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Drosophila p24 and Sec22 regulate Wingless trafficking in the early secretory pathway

Xue Li [a, b, 1], Yihui Wu [a, 1], Chenghao Shen [c], Tatyana Y. Belenkaya [d], Lorraine Ray [d], Xinhua Lin [a, d, *]

[a] State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] School of Optometry and Ophthalmology and Eye Hospital, Wenzhou Medical University, Wenzhou 325027, China
[d] Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA

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ABSTRACT

The Wnt signaling pathway is crucial for development and disease. The regulation of Wnt protein trafficking is one of the pivotal issues in the Wnt research field. Here we performed a genetic screen in *Drosophila melanogaster* for genes involved in Wingless/Wnt secretion, and identified the p24 protein family members Baiser, CHOp24, Eclair and a v-SNARE protein Sec22, which are involved in the early secretory pathway of Wingless/Wnt. We provided genetic evidence demonstrating that loss of p24 proteins or Sec22 impedes Wingless (Wg) secretion in *Drosophila* wing imaginal discs. We found that Baiser cannot replace other p24 proteins (CHOp24 or Eclair) in escorting Wg, and only Baiser and CHOp24 interact with Wg. Moreover, we showed that the v-SNARE protein Sec22 and Wg are packaged together with p24 proteins. Taken together, our data provide important insights into the early secretory pathway of Wg/Wnt.

1. Introduction

The Wnt signaling pathway is involved in many biological processes during development and in disease [1–3]. Wnt proteins are essential morphogen and secreted signal molecules that ignite the Wnt signaling pathway and induce downstream genes expression in the receiving cells [4,5]. Thus, over the past decades, great efforts have been made to clarify the synthesis, sorting, processing, and secretion of Wnt proteins in the Wnt-producing cells.

In the ER (endoplasmic reticulum), newly synthesized Wnt proteins are lipid modified by the acyltransferase Porcupine [6–8]. Porcupine catalyzes palmitoylation at the conserved cysteine and serine residues of Wnt proteins, which is essential for interactions of Wnt proteins with Wntless at the Golgi [9,10]. Following palmitoylation the multipass transmembrane Wntless escorts the Wnt protein for post-Golgi trafficking to the plasma membrane for secretion [11–15].

One regulator of Wnt secretion is the p24 family, which sorts Wnt proteins from the ER to the Golgi. Buechling and Port first identified several p24 protein family members, such as CHOp24, Eclair, Opm and p24-1, involved in Wingless/Wnt secretion [16,17]. However, there are four sub-families of p24 proteins, and the molecular mechanisms governing sorting of Wg/Wnt by p24 proteins is still unclear.

The p24 family proteins are highly conserved Type-I transmembrane proteins of approximately 24 kDa, and can be subdivided into four subfamilies including α, β, γ and δ [18]. The p24 family consists of ten members in mammals [19,20], eight in yeast [21–23] and nine in *Drosophila* [24–27]. Investigation of the p24 protein family uncovered its role in regulating the bi-directional transportation between the ER and the Golgi [28–30]. In the early secretory pathway, p24 proteins form a complex which functions as cargo selector. The complex recruits the correct subsets of trafficking machinery, packages them to escort cargoes and then cycles continuously between these compartments.

* Corresponding author. State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China. Fax: +86 10 64807970.
E-mail addresses: xinhua.lin@ioz.ac.cn, xinhua.lin@cchmc.org (X. Lin).
[1] These two authors contributed equally to this work.

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Early studies in yeast have revealed that yeast p24 proteins form functionally redundant αβγδ complexes [31]. In the study of p24 proteins escorting Wg/Wnt, Buechling found that several p24 proteins may contribute in a partially redundant manner [16]. However, Port observed a fraction of Wg escapes from the ER even in the absence of two p24 proteins, providing a hint that there might be other p24 proteins that function as Wg selectors [17].

