



Published in final edited form as:

J Cell Physiol. 2014 April ; 229(4): 512–520. doi:10.1002/jcp.24472.

Retromer Promotes Immune Quiescence by Suppressing Spätzle-Toll pathway in *Drosophila*

Bo Zhou^{*}, Eun-Young Yun^{§,†}, Lorraine Ray^{*}, Jia You^{*}, Y. Tony Ip^{#§}, and Xinhua Lin^{#,‡}

^{*}Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, and the Graduate Program in Molecular and Developmental Biology, University of Cincinnati College of Medicine, Cincinnati, OH, 45229, USA

[§]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, 01605, USA

[†]Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Suwon, 441-100, Korea

[‡]State Key Laboratory of Biomembrane and Membrane Biotechnology, and Key Laboratory of Stem Cell, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China

[#] These authors contributed equally to this work.

Abstract

The Toll and Toll-Like Receptor signaling pathways are evolutionarily conserved pathways that regulate innate immunity in insects and mammals. While efforts have been made to clarify the signal transduction events that occur during infection, much less is known about the components that maintain immune quiescence. Here we show that retromer, an intracellular protein complex known for regulating vesicle trafficking, functions in modulating the Toll pathway in *Drosophila melanogaster*. In mutant animals lacking retromer function, the Toll pathway but not JAK-STAT or IMD pathway is activated, triggering both cellular and humoral responses. Genetic epistasis and clonal analysis suggest that retromer regulates a component that acts upstream of Toll. Our data further show that in the mutant the Toll ligand Spätzle has a processing pattern similar to that of after infection. Together, the results suggest a novel function of retromer in regulating Toll pathway and innate immunity at a step that modulates ligand processing or activity.

Keywords

Retromer; Vps35; Toll; Spz; *Drosophila*

CORRESPONDENCE Tony Ip Program in Molecular Medicine, University of Massachusetts Medical School 373 Plantation Street, Room 109, Worcester, MA, 01605, USA tony.ip@umassmed.edu Xinhua Lin Division of Developmental Biology, Cincinnati Children's Hospital Medical Center 3333 Burnet Ave, Cincinnati, OH, 45229, USA xinhua.lin@ioz.ac.cn.

AUTHOR CONTRIBUTION

B. Zhou, Y.T. Ip and X. Lin designed the experiments. B. Zhou, E.Y. Yun, L. Ray and J. You performed the experiments. B. Zhou, Y.T. Ip and X. Lin wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

INTRODUCTION

The Toll and Toll-Like Receptor (TLR) signaling pathways play a conserved role in innate immunity in insects and vertebrates (Kawai and Akira, 2011). In *Drosophila*, the Toll pathway is activated through an extracellular protease cascade that detects invading pathogens (Lemaitre and Hoffmann, 2007). The protease cascade culminates in the cleavage and activation of Spätzle Processing Enzyme (SPE), which in turn cleaves and activates Spätzle (Spz) (Jang et al., 2006). As a ligand, active Spz binds and activates the transmembrane receptor Toll (Hu et al., 2004; Weber et al., 2003). The activation of Toll initiates an intracellular signal transduction pathway, which leads to the translocation of Dorsal and Dorsal-related immunity factor (DIF) into the nucleus for the transcriptional activation of downstream targets, including antimicrobial peptide genes (Belvin et al., 1995; Ip et al., 1993; Lemaitre et al., 1996; Meng et al., 1999; Rutschmann et al., 2000; Tanji et al., 2010). Upon infection, the Toll pathway amplifies the signal through enzyme cascades as well as through positive feedback loops (Jang et al., 2006; Lemaitre and Hoffmann, 2007; Lemaitre et al., 1996). In contrast, in the absence of infection, stringent controls are needed to prevent auto-activation of this pathway (Kondo et al., 2012; Levashina et al., 1999). Although the intracellular components of the Toll pathway are well characterized, the factors contributing to negative controls upstream of the Toll receptor are less known.

Retromer is a membrane-associated cytoplasmic protein complex regulating the trafficking of specific transmembrane cargos from endosomes to the trans-Golgi network (Collins, 2008). Retromer is composed of five components: Sorting nexin 1/2 (SNX1/2), SNX5/6, Vacuolar protein sorting 29 (Vps29), Vps26 and Vps35 (Collins, 2008). The complex has been shown to recycle several transmembrane proteins, thereby regulating different biological processes including Wnt secretion, apoptotic cell clearance and apical-basal polarity maintenance (Belenkaya et al., 2008; Chen et al., 2010; Cullen and Korswagen, 2012; Franch-Marro et al., 2008; Pocha et al., 2011; Port et al., 2008; Zhang et al., 2011; Zhou et al., 2011). Here we identify a novel role of retromer in suppressing the auto-activation of the Toll pathway through regulating the processing of the ligand Spz.

MATERIALS AND METHODS

Drosophila strains

dvps35¹ and UAS-*dvps35*RNAi were described previously (Belenkaya et al., 2008). *w¹¹¹⁸*, Canton-S, *tubGal4*, *cgGal4*, *Tir³*, *Spz²*, *Spz^{rm7}* (also known as *Spz⁴*), UAS-*Spz*RNAi-1 (BL28538), UAS-*Spz*RNAi-2 (BL34699) and UAS-*stat*RNAi (BL33637) were from Bloomington Stock Center. *Tl^{RxA}* was obtained from Dr. K.V. Anderson. YP1-Gal4 was described previously (Hu et al., 2004). UAS-*dvps26*RNAi (GD18396), UAS-*SPERNAi-1* (KK104906) and UAS-*SPERNAi-2* (GD30971) were from VDRC (Dietzl et al., 2007). For mosaic clone analysis, *y w hsp70-flp;FRT^{G13} ubiquitin-GFP/FRT^{G13} dvps35¹* flies were generated and heat-shocked at 37°C for 2 hours at the beginning of first instar stage. For flip-out clone analysis, *y w hsp70-flp/+;AyGal4 UAS-GFP/UAS-Toll^{10b}Myc* flies were generated and heat-shocked at 37°C for 30 minutes at the beginning of first instar stage.

