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

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Wnt proteins are secreted, lipid-modified glycoproteins that control animal development and adult tissue homeostasis. Secretion of Wnt proteins is at least partly regulated by a dedicated machinery. Here, we report a genome-wide RNA interference screen for genes involved in the secretion of Wingless (Wg), a *Drosophila* Wnt. We identify three new genes required for Wg secretion. Of these, Emp24 and Eclair are required for proper export of Wg from the endoplasmic reticulum (ER). We propose that Emp24 and Eca act as specific cargo receptors for Wg to concentrate it in forming vesicles at sites of ER export.

Keywords: cargo receptor; p24; Wnt secretion
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INTRODUCTION

Wnt signalling is crucial for animal development and controls tissue homeostasis in the adult (Logan & Nusse, 2004). Wnt proteins can function as morphogens, being produced and secreted by a defined subset of cells and subsequently spreading in the tissue to induce target gene expression in receiving cells (Neumann & Cohen, 1996; Zecca *et al*, 1996).

It has recently become clear that Wnt signalling is already tightly controlled in ligand-producing cells by regulation of protein secretion (Port & Basler, 2010). For example, post-Golgi trafficking of the *Drosophila* Wnt protein Wingless (Wg) specifically requires the conserved transmembrane protein Wntless (Banziger *et al*, 2006; Bartscherer *et al*, 2006; Goodman *et al*, 2006; Port *et al*, 2008). Why Wg requires special help for Golgi exit and how Wntless provides it is unknown. Furthermore, it is not known whether Wg also requires the help of a specialized protein machinery to travel from other compartments of the secretory pathway, such as the endoplasmic reticulum (ER).

Here, we present a genome-wide RNA interference (RNAi) screen designed to detect genes that are required for secretion of Wg in cultured cells. After careful rescreening of candidates *in vivo*, we uncovered two genes—encoding the endosomal adaptor sorting nexin 3 and the transmembrane protein Éclair—that were required for normal Wg exocytosis. Sorting nexin 3 is part of an unconventional Retromer complex that controls Wntless levels in Wg-producing cells (Harterink *et al*, 2011). Éclair is a member of the p24 protein family and we found that another member of the same family, Emp24, is also involved in regulating Wg secretion. Our results indicate that Éclair and Emp24 are required for normal exit of Wg from the ER, probably by acting as cargo receptors.

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RESULTS AND DISCUSSION

A genome-wide screen for genes required for Wg secretion

To screen for genes that are required for the secretion of Wg, we generated a *Drosophila* S2R+ cell line that stably expresses a Wg–Renilla luciferase (WgRluc) fusion protein (Fig 1A). WgRluc is secreted into the cell supernatant and is competent to trigger Wg signalling (data not shown). In addition, our reporter cells express a secreted form of Firefly luciferase (sFluc). To identify genes that are required for the secretion of Wg, but not secretion in general or cell survival, we screened for genes that if knocked down led to a reduction of WgRluc activity in the medium, without affecting sFluc activity. We used RNAi against Wntless as a positive control. Knockdown of Wntless in our reporter cells caused a substantial reduction of WgRluc activity in the medium, but did not change sFluc activity (Fig 1B). By contrast, interfering with the expression of *Drosophila inhibitor of apoptosis 1* (*diap1*), a gene necessary for cell survival, caused a reduction in both WgRluc and sFluc levels. By using this assay we screened a state-of-the-art library of double-stranded RNAs (dsRNAs) targeting more than 14,000 *Drosophila* genes (Fig 1C). We assigned 387 genes as primary hits, based on the fact that their knockdown selectively reduced WgRluc activity in the supernatant of both duplicates (supplementary Table S1 online).

Rescreening of selected candidate genes *in vivo*

High-throughput screening produces a fraction of false-positive and false-negative results. Furthermore, RNAi technology has caveats such as off-target effects (Kulkarni *et al*, 2006). Therefore, it is crucial to carefully reevaluate results obtained from large-scale RNAi screens. We rescreened selected candidates