In the early secretory pathway, cargoes are packaged by cargo selectors and transported by vesicle budding, docking, and fusion. SNARE (soluble NSF attachment protein receptor) proteins are highly conserved proteins that trigger a vesicle fusion event [32,33], and can be divided into two categories: vesicle membrane localized v-SNAREs and target compartment localized t-SNAREs. Since the Wnt secretory pathway is known to be tightly controlled by trafficking machinery, we performed genetic screen in *Drosophila melanogaster* to identify new genes involved in the Wg/Wnt secretory process. We found another p24δ protein Baiser, which interacts with Wg and co-localizes with Wg and the v-SNARE protein, Sec22.

We found that over-expression of Baiser cannot rescue defects caused by depleting other p24 proteins. We present both genetic and in vitro evidence that functional diversity of the p24 protein family members is essential to sort and process Wg/Wnt proteins. Furthermore, the ER-enriched v-SNARE Sec22 [34,35] is packaged together with Wg and p24.

2. Materials and methods

2.1. Drosophila stocks

The RNAi line, *UAS-Sec22^{RNAi}* (100766), was from the Vienna Drosophila Resource Center (http://stockcenter.vdrc.at/control/main). The following RNAi lines were generated in our laboratory: *UAS-Baiser^{RNAi}*, *UAS-CHOp24^{RNAi}*, *UAS-Eclair^{RNAi}*. Tool strains were described in Flybase including *enGal4* and *wg-lacZ*.

shRNA against Baiser, CHOp24 and Eclair were designed from DSIR. The following primers were annealed in the annealing buffer at 95 °C for 5 min, and then cloned into pWALIUM20 with NheI and EcoRI sites to generate the shRNA constructs. Primers are:

Baiser^{RNAi} top, 5′-ctagcagtGATCATCGACTACATTGCACGtagttatattcaagcataCGTGCAATGTAGTCGATGATCgcg -3′

Baiser^{RNAi} bottom, 5′-aattcgcGATCATCGACTACATTGCACGtatgcttgaatataactaCGTGCAATGTAGTCGATGATCactg -3′

CHOp24^{RNAi} top, 5′-ctagcagtGACCAGTGTCAAGCACGAACAtagttatattcaagcataTGTTCGTGCTTGACACTGGTCgcg -3′

CHOp24^{RNAi} bottom, 5′-aattcgcGACCAGTGTCAAGCACGAACAtatgcttgaatataactaTGTTCGTGCTTGACACTGGTCactg -3′

Eclair^{RNAi} top, 5′-ctagcagtGGCAGATGCGTCATCTCAAGAtagttatattcaagcataTCTTGAGATGACGCATCTGCCgcg -3′

Eclair^{RNAi} bottom, 5′-aattcgcGGCAGATGCGTCATCTCAAGAtatgcttgaatataactaTCTTGAGATGACGCATCTGCCactg -3′

2.2. Generation of Baiser mutant and Sec22 null allele

The Baiser insertion mutant (32614) was obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu), and recombined with *w*;; *FRT^{82B}/Tm6b* to generate *w*;;*FRT^{82B}Baiser/TM6b* (Fig. 2A). Homozygous mutant clones were generated by the FLP-FRT method [36]. Flies were crossed with *ywhsp-Flp*;; *FRT^{82B}ubi-GFP/Tm6b* and at 40 h post egg laying, the F1 progeny were heat shocked for 90 min at 37 °C.

Another P-element insertion line, (14846), *y^{1} P{EPgy2}EY00427 w^{67c23}*, residing upstream of the *Sec22* gene was obtained from the Bloomington Drosophila Stock Center. We obtained two deletions that are homozygous lethal from imprecise excision of a P-element insertion (Fig. 3A). The mutant were recombined with *w, FRT^{101}/Y* to generate *w, Sec22-FRT^{101}/FM7*. Homozygous mutant clones were generated by the FLP-FRT method [36]. Flies were crossed with *hs-Flp, ubi-GFP FRT^{101}*, and at 48 h post egg laying, the F1 progeny were heat shocked for 90 min at 37 °C.