Real-Time quantitative PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen). cDNA was synthesized according to a protocol as previously described (Cha et al., 2011). Real-time PCR was performed using SsoFast Evagreen Supermix (BIO-RAD) and CFX96 Real-time System (BIO-RAD). The following gene-specific primers were used:

Drosomycin, 5'-TACTTGTTTCGCCCTCTTCG-3' and 5'-GTATCTTCCGGACAGGCAGT-3'; *IMI*, 5'-CTCAGTCGTCACCGTTTTTTG-3', 5'-CATTGCACACCCTGCAATC-3'; *Diptericin*, 5'-CCGCAGTACCCACTCAATCT-3' and 5'-ACTGCAAAGCCAAAACCATC-3'; *CecropinA1*, 5'-CATCTTCGTTTTTCGTCGCTC-3' and 5'-CGACATTGGCGGCTTGTGA-3'; *AttacinA*, 5'-AGGTTCCCTAACCTCCAATC-3' and 5'-CATGACCAGCATTGTTGTAG-3'; *SPE*, 5'-CTTTTCGCTGATCGCATTTT-3' and 5'-CACCGGATTTGTCCAGTTCT-3'; *spz*, 5'-GTCCAGTTCGCCATCACTTT-3' and 5'-GGAAGCTGGTGTACCCAAAA-3'; *STAT*, 5'-CCGTTATGTGAAGAGCACA-3' and 5'-TAGCGACACACGTTTCAGGAG-3'; *dyps35*, 5'-CTTTTCTGGAGTGGAAAGCA-3' and 5'-TTCAGCAGCTCCACATACAA-3'; *ribosomal protein 49 (rp49)*, 5'-AAGCTAGCCCAACCTGCTTC-3' and 5'-GTGCGCTTCTTCACGATCT-3', 5'-ATGCTAAGCTGTCGCACAAA-3' and 5'-GTTTCGATCCGTAACCGATGT-3' or 5'-CAAGAAGCTAGCCCAACCTG-3' and 5'-AGCATATCGATCCGACTGGT-3'.

Hemocyte counts and immunostaining

Circulating hemocytes were obtained and counted as previously described (Lanot et al., 2001). Plasmatocytes and lamellocytes were identified by cell appearance. Lymph gland was dissected and stained as previously described (Qiu et al., 1998). *Drosophila* larval or adult female fat body was fixed and stained as previously described (Scott et al., 2007).

Drosophila hemocytes were fixed and stained as previously described (Lanot et al., 2001). The following primary antibodies were used: mouse anti-Dorsal (7A4, 1:5, DSHB), rabbit anti-DIF (Rutschmann et al., 2000) (1:100), rabbit anti-phosphoHistone H3 (Singh et al., 2007) (1:500), mouse anti-Cut (2B10, 1:5, DSHB), rat anti-DE-cad (DCAD2, 1:2.5, DSHB) and mouse anti-Antp (8C11, 1:5, DSHB). Alexa Fluor 488 (Molecular Probes), Cy3 and Cy5 (Jackson Immuno)-conjugated secondary antibodies were used at a dilution of 1:400. Alexa Fluor 488 Phalloidin (1:400, Molecular Probes) was used for F-actin staining. Alexa Fluor 555-conjugated Wheat Germ Agglutinin (WGA, 1:500, Molecular Probes) was used for nuclear membrane staining. DAPI (1 µg/µl, Roche) was used for nuclei staining.

Septic injury and immunoblotting

Septic injuries were performed at room temperature by pricking wandering third instar larvae with a thin tungsten needle previously dipped into a concentrated culture of *Micrococcus luteus*. Larvae were recovered at 25°C for 1 hour. For immunoblotting, *Drosophila* wandering 3rd instar larvae were collected and homogenized on ice in lysis buffer (1% Triton X-100, 50mM NaCl, 20mM Tris at pH7.5 and 1mM EDTA) plus Protease Inhibitors (Roche). Lysates were subject to centrifugation at 13,000 rpm. Supernatants were taken out carefully to avoid the lipid layer on the top, followed by a second centrifugation at the same speed. Final supernatants were mixed with Laemmli sample buffer (Bio-Rad) plus

5% 2-Mercaptoethanol (FisherBiotech). Lysates were boiled for 5 minutes and subjected to immunoblot analysis using the following primary antibodies: mouse anti-V5 (1:3000, Invitrogen) and mouse anti- β -Actin (1:5000, Abcam). Horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno) were used at a dilution of 1:3000.

Imaging and Statistical analysis

Images of third instar larvae were collected on an Olympus SZ-CTV dissection microscope. Confocal images were collected on a Zeiss LSM 510 confocal microscope with 10x dry, 20x dry, 40x oil and 63x oil objectives. All images were arranged with Adobe Photoshop. Error bars represent standard deviation. Statistical comparisons were made using Student's T-test, with P value listed in figures.

RESULTS

Loss of retromer function triggers an auto-activation of the innate immune response in *Drosophila*

Drosophila innate immunity involves the cellular immune response and the humoral immune response (Lemaitre and Hoffmann, 2007). Hyper-activation of the cellular immune response can generate melanotic aggregates (Minakhina and Steward, 2006). We noted that homozygous null mutants of *Drosophila vps35*¹ (labelled as *dvps35* mutants hereafter) generated such melanotic aggregates in the larval body cavity (Figure 1A and 1B). Similarly, when knocking down *dvps35* or another retromer component *dvps26* by RNAi (RNA interference) using the ubiquitous driver *tubulin-Gal4* (*tubGal4-dvps35RNAi*), we also observed melanotic aggregates (Figure 1C and data not shown for *vps26RNAi*). These phenotypes were observed in the absence of infections. Thus, loss of retromer activity can cause the formation of melanotic aggregates, implying that retromer may have a role in modulating *Drosophila* cellular immune response.

Upon activation of the cellular immune response, the formation of melanotic aggregates is often associated with excessive proliferation of total hemocytes, over-differentiation into lamellocytes and/or a hypertrophy of lymph gland, which is a hematopoietic organ (Harrison et al., 1995; Lemaitre and Hoffmann, 2007; Luo et al., 1995; Minakhina and Steward, 2006; Qiu et al., 1998). In *tubGal4-dvps35RNAi* larvae, the total hemocyte count and the ratio of lamellocytes were significantly enhanced compared to the wild type (Figure 1D and 1E). *tubGal4-dvps35RNAi* larvae also displayed lymph gland hypertrophy when compared to the control *tubGal4* larvae (Figure 1F and 1G). Phospho-histone H3 staining suggested a higher proliferation rate in lymph glands in *tubGal4-dvps35RNAi* larvae than that in control (Figure 1H). We also observed an expansion of the cortical zone, a reduction of the medullary zone and an absence of the posterior signalling center (Figure 1I and 1J), as marked by Cut, DE-cadherin and Antp staining, respectively. This is consistent with the increased proliferation of precursor cells in the absence of control by the signalling center, leading to lymph gland hypertrophy. Taken together, the cellular immune response was highly activated in animals lacking retromer activity.