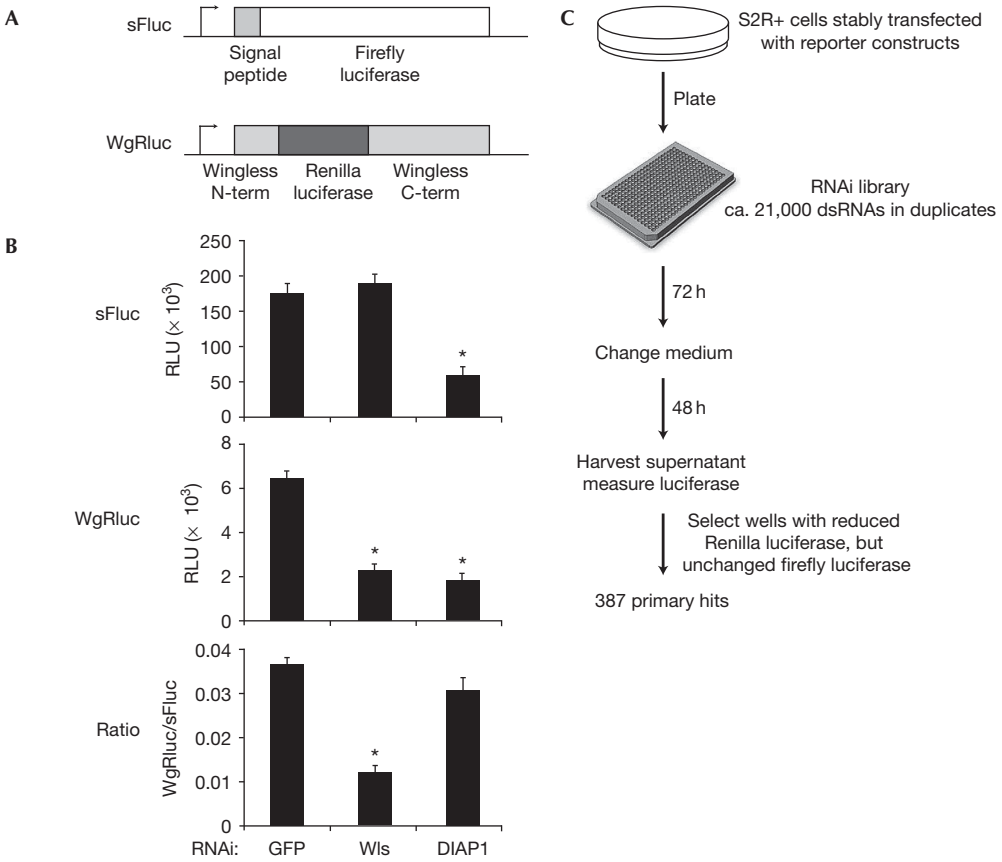


Fig 1 | A genome-wide RNAi screen for genes required for Wingless secretion. (**A**) Schematic diagram of the reporters used. The signal peptide of sFluc stems from influenza haemagglutinin. (**B**) Downregulation of Wntless reduces secretion of WgRluc, but not sFluc. Knockdown of anti-apoptotic *diap1* causes cell death and a reduction of both reporters in the media. Data represent means ± s.d. ($n = 4$), *t*-test $^*P < 0.01$ compared with GFP controls. (**C**) Summary of the screening protocol. DIAP1, Drosophila inhibitor of apoptosis 1; dsRNA, double-stranded RNA; GFP, green fluorescent protein; RNAi, RNA interference; RLU, relative luciferase units; sFluc, secreted form of Firefly luciferase; Wls, Wntless; WgRluc, Wingless–Renilla luciferase.

from our tissue culture screen in flies using a sensitized system in the eye, as well as a system in which endogenous Wg signalling in the wing is affected. Importantly, we used independent reagents to knockdown gene expression *in vivo*, reducing the likelihood that artefacts related to the previously used dsRNA, such as off-target effects, would be repeated in our rescreen. Also, we now screened in cells that naturally secrete Wg, increasing the physiological relevance of our results. To optimize the rescreening we excluded genes with well-documented functions unrelated to protein secretion from further analysis. A total of 115 genes were chosen for further analysis (supplementary Table S2 online). We screened for inhibition of Wg secretion in the *Drosophila* eye and wing (Fig 2A). Overexpression of Wg in the developing eye under the control of the *sevenless* (*sev*) promoter disturbs the normal arrangement of the ommatidial facets and gives rise to a 'rough'-eye phenotype (Brunner *et al*, 1997). This phenotype is suppressed when the secretion of the ectopic Wg protein is inhibited, as demonstrated by the effect of simultaneous knockdown of *wntless* by RNAi (Fig 2B). We expressed RNAi hairpin constructs targeting our candidate genes in the developing eye of *sev–wg*-expressing flies and screened for suppression of the rough-eye phenotype. Knockdown of 30 genes resulted in suppression of the *sev–wg* phenotype (Fig 2D; supplementary Table S2 online).

Next, we screened for potential Wg signalling components in the *Drosophila* wing. Inhibition of Wg signalling in the developing wing typically leads to the loss of wing margin tissue (Fig 2C), although this phenotype is not unique to defective Wg signalling. We expressed hairpin RNAi constructs in the wing imaginal disc using two Gal4 drivers. Whereas *nubbin–Gal4* (*nubGal4*) leads to moderate expression in the whole wing pouch, *hedgehog–Gal4 (hhGal4)* results in strong expression in only the posterior compartment of the developing wing. We identified three genes that cause loss of wing margin tissue when knocked down with *nubGal4* and eight genes when RNAi was induced by *hhGal4* (Fig 2D; supplementary Table S2 online). Only three genes showed phenotypes associated with reduced Wg secretion in more than one assay: *CG5060, sorting nexin 3 (snx3)* and *éclair* (*eca*). Unfortunately, the RNAi line used to target *CG5060* contains a stretch of 24 base pairs with perfect homology to the *Notch* gene. As reduced Notch signalling gives rise to wing margin defects, it is probably responsible for the notched phenotypes caused by the *CG5060* RNAi. We could validate the remaining two genes, *snx3* and *eca*, as new components of the Wg secretion machinery.