2.3. Drosophila immunostaining and antibodies

Fixation and immunostaining were performed following standard protocols. Primary antibodies used in this study are as follows: mouse anti-Wg (1:4; DSHB), chicken anti-β-gal (1:1000; Abcam), mouse anti-engrailed (DSHB), rat anti-Ci (1:2; DSHB), mouse anti-E-cadherin (DSHB), rabbit anti-Hh (1:200; [37]), mouse anti-Patched (DSHB), rabbit anti-Spalt (1:200; made in our laboratory according to [38]), mouse anti-V5 (1:500; Invitrogen) and rat anti-HA (1:1000; Roche).

The primary antibodies were detected by fluorescence using Alexa Fluor 488-, Cy3-and Cy5-conjugated secondary antibodies from Jackson Immuno Research Laboratories, Inc.

The Confocal fluorescence images were collected using the Zeiss LSM 780 Laser Scanning Confocal Microscope (Carl Zeiss).

3. Results

3.1. Identification of Wingless (Wg) secretion regulators

We performed a genetic RNAi screen using different tissue-specific Gal4 drivers in *Drosophila* wing discs (Fig. 1A–C). Using *enGal4* to induce RNAi expression to deplete *Baiser (CG11785), CHOp24 (CG3564)*, *Eclair (CG33104)*, or *Sec22 (CG7359)* in the posterior compartment of the wing disc, we found that endogenous Wg accumulated significantly in the Wg-producing cells (Fig. 1F–I) and the adult wing blade presented slight wing margin defects in the posterior compartment (Fig. S1F–I). However, *wg* transcription, as determined by *wg-lacZ*, was not altered (Fig.S1A–D), indicating that Wg protein trafficking is likely to be affected in the absence of these genes.

Baiser, CHOp24 and Eclair belong to different p24 protein subfamilies. The p24β member CHOp24, p24α member Eclair, and p24γ member Opm and p24-1 have already been proven to escort the Wg protein from the ER to the Golgi by Buechling and Port in 2011. However, we found Baiser, the single p24δ member in *Drosophila*, is another p24 protein involved in Wg secretion. Thus we wanted to uncover the molecular mechanism of these p24 members escorting Wg: are they playing the same role or acting partially redundantly? In addition, we observed Sec22, an ER-enriched v-SNARE protein involved in Wg secretion. Therefore, we further examined the mechanism(s) by which the protein machinery regulates the early secretory pathway of Wg/Wnt.

3.2. Baiser is a cargo selector of Wg protein

To further confirm the above results, we obtained a *Baiser* mutant, with a P-element inserted in the 5′UTR of *Baiser* (Fig. 2A). Over 90% of the homozygotes die before eclosion and the living escapers present a reduced Baiser expression to 25% of wild-type flies (Fig. 2A), smaller wing blade, and slight wing margin defects (Fig. 2B). Then we generated Baiser mutant clones by the FLP–FRT method [36] and detected significant accumulation of Wg proteins in the Wg-producing cells bearing a Baiser homozygous mutation (Fig. 2C), which is consistent with our aforementioned RNAi screen results.