Next, we examined the humoral branch of the innate immune system, which features the production of antimicrobial peptides from an immune organ, the fat body (Lemaitre and Hoffmann, 2007). We monitored the expression of several antimicrobial peptide genes using real-time PCR. A subset of antimicrobial peptide genes including *Drosomycin* (*Drs*) and *Immune-induced Molecule 1* (*IMI*) were highly expressed in whole larvae of *dvps35* mutants (Figure 2A). Furthermore, *Drs* and *IMI* were greatly induced in the dissected larval fat bodies of *dvps35* mutant larvae (Figure 2B). Consistent with null mutants, induction of *Drs* and *IMI* was also observed in whole larvae, knocking down *dvps35* by RNAi driven by *cgGal4* (fat body and hemocyte expression) or *tubGal4* (ubiquitous expression) (Figure 2C). We also used an adult female fat body driver, *YP1Gal4* to knock down *dvps35* in the adult fat bodies, and observed an induction of *Drs* and *IMI* expression after *dvps35*RNAi (Figure 2D). These results suggest that the regulation of antimicrobial peptide gene expression by *dvps35* is likely similar in both larvae and adults. Taken together, loss of *dvps35* function causes an activation of the humoral immune response in addition to the cellular response shown above.

Loss of retromer function activates specifically the Toll pathway

There are probably more than 50 antimicrobial peptide genes with diverse functions in the *Drosophila* genome. The expression of different antimicrobial peptide genes is controlled by different signaling pathways. The expression of *Drs* and *IMI* is regulated by the Toll pathway, while the expression of several other antimicrobial peptide genes including *AttacinA*, *CecropinA* and *Diptericin* is regulated by the IMD pathway (Lemaitre and Hoffmann, 2007). Although the expression of *Drs* and *IMI* was highly activated in the absence of *dvps35* function, the expression of *AttacinA*, *CecropinA* and *Diptericin* was not significantly changed (Figure 2A-2D). This argues that the Toll pathway but not the IMD pathway is activated in the absence of *dvps35* function. Consistent with this view, the activation of the Toll, but not the IMD pathway has been reported to generate many aspects of the cellular immune response observed in *tubGal4-dvps35*RNAi larvae (Figure 1) (Huang et al., 2005; Qiu et al., 1998).

To further confirm the activation of the Toll pathway, we examined the nuclear translocation of the transcription factors Dorsal and DIF. In the fat bodies and hemocytes of *tubGal4-dvps35*RNAi larvae, Dorsal was localized in the nuclei, in contrast to a cytoplasmic distribution in wild type samples (Figure 3A). Similarly, DIF was also localized in the nuclei of the fat bodies of *dvps35* mutants (Figure 3B). These results strongly suggest that abolishing *dvps35* activates the Toll pathway. We performed similar staining in the hemocyte collections. The Dorsal protein staining could be observed in the nuclei of many plasmatocytes and lamellocytes (Fig. 3C), consistent with the activation of the Toll pathway. Quantification of the result revealed that Dorsal nuclear localization in hemocytes was highly increased (Fig. 3D).

In addition to the Toll pathway, the activation of the JAK/STAT pathway can also generate melanotic aggregates (Harrison et al., 1995; Luo et al., 1995). Furthermore, crosstalk between the Toll pathway and the JAK/STAT pathway has been reported (Sackton et al., 2010). Therefore, we examined whether the generation of melanotic aggregates and the high

level expression of antimicrobial peptide genes was a result of the JAK/STAT pathway activation in *dvps35* mutants. In *tubGal4-dvps35RNAi* larvae, knocking down *stat* (*tubGal4-statRNAi-dvps35RNAi*), which encodes a transcriptional factor in the JAK/STAT pathway, neither rescued the generation of melanotic aggregates (Figure 4A), nor suppressed the overexpression of Toll-regulated antimicrobial peptide genes (*Drs* and *IM1*, Figure 4B-4C). As controls, the knock down of *vps35* and *stat* was highly efficient (Figure 4D-4E). These data argue that the JAK/STAT pathway is not required for the generation of melanotic aggregates or the induction of Toll-regulated antimicrobial peptide genes in *dvps35* mutants. Instead, there was some enhancement of the gene expression, suggesting the above mentioned crosstalk between Toll and JAK/STAT pathways.

Retromer suppressed the processing of the Toll ligand Spz

Having established a role of retromer in the Toll pathway, we next conducted epistasis analysis to determine the position of retromer action along the Toll pathway. The initial focus of our epistasis analysis was on the transmembrane receptor Toll and its ligand Spz because previous studies showed that retromer targeted transmembrane proteins (Collins, 2008). We used different alleles of *Toll* and *Spz* (*Tlr³*, *Tlr^{RxA}*, *Spz²* and *Spz^{rm7}*) and two independent lines of *Spz* RNAi to reduce the activity of these two genes. In *tubGal4-dvps35RNAi* larvae, reducing the activity of Toll (*Tlr3/Tlr^{RxA}*) efficiently suppressed the induction of Toll-regulated antimicrobial peptide genes (Figure 5A and 5B). Similarly, reducing the activity of Spz (*Spz²/Spz^{rm7}* and *Spz* RNAi) also gave rise to an efficient suppression in *tubGal4-dvps35RNAi* larvae (Figure 5C-5H). It is note worthy that the knock down of *dvps35* caused a modest increase of Spz transcript (Figure 5G). This is consistent with previous observations that many of the Toll pathway components are up-regulated when the pathway is activated (Ip et al., 1993; Lemaitre et al., 1996). Overall, both the receptor and the ligand of the Toll pathway are required for the activation of downstream events in the absence of *dvps35* activity.

Next we investigated components upstream of the ligand. SPE is the immediate upstream protease of Spz in the Toll pathway during the immune response (Jang et al., 2006). We conducted epistasis analysis between *dvps35* and *SPE*. In *tubGal4-dvps35RNAi* larvae, knocking down *SPE* by two independent RNAi lines (driven by *tub-Gal4*) did not suppress the induction of Toll-regulated antimicrobial peptide genes (Figure 5I-5L). We noted that again there was an enhanced expression of *SPE* in *tubGal4-dvps35RNAi* (compared to *tubGal4*, Figure 5K), suggesting a positive feedback regulation of the *SPE* gene after activation of Toll (Jang et al., 2006; Lemaitre et al., 1996). These data demonstrate that *SPE* is not required for the activation of the Toll pathway in the absence of *dvps35* activity in the absence of infection. Collectively, the above data indicate that retromer regulates Spz independently of the upstream proteolytic cascade.