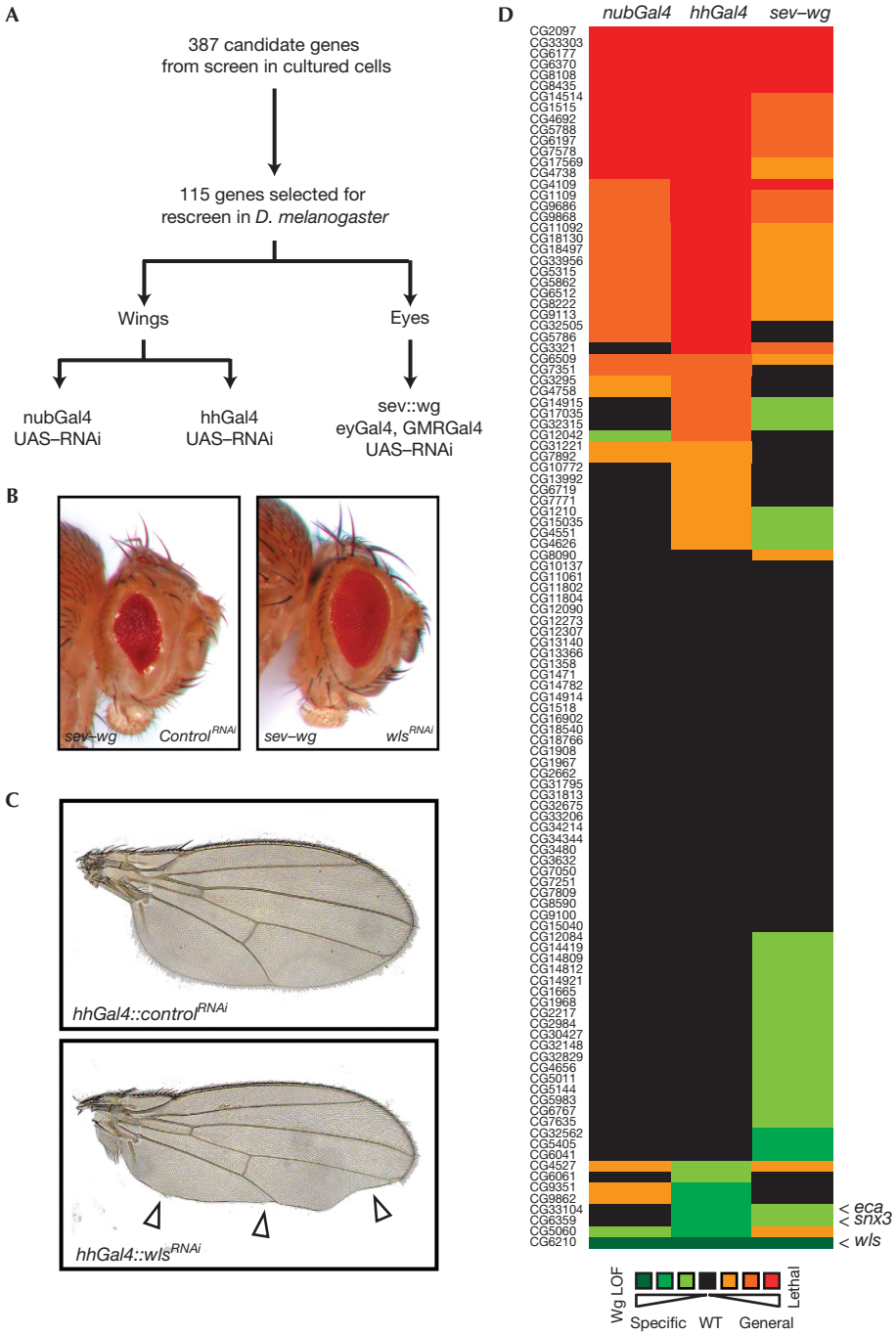


Fig 2 | Testing candidate genes for an effect on Wingless secretion *in vivo*. (**A**) Schematic illustration of the rescreening procedure. (**B**) Eyes of *sev–wg*-expressing flies coexpressing *UAS–lacZ RNAi* or *UAS-wls RNAi* induced by *GMR–Gal4* and *eyGal4*. Inhibition of Wg secretion by reducing Wntless levels leads to a suppression of the *sev–wg*-induced rough-eye phenotype. (**C**) Wings of flies expressing *UAS–lacZ RNAi* or *UAS–wls RNAi* induced by *hhGal4* in the posterior compartment of the developing wing. Strongly reduced Wg secretion caused by *wls* knockdown leads to loss of wing margin tissue (arrow heads). (**D**) Graphical summary of the results from the *in vivo* rescreen. For details, refer to supplementary Table S2 online. *eca*, *éclair*; *hhGa14*, *hedgehog–Gal4*; RNAi, RNA interference; nubGal4, nubbin–Gal4; *sev*, *sevenless*; *snx3*, *sorting nexin 3*; UAS, upstream activating sequence; LOF, loss of function; Wg, Wingless; *wls*, *wntless*; WT, wild type.

Snx3 is part of an unconventional retromer complex that regulates Wntless levels and thereby promotes Wg secretion (Harterink *et al*, 2011). Éclair is a member of the p24 protein family that is involved in ER-to-Golgi trafficking, and thus potentially represents the first component in this early step of Wnt secretion. We then focused on the role of p24 proteins in Wg secretion.