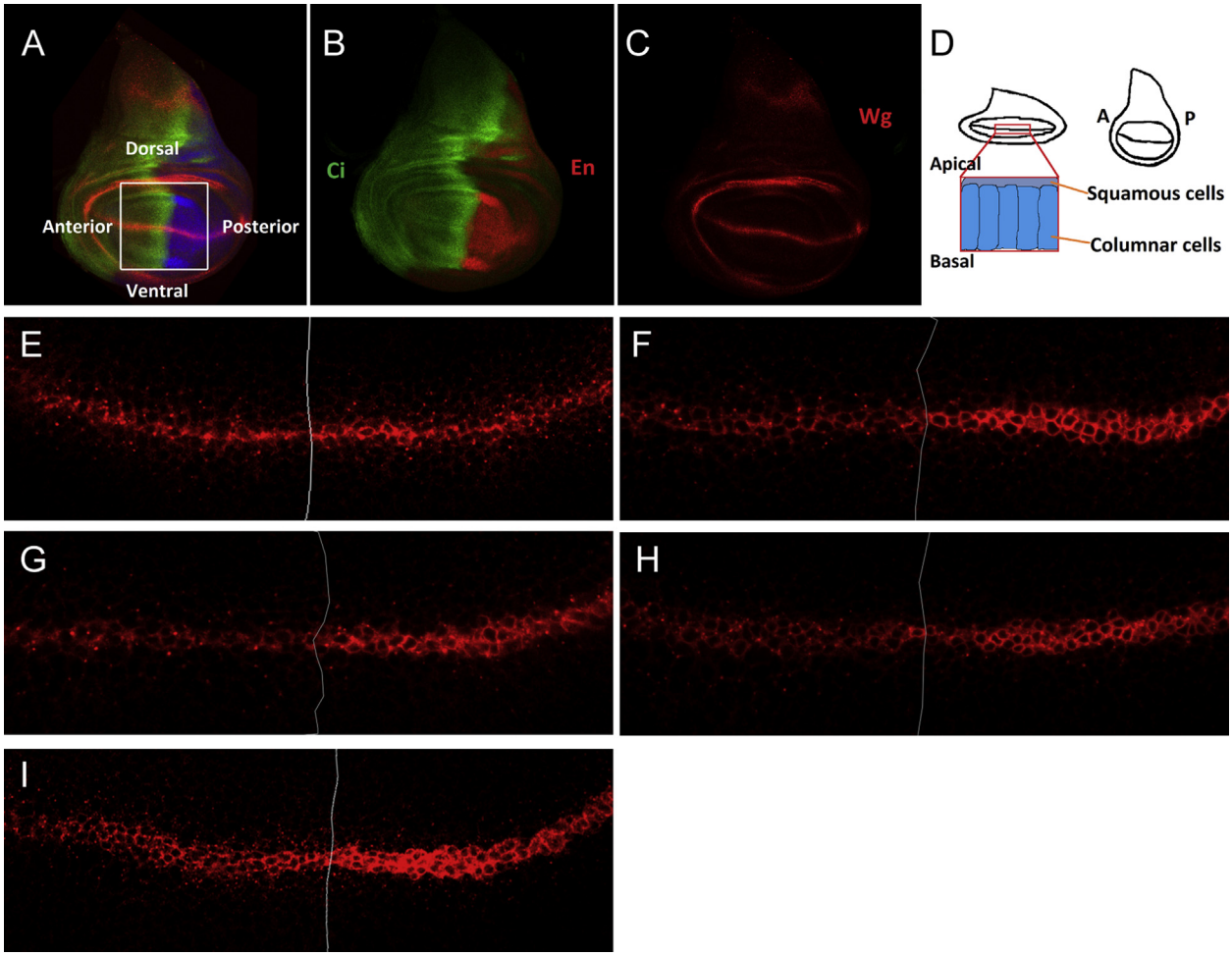


Fig. 1. RNAi screen identify Wingless secretion regulators. Schematic illustration of the *Drosophila* wing imaginal disc is shown in (A). *ci* (*cubitusinterruptus*, Green) and *en* (*engrailed*, Red) expression pattern in *Drosophila* wing disc are shown in (B). *Wingless* expression pattern is shown in (C) and a longitudinal section of Wg-producing cells from a Confocal Z-stack of Wing disc is shown in (D). Wild-type Wingless antibody staining is shown in (E). (F–I) Expression of *UAS-Baiser*^{RNAi}, *UAS-CHOp24*^{RNAi}, *UAS-Eclair*^{RNAi} and *UAS-Sec22*^{RNAi} were induced by *enGal4*, Wg accumulates in the posterior Wg-producing cells of wing disc. Wing discs are oriented anterior left, dorsal up.