It is somewhat surprising that the epistasis analysis placed retromer function at the ligand level because the most logical target for retromer is the transmembrane protein Toll. To further investigate if retromer acts at the receptor level or above, we tested whether deleting *dvps35* could activate the Toll pathway in the same cells. If retromer regulates the receptor, one would predict a cell autonomous activation of the Toll pathway. We generated

homozygous mutant cell clones of *dvps35* in fat bodies, marked by the absence of GFP. In these mutant cells, we were not able to observe a nuclear localization of Dorsal (Figure 6A). In a parallel experiment where we induced flip-out clones to allow the expression of a gain-of-function Toll (Toll^{10b}, marked by GFP expression), there was a clear nuclear accumulation of Dorsal (Figure 6B). These results suggest that *dvps35* is not required cell autonomously to regulate the Toll pathway activity. Instead, *dvps35* may regulate a step upstream of the receptor Toll.

Taken together, the genetic epistasis suggests that retromer regulates a component between Toll and SPE. A logical speculation is that retromer regulates Spz, thereby modulating the Toll pathway activity. To test this idea, we monitored the processing/maturation of Spz, by using a transgenic construct that contains the V5 epitope tag. Spz maturation is probably a multi-step process (Weber et al., 2007), but the final cleaved product is a 16 kD C-terminal fragment of 106 amino acids that binds to Toll (Hu et al., 2004; Lemaitre and Hoffmann, 2007; Weber et al., 2003). Nonetheless, multiple processed forms with sizes smaller than the full-length 37 kD precursor but larger than the 16 kD final product were previously observed during both early embryogenesis and infections correlating with the activation of the Toll pathway (Levashina et al., 1999; Morisato and Anderson, 1994). Here we observed a similar cleavage pattern of Spz during infections and in *tubGal4-dvps35RNAi*. In larvae with Gram⁺ bacterial infections, which activate the Toll pathway, Spz was mainly cleaved into two bands migrating around 30 kD (indicated by the bracket in Figure 6C). The putative active form of Spz (16 kD, indicated by an * in Figure 6C) was present at a very low level in the control and was not substantially enhanced after Gram⁺ bacterial infection. The similar two-band pattern around the 30 kD range was also seen in *tubGal4-dvps35RNAi* larvae, with no detectable 16 kD form of Spz. The very low or undetectable level of the 16 kD form of Spz observed during infections or in *tubGal4-dvps35RNAi* is possibly due to a rapid degradation when in complex with Toll. The two bands of Spz at 30 kD might represent more stable forms during Spz processing/maturation. Based on these observations, we propose that in the absence of *dvps35* activity, Spz undergoes protein cleavage similar to that occurs during infections. Therefore, retromer may play a role in restricting the processing of Spz into the mature ligand prior to infection.

DISCUSSION

Here we have identified a role of retromer in negatively regulating the Toll pathway to maintain immune quiescence. In the absence of infection, the loss of retromer activity alone is capable of activating the Toll pathway and launching both the cellular and humoral immune responses. Furthermore, genetic epistasis and mosaic analysis suggest that retromer acts upstream of Toll and downstream of SPE, and we uncovered a retromer function in restricting the processing/maturation of Spz. In summary, retromer plays a critical role in suppressing the auto-activation of the innate immune system through Spz in the Toll pathway in *Drosophila*.

Based on previous knowledge that retromer regulates trafficking of transmembrane proteins, one can envisage that retromer normally may regulate the Toll pathway in one of the four following ways: (1) in Toll-responsive cells to transport the Toll-Spz complex for

destruction; (2) in Spz secreting cells to suppress the release of active Spz; (3) in certain cells to assist the clearance of active Spz in the hemolymph; or (4) in certain cells to repress Spz through an indirect effect of other yet to be identified components. In the *dyps35* mutant clonal cells in fat bodies we did not observe increased Dorsal nuclear localization, indicating that retromer is not simply regulating the Toll pathway cell-autonomously. Our epistasis analysis suggests that retromer acts between Toll and SPE. Even though Spz is the only known component in between, there can be many other proteins that regulate the processing, maturation, trafficking or degradation of Spz in normal flies in order to restrict the activity of the Toll signaling pathway prior to infections. The full mechanism of Spz maturation is not yet unveiled and the retromer function in this process requires further investigation. Although we cannot exclude the possibility that retromer has an indirect effect on Spz, we favor a function of retromer in anti-release and/or clearance of active Spz (Figure 6D). Retromer has been shown to target transmembrane proteins. It will be intriguing to identify the transmembrane target of retromer in the context of regulating Spz and explore the mechanism of how this transmembrane target suppresses the release and/or assists the clearance of active Spz. Equally important is to examine whether modulating retromer-dependent Spz maturation is part of the activation mechanism of the Toll pathway during infections.

ACKNOWLEDGEMENTS

We thank Dr. K.V. Anderson, the Bloomington Stock Center and VDRC for *Drosophila* stocks; Dr. S. Hou and the Iowa Developmental Studies Hybridoma Bank (DSHB) for antibodies; Q. Tao, S. Cha, C. Liu and Y. Feng for technical assistance; Drs. T. Belenkaya and S. Wasserman for comments on the manuscript. This work was supported by a grant from NIH (R01DK83450) to Y.T.I., NIH grants (2R01 GM063891 and 1R01GM087517) to X.L. as well as grants from the National Basic Research Program of China (2011CB943901) and National Natural Science Foundation of China (31101036) to X.L.. Y.T.I. is a member of the UMass DERC (DK32520), a member of the UMass CCTS (UL1TR000161) and a member of the Guangdong Innovative Research Team Program (No. 201001Y0104789252).

REFERENCES

- Belenkaya TY, Wu Y, Tang X, Zhou B, Cheng L, Sharma YV, Yan D, Selva EM, Lin X. The retromer complex influences Wnt secretion by recycling wntless from endosomes to the trans-Golgi network. *Dev Cell*. 2008; 14(1):120–131. [PubMed: 18160348]
- Belvin MP, Jin Y, Anderson KV. Cactus protein degradation mediates *Drosophila* dorsal-ventral signaling. *Genes Dev*. 1995; 9(7):783–793. [PubMed: 7705656]
- Cha SW, Tadjudje E, Wylie C, Heasman J. The roles of maternal Vangl2 and aPKC in *Xenopus* oocyte and embryo patterning. *Development*. 2011; 138(18):3989–4000. [PubMed: 21813572]
- Chen D, Xiao H, Zhang K, Wang B, Gao Z, Jian Y, Qi X, Sun J, Miao L, Yang C. Retromer is required for apoptotic cell clearance by phagocytic receptor recycling. *Science*. 2010; 327(5970):1261–1264. [PubMed: 20133524]
- Collins BM. The structure and function of the retromer protein complex. *Traffic*. 2008; 9(11):1811–1822. [PubMed: 18541005]
- Cullen PJ, Korswagen HC. Sorting nexins provide diversity for retromer-dependent trafficking events. *Nat Cell Biol*. 2012; 14(1):29–37. [PubMed: 22193161]
- Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oettel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ. A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature*. 2007; 448(7150):151–156. [PubMed: 17625558]