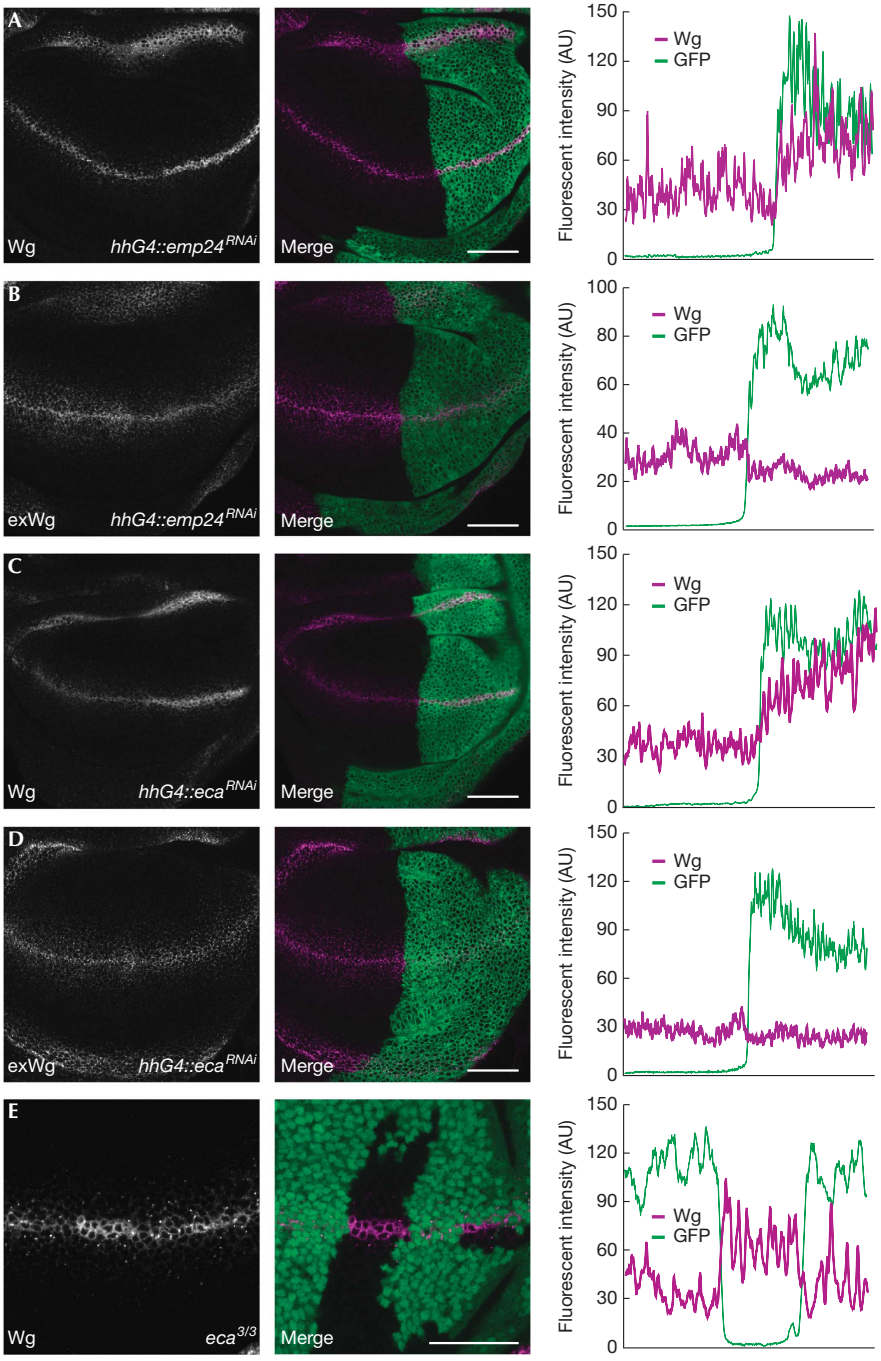


Fig 3 | Emp24 and Éclair regulate Wingless secretion. (**A–D**) Downregulation of either *emp24* (**A**,**B**) or *eca* (**C**,**D**) results in accumulation of Wg in producing cells. RNAi was expressed under the control of *hhGal4* in the posterior compartment, which is marked by expression of *UAS–CD8–GFP*. Total Wg is shown in (**A**,**C**), whereas exclusively extracellular Wg is stained in **B**,**D**. (**E**) Clones of cells homozygous mutant for Éclair were induced by mitotic recombination. Wg protein accumulates inside Wg-expressing cells, as shown by staining total Wg protein. Quantification of the fluorescent signal in cells along the dorsal–ventral boundary is shown in the right panel. Scale bars, 50 μm. AU, arbitrary units; *eca*, éclair; GFP, green fluorescent protein; *hhGa14*, *hedgehog–Gal4*; RNAi, RNA interference; UAS, upstream activating sequence; Wg, Wingless.

The p24 proteins Éclair and Emp24 regulate Wg secretion

Éclair belongs to the evolutionarily conserved family of p24 transmembrane proteins that function in the early secretory pathway. As previous work has suggested that p24 proteins might function as hetero-oligomers or dimers (Fullekrug *et al*, 1999; Marzioch *et al*, 1999; Jenne *et al*, 2002), we tested all *Drosophila* p24 genes for a function in Wg secretion. There are nine p24 family members in flies: *eclair*, *CG33105*, *emp24*, *CG9308*,