3.3. p24 protein family members are functionally diverse

To further understand the molecular mechanism in which the p24 proteins escort Wg, we transfected *Drosophila* S2 cells with Eclair, Baiser and Wg, and found that the two p24 proteins co-localize with the Wg protein precisely (Fig. 2D). Moreover, upon immunoprecipitation of the Baiser-V5 protein from transfected cells, Wg was detected by western blotting in the immunoprecipitate, indicating that there is an interaction between Baiser and Wg (Fig. 2E), and that Baiser is a cargo selector of Wg.

Surprisingly, we found that both Baiser and CHOp24 interact with Wg but Eclair does not (Fig. 2E), suggesting that these p24 proteins are functionally diverse. Furthermore, over-expression of Baiser-V5 cannot rescue the Wg secretion defect caused by depleting CHOp24 or Eclair (Fig. 2F–H), arguing that these p24 members are playing different roles in the process of escorting Wg, and cannot be replaced by each other.

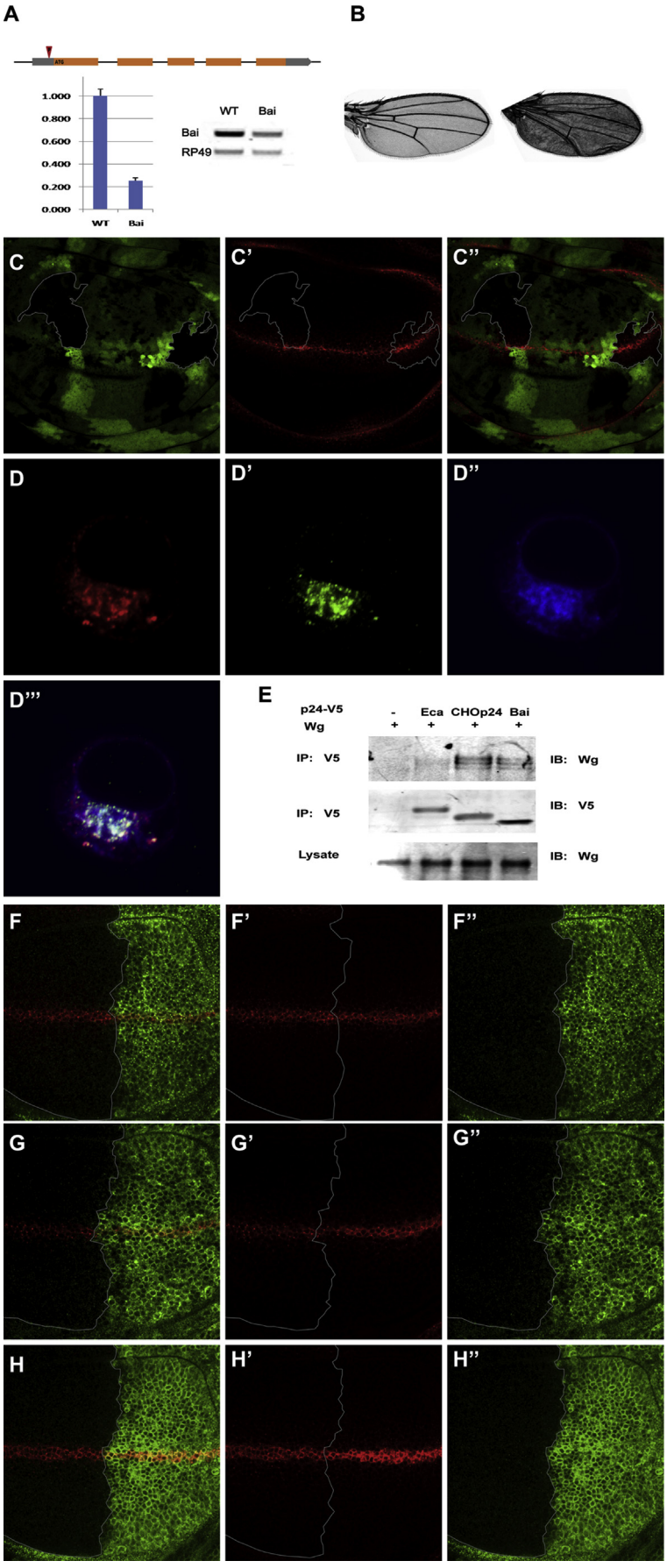
Thus in the process of escorting the Wg protein for anterograde transportation from the ER to the Golgi, the p24 proteins interact with Wg and get the cargo packaged. Several p24 proteins, including Baiser, CHOp24, Eclair, Opm and p24-1 [16,17], are involved in this process. These p24 family members are functionally diverse and cannot be replaced by each other, probably function in a partially redundant manner and/or by forming diverse complexes [31].