- Franch-Marro X, Wendler F, Guidato S, Griffith J, Baena-Lopez A, Itasaki N, Maurice MM, Vincent JP. Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. *Nat Cell Biol.* 2008; 10(2):170–177. [PubMed: 18193037]
- Harrison DA, Binari R, Nahreini TS, Gilman M, Perrimon N. Activation of a *Drosophila* Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. *EMBO J.* 1995; 14(12):2857–2865. [PubMed: 7796812]
- Hu X, Yagi Y, Tanji T, Zhou S, Ip YT. Multimerization and interaction of Toll and Spatzle in *Drosophila*. *Proc Natl Acad Sci U S A.* 2004; 101(25):9369–9374. [PubMed: 15197269]
- Huang L, Ohsako S, Tanda S. The lesswright mutation activates Rel-related proteins, leading to overproduction of larval hemocytes in *Drosophila melanogaster*. *Dev Biol.* 2005; 280(2):407–420. [PubMed: 15882582]
- Ip YT, Reach M, Engstrom Y, Kadalayil L, Cai H, Gonzalez-Crespo S, Tatei K, Levine M. Dif, a dorsal-related gene that mediates an immune response in *Drosophila*. *Cell.* 1993; 75(4):753–763. [PubMed: 8242747]
- Jang IH, Chosa N, Kim SH, Nam HJ, Lemaitre B, Ochiai M, Kambris Z, Brun S, Hashimoto C, Ashida M, Brey PT, Lee WJ. A Spatzle-processing enzyme required for toll signaling activation in *Drosophila* innate immunity. *Dev Cell.* 2006; 10(1):45–55. [PubMed: 16399077]
- Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity.* 2011; 34(5):637–650. [PubMed: 21616434]
- Kondo T, Kawai T, Akira S. Dissecting negative regulation of Toll-like receptor signaling. *Trends Immunol.* 2012; 33(9):449–458. [PubMed: 22721918]
- Lanot R, Zachary D, Holder F, Meister M. Postembryonic hematopoiesis in *Drosophila*. *Dev Biol.* 2001; 230(2):243–257. [PubMed: 11161576]
- Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. *Annual review of immunology.* 2007; 25:697–743.
- Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell.* 1996; 86(6):973–983. [PubMed: 8808632]
- Levashina EA, Langley E, Green C, Gubb D, Ashburner M, Hoffmann JA, Reichhart JM. Constitutive activation of toll-mediated antifungal defense in serpin-deficient *Drosophila*. *Science.* 1999; 285(5435):1917–1919. [PubMed: 10489372]
- Luo H, Hanratty WP, Dearolf CR. An amino acid substitution in the *Drosophila* hopTum-1 Jak kinase causes leukemia-like hematopoietic defects. *EMBO J.* 1995; 14(7):1412–1420. [PubMed: 7729418]
- Meng X, Khanuja BS, Ip YT. Toll receptor-mediated *Drosophila* immune response requires Dif, an NF-kappaB factor. *Genes Dev.* 1999; 13(7):792–797. [PubMed: 10197979]
- Minakhina S, Steward R. Melanotic mutants in *Drosophila*: pathways and phenotypes. *Genetics.* 2006; 174(1):253–263. [PubMed: 16816412]
- Morisato D, Anderson KV. The spatzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the *Drosophila* embryo. *Cell.* 1994; 76(4):677–688. [PubMed: 8124709]
- Pocha SM, Wassmer T, Niehage C, Hoflack B, Knust E. Retromer controls epithelial cell polarity by trafficking the apical determinant Crumbs. *Curr Biol.* 2011; 21(13):1111–1117. [PubMed: 21700461]
- Port F, Kuster M, Herr P, Furger E, Banziger C, Hausmann G, Basler K. Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nat Cell Biol.* 2008; 10(2):178–185. [PubMed: 18193032]
- Qiu P, Pan PC, Govind S. A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. *Development.* 1998; 125(10):1909–1920. [PubMed: 9550723]
- Rutschmann S, Jung AC, Hetru C, Reichhart JM, Hoffmann JA, Ferrandon D. The Rel protein DIF mediates the antifungal but not the antibacterial host defense in *Drosophila*. *Immunity.* 2000; 12(5):569–580. [PubMed: 10843389]
- Sackton TB, Lazzaro BP, Clark AG. Genotype and gene expression associations with immune function in *Drosophila*. *PLoS Genet.* 2010; 6(1):e1000797. [PubMed: 20066029]

- Scott RC, Juhasz G, Neufeld TP. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr Biol.* 2007; 17(1):1–11. [PubMed: 17208179]
- Singh SR, Liu W, Hou SX. The adult *Drosophila* malpighian tubules are maintained by multipotent stem cells. *Cell Stem Cell.* 2007; 1(2):191–203. [PubMed: 18371350]
- Tanji T, Yun EY, Ip YT. Heterodimers of NF-kappaB transcription factors DIF and Relish regulate antimicrobial peptide genes in *Drosophila*. *Proc Natl Acad Sci U S A.* 2010; 107(33):14715–14720. [PubMed: 20679214]
- Weber AN, Gangloff M, Moncrieffe MC, Hyvert Y, Imler JL, Gay NJ. Role of the Spatzle Pro-domain in the generation of an active toll receptor ligand. *J Biol Chem.* 2007; 282(18):13522–13531. [PubMed: 17324925]
- Weber AN, Tauszig-Delamasure S, Hoffmann JA, Lelievre E, Gascan H, Ray KP, Morse MA, Imler JL, Gay NJ. Binding of the *Drosophila* cytokine Spatzle to Toll is direct and establishes signaling. *Nat Immunol.* 2003; 4(8):794–800. [PubMed: 12872120]
- Zhang P, Wu Y, Belenkaya TY, Lin X. SNX3 controls Wingless/Wnt secretion through regulating retromer-dependent recycling of Wntless. *Cell Res.* 2011; 21(12):1677–1690. [PubMed: 22041890]
- Zhou B, Wu Y, Lin X. Retromer regulates apical-basal polarity through recycling Crumbs. *Dev Biol.* 2011; 360(1):87–95. [PubMed: 21958744]

