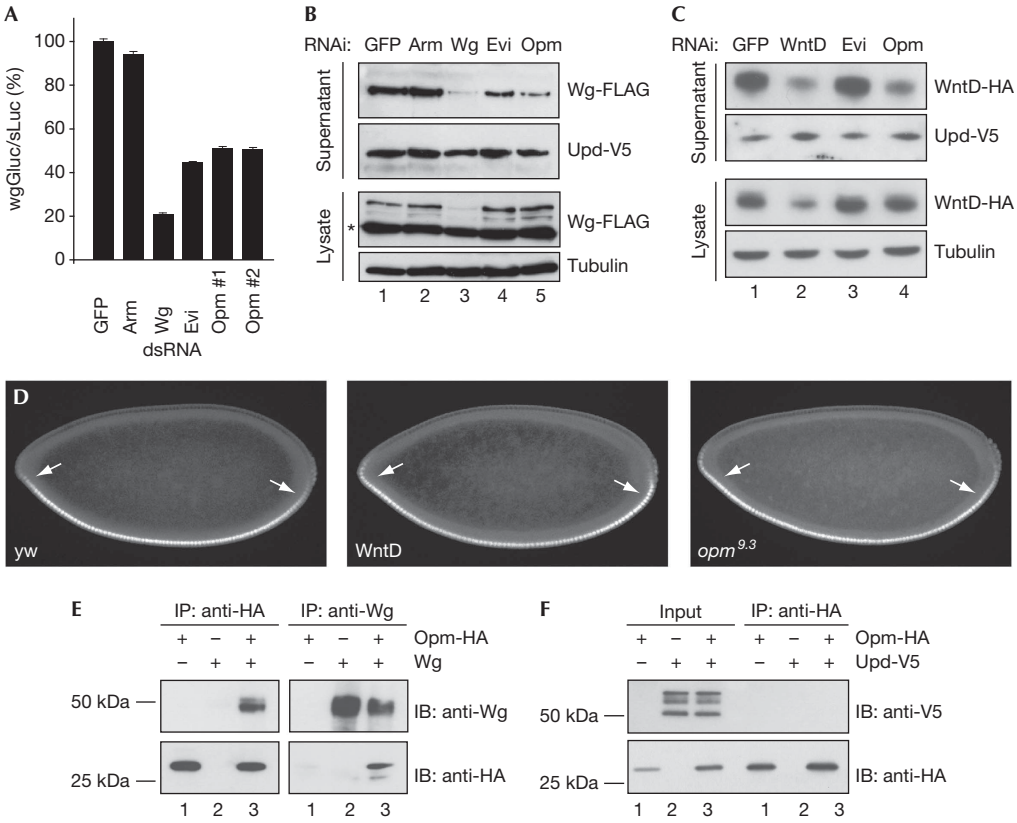


Fig 5 | Opm is required for Wnt secretion. (**A**) Wingless (Wg)–Gluc secretion assay normalized to sLuc (Wg–Gluc/sLuc). Data are shown as mean ± s.d. (*$P<0.0001$, Student's *t*-test, $n=4$). (**B**) Western blot of Wg-FLAG in supernatants and lysates of S2 wg-FLAG cells in the presence of the indicated double-stranded RNAs (dsRNAs); Unpaired (Upd)-V5 serves as loading control. The asterisk indicates a non-specific band observed in all lysates. (**C**) Wnt inhibitor of Dorsal (WntD) secretion assay in S2 cells transfected with WntD-HA, in the presence of the indicated dsRNAs. (**D**) Lateral view of stage 4 embryos of the indicated genotypes stained for anti-Dorsal, anterior to the left ($n>30$ for each genotype). Note the extension of nuclear Dorsal at the termini for WntD (94% of embryos, $P<0.001$) and *opm*$^{9.3}$ (77% of embryos, $P<0.001$, χ^2-test). (**E**,**F**) Co-IPs of Opm-HA and Wg or Upd-V5 from lysates of transfected S2 cells. Evi, Evenness interrupted; GFP, green fluorescent protein; Gluc, Gaussia luciferase; HA, haemagglutinin; IB, immunoblot; IP, immunoprecipitation; Luc, secreted version of firefly luciferase; opm, opossum; RNAi, RNA interference; Upd, unpaired; yw, yellow white control embryos.

(*opm*$^{9.3}$; 13A on the X chromosome). Despite the fact that offspring of homozygous *opm*$^{9.3}$ mutant females die early during embryogenesis due to severe patterning defects (supplementary Fig S4A,B online), *opm*$^{9.3}$ homozygous mutants are viable, suggesting a strong maternal contribution, which has indeed been reported for most of the p24 proteins (Boltz *et al*, 2007). Consistently, we found that one wild-type allele of *opm* is sufficient to rescue the embryonic lethality of progeny of *opm*$^{9.3}$/*opm*$^{9.3}$ females. However, the phenotype of *opm*$^{9.3}$ seems to be more complex and does not resemble the classical Wg loss-of-function phenotype, indicating that its function might affect more processes and is possibly redundant with other p24 proteins.