3.4. Sec22 is involved in Wg secretion

Upon our screen, we identify an ER-enriched v-SNARE protein Sec22 involved in Wg trafficking, and thus we generated two Sec22 mutants (Fig. 3A). Consistent with the RNAi results, Wg protein accumulates significantly in the Sec22 homozygous mutant clones (Fig. 3C). We fed *Drosophila* S2 cells with dsRNA of Sec22 and examined the Wg protein level in the supernatant and lysate. We observed a significant reduction of the secreted Wg in the supernatant and an increased level of Wg in the cell lysate (Fig. 3B), indicating that depletion of Sec22 impedes Wg secretion.

3.5. The v-SNARE Sec22 is packaged together with Wg and p24

To further examine the molecular mechanism by which Sec22 regulates Wg trafficking, we transfected *Drosophila* S2 cells with Baiser, Sec22, and Wg, and found that Sec22 co-localized with Baiser and Wg precisely, suggesting that Wg and Sec22 are packaged together with Baiser. Thus, during the early secretory phase of Wg, the v-SNARE Sec22 is packaged together with p24 proteins to escort Wg for anterograde transportation. These data demonstrate that the molecular machinery involved in Wg trafficking are diverse.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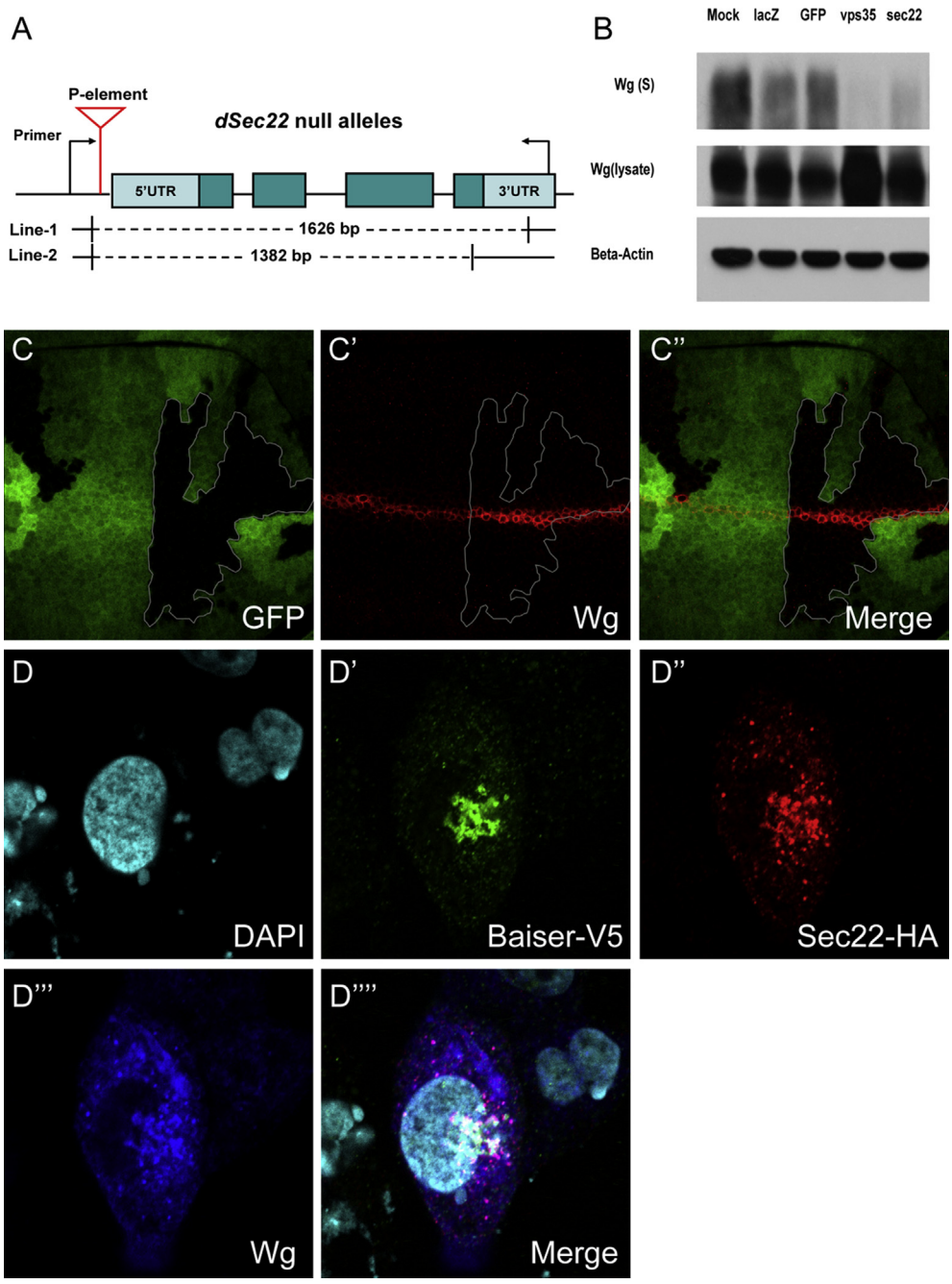