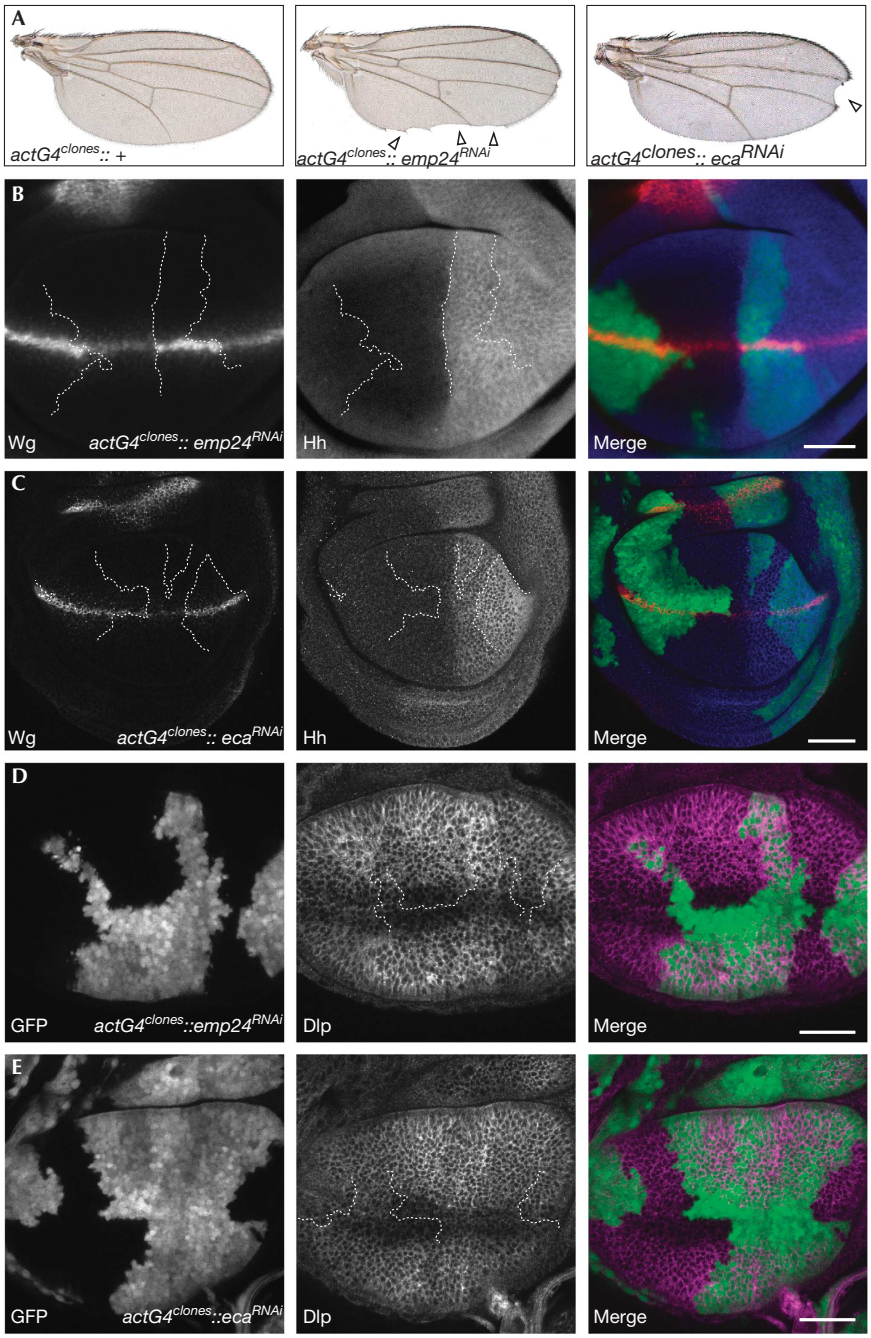


Fig 4 | Emp24 and Éclair are not required for general protein secretion in *Drosophila*. (**A**) Clones of cells expressing RNAi hairpins against *emp24* or *eca* under the control of *actin-Gal4* frequently lead to typical Wg loss-of-function phenotypes in adult wings (arrow heads). (**B**,**C**) In such clones, Wg accumulates in the *wg*-expression domain, whereas in Hh-expressing cells Hh does not accumulate (**D**,**E**). Clones induced by the same method do not accumulate Dlp along the dorsal–ventral boundary. However, slight Dlp accumulation is observed in *emp24RNAi* clones outside the Wg-expression domain. Clone boundaries are indicated by lines in **B**–**E**. Scale bars, 50 μm. Dlp, Dally-like protein; *eca*, *éclair*; GFP, green fluorescent protein; Hh, hedgehog; RNAi, RNA interference; Wg, Wingless.

baiser, *CG1967*, *CG9053*, *CG31787* and *logjam* (supplementary Fig S1 online). We were unable to detect transcripts from *CG33105*, *CG9308* and *CG31787* in wing imaginal discs by reverse transcription–PCR, suggesting that these genes are not expressed in this tissue (data not shown). This was supported by sequencing of total RNA from purified wing imaginal discs (C. Bauer & K. Basler, unpublished data). We used transgene-mediated RNAi to individually downregulate the expression of the six remaining p24 genes (supplementary Fig S2 online). Knockdown of *baiser*, *CG1967*, *CG9053* or *logjam* in the

developing wing or eye did not give rise to phenotypes indicative of reduced Wg secretion. However, downregulation of either *eclair* or *emp24* expression moderately suppressed the *sev–wg*-induced rough-eye phenotype and frequently led to loss of wing margin tissue (supplementary Fig S3 online), suggesting that these genes are required for Wg secretion. To confirm that *eclair* and *emp24*, but not the other p24 genes, regulate Wg exocytosis we knocked down the expression of each gene in the posterior compartment of wing imaginal discs and stained for endogenous Wg protein. Knockdown of *baiser*, *logjam*, *CG1967* and *CG9053* did not result in any alterations in Wg staining (supplementary Fig S3 online). By contrast, Wg staining was stronger in cells with downregulated *eclair* or *emp24* expression (Fig 3A,C). The increased levels of anti-Wg staining resulted from an accumulation of Wg inside the producing cells; staining exclusively extracellular Wg showed a reduction of Wg on the cell surface of $eclair^{RNAi}$ or $emp24^{RNAi}$ cells (Fig 3B,D). Elevated levels of Wg did not result from an increase in *wg* transcription, as expression of a *wg–lacZ* reporter was unchanged (supplementary Fig S4 online). Simultaneous knockdown of *eclair* and *emp24* did not increase the defect in Wg secretion (data not shown). To confirm the role of p24 proteins in Wg secretion independent from RNAi, we used the ethylmethane sulphonate-induced allele $eclair^{3R\text{-}45\text{-}33}$. We generated $eclair^{3R\text{-}45\text{-}33}/eclair^{3R\text{-}45\text{-}33}$ homozygous mutant clones in wing imaginal discs and stained for endogenous Wg protein. Mutant cells accumulated Wg protein, as observed with our RNAi lines (Fig 3E). We therefore conclude that the p24 proteins Éclair and Emp24 are required for proper Wg secretion.