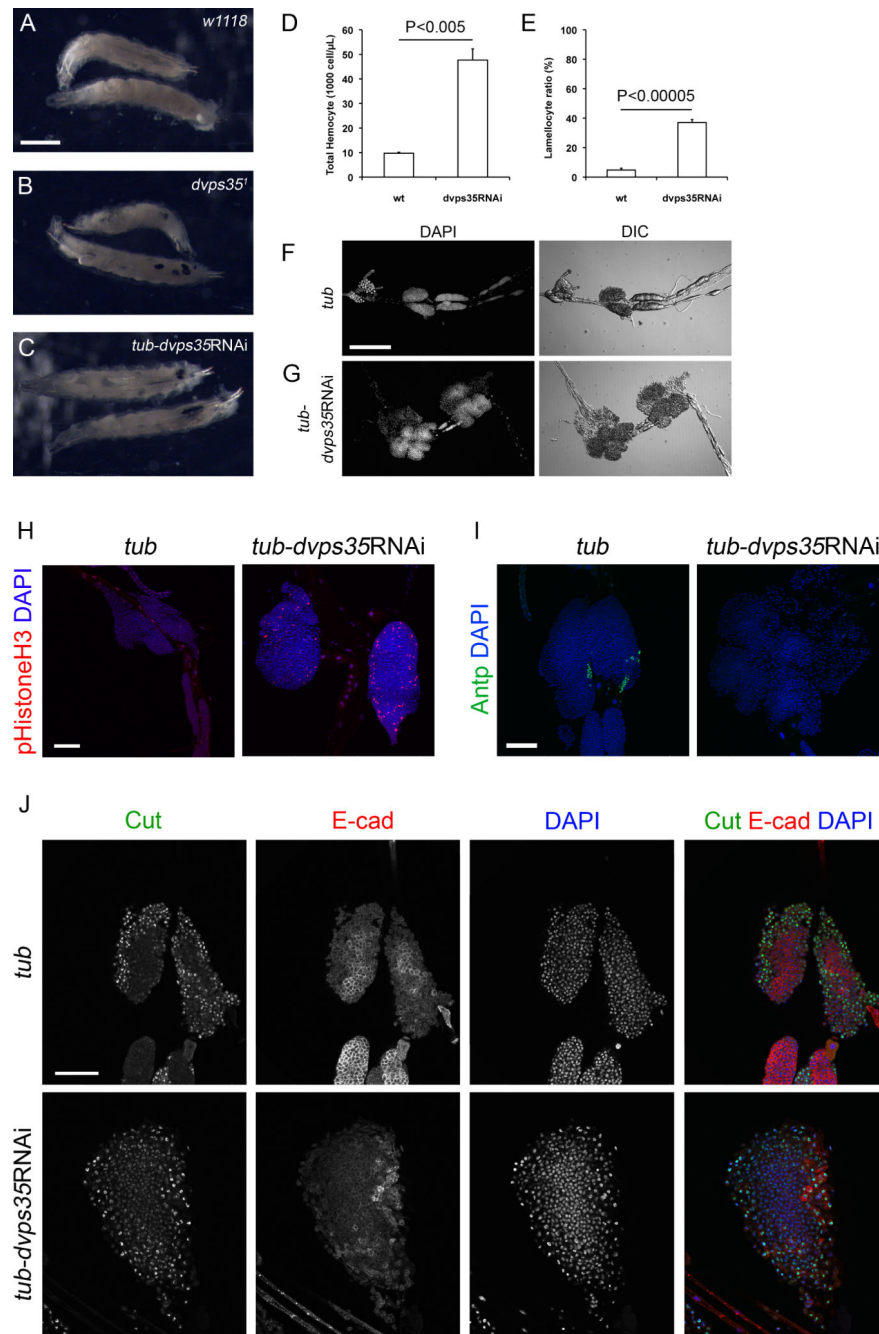


Figure 1. Loss of retromer activity activates the cellular immune response in *Drosophila* prior to infections

(A-C) Third instar larvae of (A) wild type, (B) *dvps35* mutants and (C) *tubGal4-dvps35RNAi*. Scale bar, 1 mm. (D) Total hemocyte count of wild type and *tubGal4-dvps35RNAi* third instar larvae. (E) Lamellocyte ratio in total hemocytes of wild type and *tubGal4-dvps35RNAi* third instar larvae. (F and G) Lymph glands of (F) *tub-Gal4* and (G) *tub-Gal4-dvps35RNAi* third instar larvae. Anterior, left. Scale bar, 200 μm. (H) phospho-Histone H3 and DAPI staining in lymph glands of *tub-Gal4* and *tub-dvps35RNAi* third instar larvae. (I) Antp and DAPI staining in lymph glands of *tub-Gal4* and *tubGal4-*

dvps35RNAi third instar larvae. **(J)** Cut, DE-cadherin and DAPI staining in lymph glands of *tub-Gal4* and *tubGal4-dvps35RNAi* third instar larvae. Anterior to the top in all images in H-J. Scale bar in H-J, 50 μm .

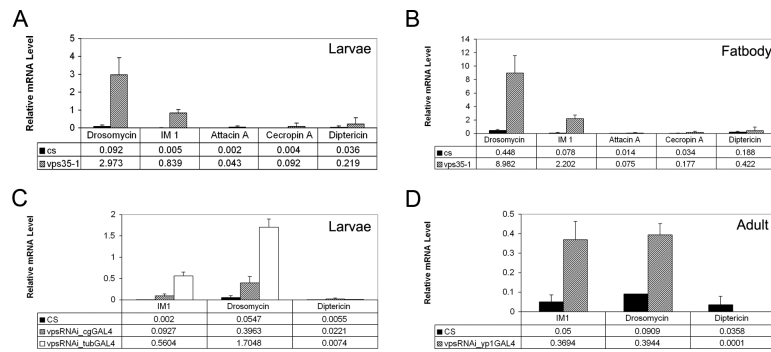


Figure 2. Loss of retromer activity activates the humoral response regulated by Toll (A and B) mRNA expression level assayed by q-PCR of Toll or IMD pathway-regulated antimicrobial peptide genes in (A) whole larvae or (B) larval fat body of wild type and *dvps35* mutants. CS, Canton-S. *Drosomycin* and *IM1* are Toll-regulated genes. *AttacinA*, *CecropinA* and *Diptericin* are IMD-regulated genes. *Ribosomal protein 49 (rp49)* served as internal control. (C and D) Expression level of Toll or IMD pathway-regulated antimicrobial peptide genes in (C) larvae or (D) adult of wild type, *cgGal4-dvps35RNAi*, *tubGal4-dvps35RNAi* and *yt1Gal4-dvps35RNAi*.