Fig. 3. Sec22 regulates Wg trafficking and is packaged together with Wg and p24. Two mutant alleles of *Sec22* were generated through P-element-mediated imprecise excision (A). *Drosophila* S2 cell was fed with dsRNA of *Sec22*, *Vps35*, *GFP* and *lacZ*. Wg protein in the supernatant (S) and in the cell lysate (lysate) were detected (B). Wg accumulates (C′) in the Wg-producing cells bearing *Sec22* homozygous mutant clones marked by the absence of GFP (C). Baiser-V5 (D′), Sec22-HA (D″) and Wg (D‴) co-localize precisely (D⁗) and DAPI is shown in (D).

4. Discussion

4.1. The Wg trafficking regulators

Wingless (Wg)/Wnt signaling plays crucial roles in development, tissue homeostasis, and diseases, thus the appropriate regulation of Wnt secretion is essential in Wnt signaling. Our genetic screening among genes of the secretory pathway organelles, identified Baiser, CHOp24, Eclair and Sec22 as genes involved in the regulation of Wnt secretion. We conducted further experiments to clarify the molecular mechanism(s) by which these regulators escort Wg protein.

Fig. 2. Baiser is a direct cargo selector that escorts Wg. The *Baiser* insertion mutant detail and the real-time quantitative PCR results of *Baiser* homozygotes are shown in (A). Wing blade of *wt* (left) and *Baiser* (right) homozygous escaper (B). Wg accumulates (C′) in the Wg-producing cells bearing *Baiser* homozygous mutant clones marked by the absence of GFP (C). Wg (D″) co-localizes with Baiser (D′) and Eclair (D) in S2 cells. Wg can be immunoprecipitated with Baiser, CHOp24 but not Eclair (E). Over-expression of *Baiser-V5* driven by *enGal4* can rescue the Wg secretion defect caused by *UAS-Baiser*^{RNAi} (F), but not *UAS-CHOp24*^{RNAi} (G) or *UAS-Eclair*^{RNAi} (H). Wing discs are oriented anterior left, dorsal up.