We next tested whether the secretion of Wg is affected in mitotic *opm* mutant clones in wing imaginal discs. Total Wg protein was increased in large clones of *opm* mutant tissues compared with adjacent wild-type tissues, and Sens was reduced, although residual Sens remained detectable (supplementary Fig S4C,D online), confirming that Wg is retained in the absence of *opm*. However, the block of Wg secretion is incomplete compared with the strong phenotype of *evi* mutants (Banziger *et al*, 2006; Bartscherer *et al*, 2006). Furthermore, we did not observe a significant change in the expression of Sens in small or medium-sized *opm*$^{9.3}$ mutant clones, indicating a potential rescue effect from wild-type tissue or a partial redundancy with other p24 proteins.

Opm is required for Wg secretion

To further confirm a role of Opm in Wg secretion, we analysed the effect of *opm* depletion on a Wg–Gaussia luciferase (Wg–Gluc) fusion protein and secreted Wg protein. Opm-RNAi reduced both secretion of Wg–Gluc and FLAG-tagged Wg, whereas the *Drosophila* JAK/STAT ligand Unpaired (Upd) was secreted normally (Fig 5A,B; supplementary Fig S5A online), suggesting a specific requirement for Opm in Wg, but not Upd, secretion.

We next analysed the secretion of WntD, a distant member of the *Drosophila* Wnt family, that is not palmitoylated as other Wnts and is secreted in an Evi-independent manner (Gordon *et al*, 2005; Ching *et al*, 2008). In contrast to Evi-depleted cells, WntD secretion was significantly impaired in *opm*-deficient cells (Fig 5C; supplementary Fig S5B,C online), indicating that *opm* is required

for Wg and WntD secretion. We confirmed this by comparing *opm*$^{9.3}$ and WntD early embryonic phenotypes. *opm*$^{9.3}$ mutants phenocopy the extended gradient of nuclear Dorsal of WntD mutants (Gordon *et al*, 2005) and the altered expression of the terminal gap genes *tailless* and *huckebein* (Fig 5D; supplementary Fig S5D online). We speculate that these defects in WntD signalling might also mask a Wg loss-of-function phenotype, resulting in a complex, partially overlapping phenotype in *opm* mutants.

As Opm mainly localized to the ER and ERGIC functioning in the ligand-secreting cell, we proposed that it might interact with Wnt proteins. Co-immunoprecipitation experiments in transfected cells supported our model that Opm interacts with Wg, although this interaction might be indirect, leading to an altered Wg localization and significantly smaller amounts of plasma membrane-bound Wg in *opm*-deficient cells (Fig 5E; supplementary Fig S5E online). We were not able to demonstrate a biochemical interaction of Opm with WntD, Por or Evi. Opm did not interact with the secreted JAK/STAT ligand Upd or with a control ER-resident transmembrane protein (Fig 5F; supplementary Fig S5F online). However, we cannot exclude a requirement of p24 proteins in transporting other signalling components, which remains to be investigated.

DISCUSSION

Different models have been proposed for cargo transport along the exocytic pathway. The bulk flow model proposes a passive transport of proteins, that is, unless retention mechanisms interfere. In contrast, other models require receptor-mediated export from the ER, where retention is the default unless cargo is associated with specific receptors that mediate sorting into transport vesicles.

The data presented here favour the latter view that Wnt proteins require receptors from the p24 protein family, a class of highly conserved transmembrane proteins, to exit from the ER. We identified several p24 proteins involved in Wnt transporting and show that Opm is required for secretion of the *Drosophila* Wnt proteins Wg and WntD. We also show that the human homologue of Opm is required for Wnt signalling. Similarly, at least two other p24 proteins, CHOp24 and p24-1, are required for Wg secretion, as knockdown of these p24 proteins phenocopies previously reported Evi loss-of-function phenotypes, such as cell-autonomous retention of Wg in secreting cells. Our results indicate that Wnt secretion relies on a specialized anterograde route with several p24 proteins functioning as highly conserved cargo receptors. The requirement of several *Drosophila* p24 proteins for Wnt secretion was also demonstrated in a recent report by Port *et al* (2011). p24 proteins are one of the first examples of Wnt growth factor-specific cargo receptor of the early secretory pathway. However, it remains to be investigated whether these p24 proteins function as complexes as previously proposed (Carney & Bowen, 2004).