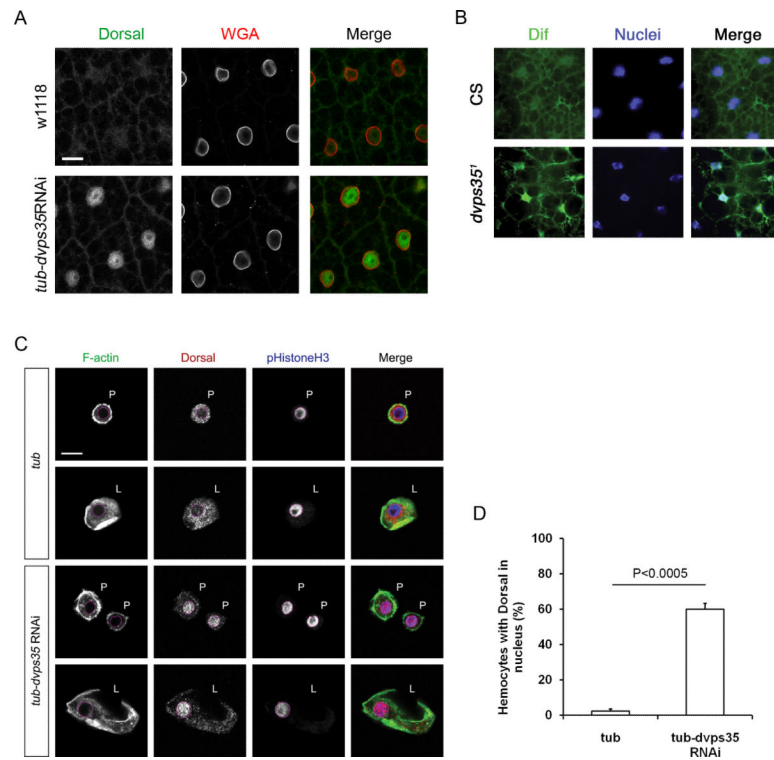


Figure 3. Loss of retromer activity activates the Toll but not IMD pathway

(A) Staining of Dorsal and nuclear membrane (WGA) in the fat body of wild type (*w1118*) and *tubGal4-dvps35RNAi* third instar larvae. Scale bar, 20 μ m. (B) Staining of DIF and DAPI in the adult fat body of wild type (CS) and *dvps35* mutant. CS, Canton-S. (C) Staining of F-actin, Dorsal and phospho-Histone H3 in hemocytes of *tubGal4* and *tubGal4-dvps35RNAi* third instar larvae. P, plasmatocytes; L, lamellocytes; red dotted circles, nuclei. Scale bar, 10 μ m. (D) Ratio of hemocytes with Dorsal in the nuclei in *tubGal4* and *tubGal4-dvps35RNAi* third instar larvae.

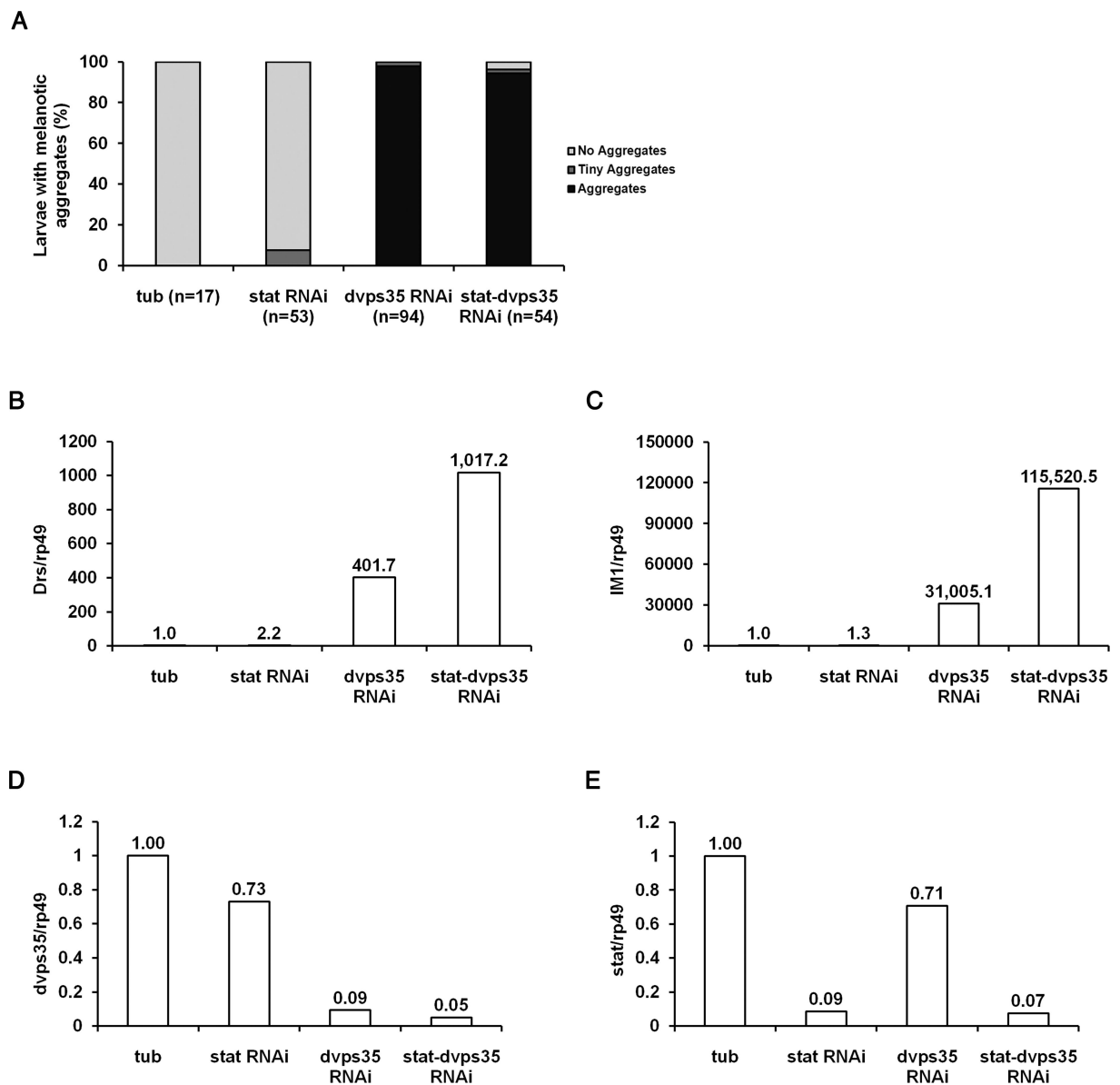


Figure 4. The JAK-STAT pathway is not essential for the generation of melanotic aggregates or the activation of the Toll pathway in the absence of dVps35 activity

(A) Ratio of third instar larvae with melanotic aggregates in *tubGal4*, *tubGal4-statRNAi*, *tubGal4-dvps35RNAi* and *tubGal4-statRNAi-dvps35RNAi*. (B-E) Expression level of (B) *Drs*, (C) *IMI1*, (D) *dvps35* and (E) *stat* in third instar larvae of *tubGal4*, *tubGal4-statRNAi*, *tubGal4-dvps35RNAi* and *tubGal4-statRNAi-dvps35RNAi*.