Multiple molecular machines are assembled and packaged together to transport cargoes. During the process of escorting Wg protein, p24 proteins interact with Wg, function as cargo selectors and package Wg together with the v-SNARE Sec22 for anterograde transportation.

4.2. The functional diversity of p24 family members

Previous study in yeast uncovered that the different p24 subfamily members form diverse complexes, thus playing multiple roles in escorting cargoes [31]. Consistent with the yeast work, we found that different p24 proteins play different roles and cannot be replaced by each other, indicating the functional diversity of the p24 family.

Baiser, CHOp24, Eclair, Opm and p24-1 are essential for the p24 complex activity in escorting Wg. These p24 members shuttle between ER and Golgi to facilitate the translocation of Wg. Upon our work, deletion of Baiser inhibited p24 activity and impeded Wg secretion. However, over-expression of Baiser cannot relieve the p24 activity inhibition caused by loss of other p24 proteins, arguing that p24 family members are not redundant but indispensable in escorting Wg.

4.3. The SNARE complex involved in Wg secretion

We identified Sec22 as an important regulator of Wg anterograde transportation. However, Sec22 encodes a conserved v-SNARE, which will form a SNARE complex with the cis-Golgi-localized t-SNARE. Thus it is likely that the conserved SNARE complex components Sed5, Bos1 and Bet1 [39–41] are involved in Wg trafficking.

Furthermore, on the pathway of Wg trafficking, docking and fusion between vesicles and the target membrane require the SNARE complex. It is reasonable to speculate that there are other SNARE complex components essential for Wg transportation. It is important to determine other molecular machinery such as Tethers, Rabs, Arfs, and COPs involved.

The synthesis, sorting, processing, and secretion of Wg proteins take place in different compartments and pathways. Multiple regulators determine Wg/Wnt trafficking and signaling. Upon our screen, we identified p24 and Sec22 as regulators in the early secretory pathway of Wg trafficking. The aforementioned experiments have provided compelling evidence that p24 proteins such as Baiser and CHOp24 sort Wg by direct interaction, and package it together with other molecular machinery, such as the v-SNARE Sec22. Then the cargo and cargo selector are assembled for anterograde transportation from the ER to the Golgi (Fig. 4).

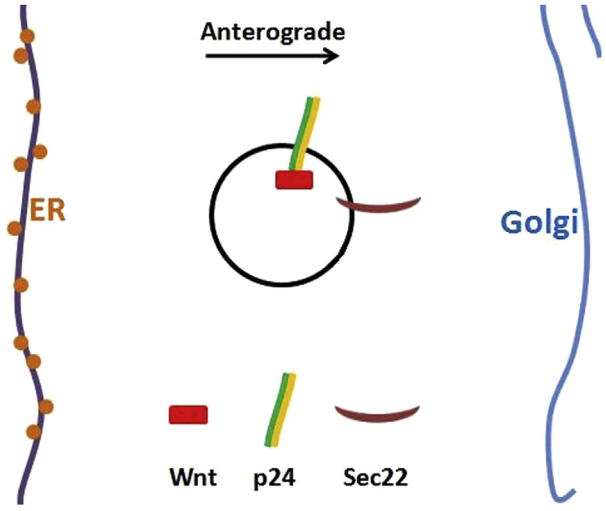

Fig. 4. p24 and Sec22 escort Wg for anterograde transportation. Wg, p24 and Sec22 are packaged together for Wg anterograde transportation from ER to Golgi. P24 proteins function as the cargo selector and Sec22 functions as the v-SNARE.

We demonstrate that the p24 proteins are functionally diverse by forming complexes and playing different roles in this process. Furthermore, the v-SNARE Sec22 is involved in this process and gives us a hint that other SNARE complex components are potential regulators of Wg/Wnt trafficking. Our work provides insight into the molecular machinery shuttling between ER and Golgi to escort Wg/Wnt for secretion.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.151.

Transparency document

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