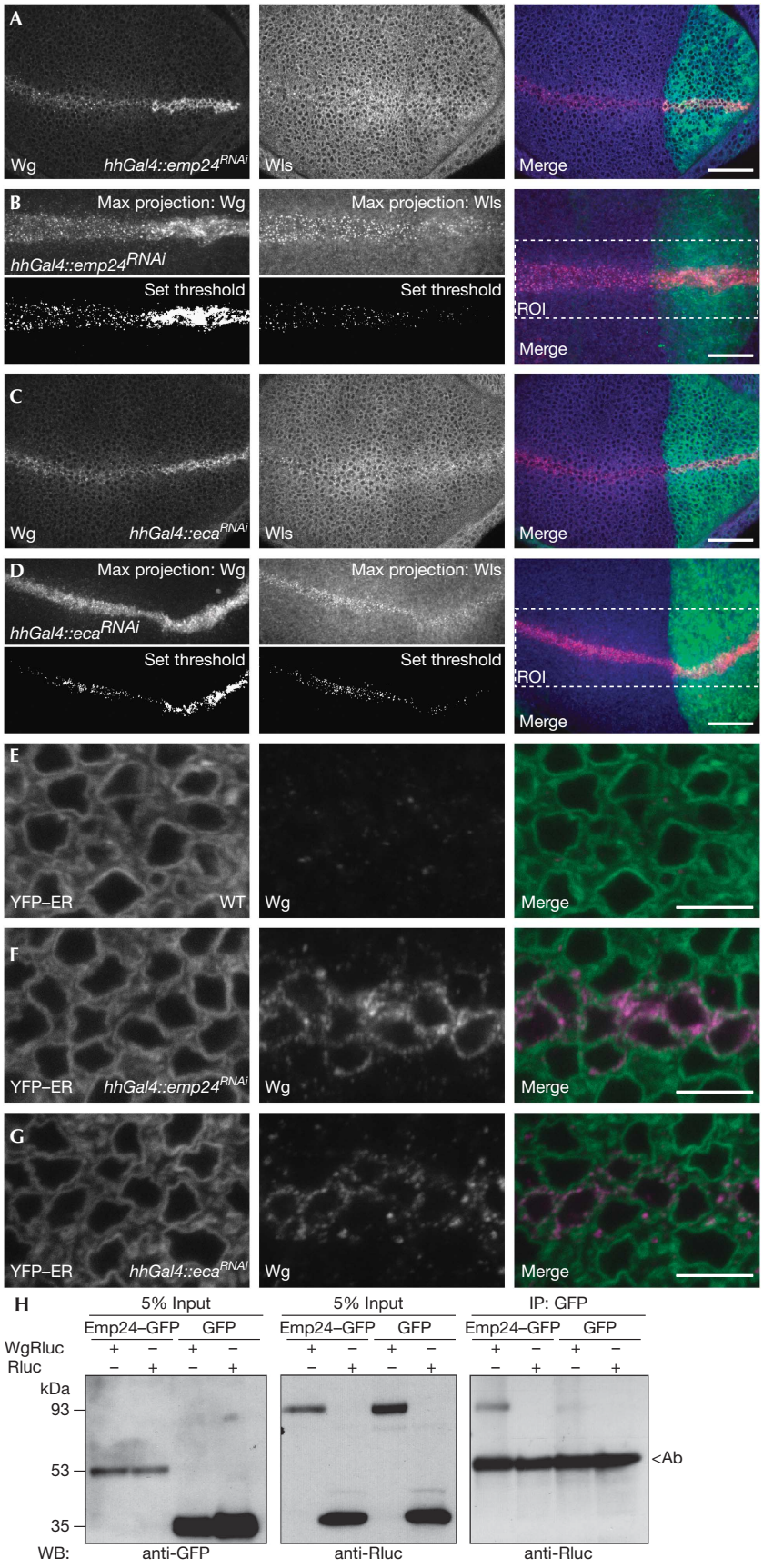
Éclair and Emp24 govern ER exit of Wg

How might Éclair and Emp24 regulate Wg secretion? p24 proteins have been shown to act as cargo receptors for ER-to-Golgi transport of glycosyl phosphatidylinositol (GPI)-anchored proteins, but have also been shown to be important for biogenesis of retrograde COPI (coat protein complex I) vesicles at the *cis*-Golgi (Muniz *et al*, 2000; Aguilera-Romero *et al*, 2008). Whereas inhibition of COPI vesicle biogenesis would lead to a block of protein transport, because essential factors that mediate ER exit become mislocalized to the Golgi, loss of cargo receptors will specifically affect transport of their target proteins. Therefore, reduced Wg secretion in the absence of Éclair and Emp24 could be due to three reasons: (1) impaired vesicle biogenesis results in an overall block of ER-to-Golgi transport; (2) Éclair and Emp24 regulate transport of proteins essential for Wg secretion or Wg-binding proteins; (3) Éclair and Emp24 act as cargo receptors for Wg itself.