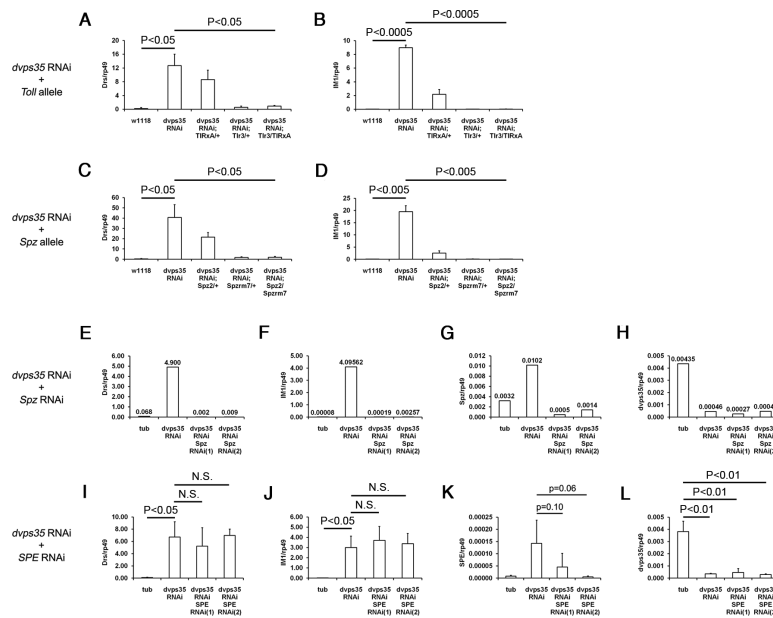


Figure 5. Genetic epistasis analysis places the action of retromer at the level of Spz (A and B) Expression level of (A) *Drs* and (B) *IMI* in third instar larvae of wild type and *tubGal4-dvps35*RNAi with different combinations of *Toll* hypomorphic (*Tr³*) or null (*Tr^{RxA}*) alleles. (C and D) Expression level of (C) *Drs* and (D) *IMI* in third instar larvae of wild type and *tubGal4-dvps35*RNAi with different combinations of *Spz* null alleles (*Spz²* and *Spz^{tm7}*). (E-H) Expression level of (E) *Drs*, (F) *IMI*, (G) *Spz* and (H) *dvps35* in third instar larvae of *tubGal4*, *tubGal4-dvps35*RNAi, *tubGal4-dvps35*RNAi-*Spz*RNAi(1) and *tubGal4-dvps35*RNAi-*Spz*RNAi(2). (I-L) Expression level of (I) *Drs*, (J) *IMI*, (K) *SPE* and (L) *dvps35* in third instar larvae of *tubGal4*, *tubGal4-dvps35*RNAi, *tubGal4-dvps35*RNAi-*SPE*RNAi(1) and *tubGal4-dvps35*RNAi-*SPE*RNAi(2).

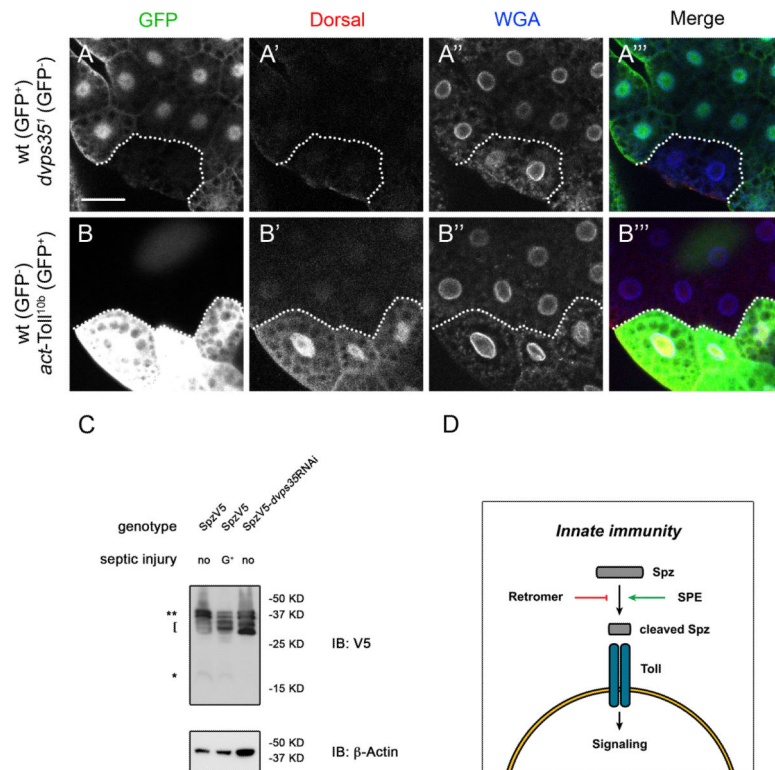


Figure 6. Retromer regulates the maturation/processing of the ligand Spz
(A-A''') Staining of GFP, Dorsal and nuclear membrane (WGA) in larval fat bodies containing mosaic clones of *dvps35* mutant cells. *dvps35* mutant cells are marked by absence of GFP. **(B-B''')** Staining of GFP, Dorsal and nuclear membrane (WGA) in larval fat bodies containing flip-out clones of cells expressing Toll^{10b} protein. Ectopic expressing cells are marked by GFP. Scale bar in A and B, 50 μ m. **(C)** Immunoblotting of V5 and β -Actin in third instar larvae of *tubGal4-SpzV5* (no infection), *tubGal4-SpzV5* (infection with Gram⁺ bacteria) and *tubGal4-SpzV5-dvps35RNAi* (no infection). Double asterisks, full length Spz. Bracket, two-band pattern around the 30 kD range of cleaved Spz. Asterisk, 16 kD C-terminal fragment of Spz. **(D)** A model illustrating retromer suppresses the activation of Spz independently of SPE.