Until now, the biological function of p24 proteins has been elusive, and only few studies have characterized p24 mutant phenotypes. Whereas in yeast and *Caenorhabditis elegans* p24 proteins have no effect on viability and morphology, two *Drosophila* p24 proteins have been shown to be maternal-effect lethal and are required for the activity of maternally expressed thickvein (Bartoszewski *et al*, 2004). Our data indicate that several p24 proteins might be involved in transporting Wnt proteins or might function synergistically in accordance with a proposed oligomeric behaviour (Carney & Bowen, 2004). Recent studies in yeast have implicated p24 proteins in the export of glycosyl phosphatidylinositol-anchored proteins, linking the cargo protein to the COPII coat (Castillon *et al*, 2011). Whether a similar or divergent mechanism underlies the export of Wnt proteins remains to be investigated. Although we could show a biochemical interaction of Opm and Wg, we have so far failed to detect a similar interaction with WntD. Therefore, we cannot at present exclude the possibility that the effect on the secretion of WntD might be indirect.

Although several p24 proteins seem to be affected by RNAi, only a single p24 protein seems to be altered in *opm*$^{9.3}$ mutants, possibly explaining the weak Wg loss-of-function phenotype. In addition, the observed phenotypes are milder than *evi* loss of function, indicative of an incomplete block of Wg secretion. We speculate that p24 proteins function partially redundantly, and that more p24 proteins contribute to Wg secretion in a tissue-specific manner, which would be consistent with the report by Port *et al* (2011). It is also likely that a regulatory compensation mechanism balances p24 levels as suggested previously (Boltz & Carney, 2008; Takida *et al*, 2008).

In summary, our data support a model in which Wnt proteins follow a specific route of secretion and require specialized transport components upstream of the Golgi network. Our data also indicate that temporally and spatially precisely coordinated Wnt activity can only be achieved by receptor-mediated recruitment of Wnt proteins into the secretory pathway. p24 proteins seem to have a crucial role by functioning as cargo receptors for Wnt proteins, and might serve as a model for specific receptor-mediated secretory routes of other growth factors.

METHODS

Cell culture. *Drosophila* S2R+, Kc_{167}, S2 hs-wg and S2 wg-FLAG cells were maintained in Schneider's medium supplemented with 10% FCS and 50 $\mu g^{-1}\mu l^{-1}$ penicillin/streptomycin. S2 hs-wg and S2 wg-FLAG cells were selected with 125 $\mu g^{-1}\mu l^{-1}$ hygromycin. Cells were transfected using Effectene (Qiagen). HEK293T cells were maintained in DMEM (Gibco) supplemented with 10% FCS and 50 $\mu g^{-1}\mu l^{-1}$ penicillin/streptomycin (Invitrogen). Cells were transfected with 50 ng DNA and 50 nM siRNA in 384-well plates using FuGene (Roche) and Dharmafect1 (Dharmacon), respectively.
RNAi screening. For the initial screen, 10^4 S2R+ cells were seeded on 250 ng dsRNA in 384-well plates. After 24 h, dTCF-Luc, pRP128-RL and pAc-wg were transfected. Cells were grown at 25 °C for 4 days to allow protein depletion. The screen was performed in duplicates. Data analysis was performed using Bioconductor/R and cellHTS.
RNAi experiments. dsRNAs were designed using GenomeRNAi (Horn *et al*, 2007). Each RNAi experiment was conducted at least three times in quadruplicate for each dsRNA (for primer sequences see supplementary Table S3 online). For the paracrine signalling assay, S2R+ cells treated with dsRNAs for 5 days and transfected with the reporter plasmids were mixed in a 1:3 ratio with S2 hs-wg cells that were activated by heat shock at 37 °C for 45 min 24 h before readout.

RNAi experiments in human cells were conducted as described in Bartscherer *et al* (2006), using the following siRNAs: β-catenin

(M-003482-00), hEvi (M-018728-01), TMED5 (M-007854-01) and non-targeting siRNA (siCon, D-001206-13-05; Dharmacon).

Secretion assays and immunoprecipitation. The Wg and WntD secretion assays were performed as described in Bartscherer *et al* (2006). Antibodies used were mouse-α-FLAG-HRP (Sigma), rabbit-α-V5 (Rockland), mouse-α-HA (Cell Signaling), α-Wg (DSHB), mouse-α-tubulin (Sigma), α-rabbit-HRP and α-mouse-HRP (TrueBlot ULTRA, eBiosciences). For immunoprecipitation, cells were processed as described in Belenkaya *et al* (2002).

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank T. Horn, C. Kalla, A. Ragab and K. Bartscherer for support and critical comments on the manuscript. We are particularly grateful to Z. Paroush and R. Helman for critical discussions and support with the analysis of the embryonic WntD phenotype. We thank A. Teleman, B. Thompson, B. Dixon, K. Basler, N. Perrimon, H. Bellen, R. Nusse and M. Mlodzik for providing reagents. Support by the DKFZ Light Microscopy Facility is gratefully acknowledged. This work was supported by a Marie-Curie Excellence grant from the European Commission and by the Deutsche Forschungsgemeinschaft (FOR1036).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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