To address the first possibility, we analysed whether Éclair and Emp24 are also required for the secretion of two other morphogens that are expressed in wing imaginal discs: Hedgehog and Decapentaplegic (Dpp). We expressed RNAi hairpin constructs targeting *eclair* or *emp24* in clones of cells using *actGal4*. These clones accumulate Wg and frequently give rise to Wg loss-of-function phenotypes in the adult wing (Fig 4A–C). By contrast, endogenous Hedgehog protein did not accumulate in RNAi-expressing clones, suggesting that it is insensitive to reduced levels of Éclair and Emp24 (Fig 4B,C). We then analysed whether Éclair or Emp24 are involved in Dpp signalling in wing imaginal discs, as has been suggested in *Drosophila* embryos (Bartoszewski *et al*, 2004). As antibodies that detect endogenous Dpp do not exist, we monitored the expression of two sensitive downstream events of Dpp signalling, phosphorylation of Mad protein and expression of the target gene *brinker*. Knockdown of *eclair* or *emp24* had no effect on phosphorylation of Mad or expression of *brinker*, suggesting that Dpp signalling is not detectably altered in the wing imaginal discs (supplementary Fig S5 online). Together, these results show that neither Éclair or Emp24 are required for general protein secretion.

The second hypothesis predicts that Éclair and Emp24 affect Wg secretion indirectly by regulating a Wg-interacting protein. The acetyltransferase Porcupine is involved in Wg secretion by regulating the post-translational modification of Wg. However, Porcupine is thought to reside permanently in the ER and is therefore unlikely to be affected by ER-to-Golgi trafficking defects. By contrast, the Wg-interacting proteins Dally, Dally-like protein (Dlp) and Wntless transverse the secretory pathway, and their ER-to-Golgi transport could be regulated by Éclair and Emp24. In this case, in the absence of Éclair or Emp24 Dally, Dlp or Wntless might accumulate in the ER, where they could bind to and sequester Wg. Dally and Dlp are GPI-anchored proteins, making them likely candidates for an interaction with p24 s. We focused on Dlp, because an existing antibody allows the detection of endogenous protein in wing imaginal discs. Dlp protein levels are low in Wg-expressing cells and increase further away from the dorsal–ventral boundary. We reduced the levels of Éclair or Emp24 by using RNAi in clones of cells and stained for endogenous Dlp. Knockdown of Éclair or Emp24 did not lead to an increase of Dlp levels in Wg-expressing cells (Fig 4D,E). Another protein that interacts with Wg in the secretory pathway is Wntless (Banziger *et al*, 2006). We asked whether a reduction of Éclair or Emp24 sequesters Wntless in the ER. Again, we did not detect any increase of endogenous Wntless in cells with knocked down *eclair* or *emp24* (Fig 5A,C). Instead, the punctuate staining of Wntless in Wg-producing cells was slightly reduced in RNAi-expressing cells (Fig 5B,D). We have previously shown that these punctuae represent an accumulation of Wntless in the Golgi that is induced by Wg (Port *et al*, 2008). Lowering Wg levels in secreting cells reduces Wntless levels in the Golgi, whereas providing more Wg leads to Golgi accumulation of Wntless. The fact that loss of Éclair or Emp24 causes a reduction rather than increase in Wntless levels in the Golgi indicates that less Wg reaches this compartment. Hence, the overall higher level of Wg in these cells probably reflects an accumulation in the ER. Consistent with this, we found that in cells expressing RNAi targeting *eclair* or *emp24*, Wg colocalizes extensively with the ER marker YFP–KDEL (Fig 5E–G).

Together, these results argue for the third model, in which Éclair and Emp24 act as cargo receptors for Wg protein. Such a mode of action requires a physical interaction between the cargo and its receptor, and we therefore tested whether Wg could be coimmunoprecipitated with either Éclair or Emp24. Cargo–cargo receptor interactions are typically transient and have low affinity, making experimental validation of physical binding challenging. We used an established crosslinking protocol in cultured cells that was previously used to show interactions between p24 proteins and their protein cargo (Muniz *et al*, 2000). We failed to detect any binding of Éclair to Wg (data not shown). By contrast, we observed a robust and specific interaction between Emp24 and Wg (Fig 5H). The highly conserved mammalian orthologues of



◀ **Fig 5** | Emp24 and Éclair directly control endoplasmic reticulum export of Wingless. Knockdown of *emp24* (**A,B**) or *eca* (**C,D**) does not cause any accumulation of Wntless. Instead, Wg-induced accumulation of Wntless along the dorsal–ventral boundary is reduced (**B,D**). A single confocal section is shown in **A** and **C**. Maximum intensity projections are shown in **B** and **D** to visualize spots of intense Wntless staining, and thresholded views of the same images are provided to compare the areas of most intense staining. Cells expressing RNAi hairpin constructs are marked in green in the merge images. (**E–G**) Wg accumulates in the ER in the absence of Emp24 or Éclair. RNAi constructs were expressed under the control of *hhGal4* together with a *UAS–YFP–ER* marker. Samples were stained in parallel and imaged with identical settings, and single confocal views of similar regions of the wing disc are shown. (**H**) WgRluc specifically coimmunoprecipitates with Emp24–GFP. The indicated proteins were expressed in S2R+ cells, crosslinked and immunoprecipitated with specific antibodies. Scale bars, 50 μm in **A–D** and 5 μm in **E–G**. Ab, antibody; ER, endoplasmic reticulum; *eca*, *éclair*; GFP, green fluorescent protein; *hhGa14*, *hedgehog–Gal4*; IP, immunoprecipitation; RNAi, RNA interference; Rluc, Renilla luciferase; ROI, region of interest; UAS, upstream activating sequence; WB, Western blot; Wg, Wingless; WgRluc, Wg–Renilla luciferase; Wls, Wntless; WT, wild type; YFP, yellow fluorescent protein.

Éclair and Emp24 (TMED4/p25 71% identity, 84% similarity; TMED2/p24 63% identity, 80% similarity) have been demonstrated in several instances to form heteromeric complexes (Fullekrug *et al*, 1999; Marzioch *et al*, 1999; Jenne *et al*, 2002). Therefore, it is reasonable to propose that Éclair and Emp24 act together in a complex and Emp24 provides the interaction interface with Wg. For technical reasons, we probably did not detect the entire complex in our pull-downs: epitope-tagged Éclair protein was poorly expressed in S2 cells.

Together, our data support a model in which Éclair and Emp24 form a complex that recruits Wg into forming COPII vesicles at ER exit sites. Without this active recruitment Wg accumulates in the ER and Wg secretion is impeded. However, a fraction of Wg escapes the ER even in the absence of Éclair and Emp24, probably by bulk flow facilitated by the elevated levels of Wg, or by other cargo receptors that act in a partly redundant fashion. Until now, p24 proteins have been shown to act as cargo receptors for GPI-linked proteins in yeast and mammalian cells (Takida *et al*, 2008; Castillon *et al*, 2009). Our work shows that the Wnt protein Wg also depends on p24 proteins for ER export. A recent study in mammalian cells has shown that p24s and GPI-linked proteins partition into raft-like membrane microdomains in the ER, and clustering in these domains was found to be essential for their interaction (Bonnon *et al*, 2010). Interestingly, Wnt proteins are post-translationally modified by two different lipid adducts (Willert *et al*, 2003; Takada *et al*, 2006), and thereby become associated with detergent-resistant membrane microdomains (Zhai *et al*, 2004). Lipid modification of Wnts is important for protein secretion, although the details remain controversial (Port & Basler, 2010). It is tempting to speculate that lipid-modified Wnts and GPI-anchored proteins might be targeted to the same membrane microdomains. Stable association with these domains might depend on different members of the p24 family, which also provide the cytoplasmic signal to recruit coat proteins for vesicle formation (Dominguez *et al*, 1998). It will be important to untangle the exact role of the post-translational modification of Wnts for ER export, as well as other steps along the secretion route.

METHODS

Genome-wide RNAi screen. The WgRluc construct was cloned by inserting the Renilla luciferase coding sequence between codons R32 and S36 of the Wg coding sequence (Zecca *et al*, 1996). S2R+ cells constitutively expressing secreted WgRluc and sFluc were produced by cotransfection of the corresponding plasmids and selection with blasticidine. The screen was performed at the *Drosophila* RNAi Screening Center (Harvard Medical School). In brief, 20 μl of cells (~15,000 cells) in serum-free Schneider's medium were seeded in 384-well plates containing 0.1 μg dsRNA and incubated for 1 h. Medium was supplemented with 20 μl Schneider's medium containing 20% fetal calf serum and incubated for 3 days at 25 °C. The plates were then centrifuged and the medium was aspirated and replaced by Schneider's medium containing 10% fetal calf serum. After incubation for 2 days further, plates were centrifuged and 10 μl medium was transferred to new plates. Activity of WgRluc and sFluc in the cell supernatant was assayed by using Dual-Glow luciferase substrate (Promega) and a plate reader (Analyst GT; Molecular Devices).

***Drosophila* stocks, *in vivo* RNAi and immunohistochemistry.** Fly lines expressing RNAi hairpins were obtained from the Vienna *Drosophila* RNAi Center. To target *emp24* and *eclair* we used lines 100274 and 101388, but lines 7038 and 49749 showed similar phenotypes. RNAi lines were crossed to the following fly strains: *w, UAS–CD8–GFP;;hhGal4/Tm6b* and *w; nubGal4* and w; eyGal4, GMRGal4; *sev–wg* and *w, hsp–Flp, UAS–CD8–GFP; sp/cyo; act>stop>Gal4.* For ER colocalization *hhGal4>UAS–YFP–ER* flies (from S. Eaton, Dresden) were crossed to the respective RNAi strains or wild-type controls. Larva were collected at the late third-instar stage and dissected in PBS. Wing imaginal discs were stained using standard procedures (Port *et al*, 2008). Images were collected on a Zeiss LSM 710 confocal microscope using the sequential scanning mode. Images were processed using ImageJ and Adobe Photoshop. Quantification of fluorescent intensities was performed using the Plot profile function of ImageJ.

Crosslinking and coimmunoprecipitation. *Drosophila* S2R+ cells were transfected with Fugene HD (Roche) according to the manufacturer's instructions. After 48 h, cells were washed twice with PBS and then incubated with 1 mM dithiobis(succinimidyl-propionate) (Pierce) for 20 min at 20 °C. The crosslinking reaction was quenched by addition of glycine (50 mM final, 5 min, 20 °C). Cells were lysed on ice for 30 min in 150 mM NaCl, 50 mM Tris–HCl (pH 8.0), 1% NP-40, 0.5% deoxycholic acid, protease inhibitor cocktail (Complete Mini, Roche). Lysate was incubated with green fluorescent protein antibody-conjugated protein-G sepharose beads. Beads were washed three times with lysis buffer and then immunoprecipitates were eluted by boiling in reducing SDS loading buffer. Samples were separated by SDS–PAGE, transferred onto nitrocellulose membranes and probed with specific antibodies.

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

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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