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

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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.

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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 (Weber et al., 2003; Hu et al., 2004). 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 (Ip et al., 1993; Belvin et al., 1995; 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 (Lemaitre et al., 1996; Jang et al., 2006; Lemaitre and Hoffmann, 2007). In contrast, in the absence of infection, stringent controls are needed to prevent auto-activation of this pathway (Levashina et al., 1999; Kondo et al., 2012). 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; Franch-Marro et al., 2008; Port et al., 2008; Chen et al., 2010; Pocha et al., 2011; Zhang et al., 2011; Zhou et al., 2011; Cullen and Korswagen, 2012). 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

$dvps35^1$ and UAS-$dvps35$RNAi were described previously (Belenkaya et al., 2008). w^{1118}, Canton-S, tubGal4, cgGal4, Tl^{r3}, Spz^2, Spz^{rm7} (also known as Spz^4), UAS-SpzRNAi-1 (BL28538), UAS-SpzRNAi-2 (BL34699), and UAS-$stat$RNAi (BL33637) were from Bloomington Stock Center, Bloomington, IN. 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 (Vienna, Austria) (Dietzl et al., 2007). For mosaic clone analysis, $y\ w\ hsp70$-$flp;FRT^{G13}\ ubiquitin$-$GFP/FRT^{G13}\ dvps35^1$ flies were generated and heat-shocked at 37°C for 2 h 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 min at the beginning of first instar stage.

Real-time quantitative PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, Grand Island, NY). 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, Hercules, CA) and CFX96 Real-time System (BIO-RAD). The following gene-specific primers were used:

Drosomycin, 5′-TACTTGTTCGCCCTCTTCG-3′ and 5′-GTATCTTCCGGACAGGCAGT-3′; *IM1*, 5′- CTCAGTCGTCA CCGTTTTTG-3′ and 5′-CATTGCACACCCTGCAATC-3′; *Diptericin*, 5′-CCGCAGTACCCACTCAATCT-3′ and 5′-ACTGC AAAGCCAAAACCATC-3′; *CecropinA1*, 5′-CATCTTCGTTTTC GTCGCTC-3′ and 5′-CGACATTGGCGGCTTGTTGA-3′; *AttacinA*, 5′-AGGTTCCTTAACCTCCAATC-3′ and 5′-CATGAC CAGCATTGTTGTAG-3′; *SPE*, 5′-CTTTTCGCTGATCGCATT TT-3′ and 5′-CACCGGATTTGTCCAGTTCT-3′; *spz*, 5′-GTCCA GTTCGCCATCACTTT-3′ and 5′-GGAAGCTGGTGTACCCAA AA-3′; *STAT*, 5′-CCGGTTATGTGAAGAGCACA-3′ and 5′-TAG CGACACACGTTCAGGAG-3′; *dvps35*, 5′-CTTTTCTGGAGTG GAAAGCA-3′ and 5′-TTCAGCAGCTCCACATACAA-3′; *ribosomal protein 49* (*rp49*), 5′-AAGCTAGCCCAACCTGCTTC-3′ and 5′-GTGCGCTTCTTCACGATCT-3′, 5′-ATGCTAAGCTG TCGCACAAA-3′ and 5′-GTTCGATCCGTAACCGATGT-3′ or 5′-CAAGAAGCTAGCCCAACCTG-3′ and 5′-AGCATATCGAT CCGACTGGT-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, Iowa City, IA), 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, Grand Island, NY), Cy3 and Cy5 (Jackson Immuno, West Grove, PA)-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, Basel, Switzerland) 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 h. For immunoblotting, *Drosophila* wandering third instar larvae were collected and homogenized on ice in lysis buffer (1% Triton X-100, 50 mM NaCl, 20 mM Tris at pH 7.5, and 1 mM 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, Waltham, MA). Lysates were boiled for 5 min and subjected to immunoblot analysis using the following primary antibodies: mouse anti-V5 (1:3,000, Invitrogen) and mouse anti-β-Actin (1:5,000, Abcam, Cambridge, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno) were used at a dilution of 1:3,000.

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 10× dry, 20× dry, 40× oil, and 63× 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 (Fig. 1A,B). Similarly, when knocking down $dvps35$ or another retromer component $dvps26$ by RNA interference (RNAi) using the ubiquitous driver *tubulin*-Gal4 (tubGal4-$dvps35$RNAi), we also observed melanotic aggregates (Fig. 1C and data not shown for $vps26$RNAi). 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
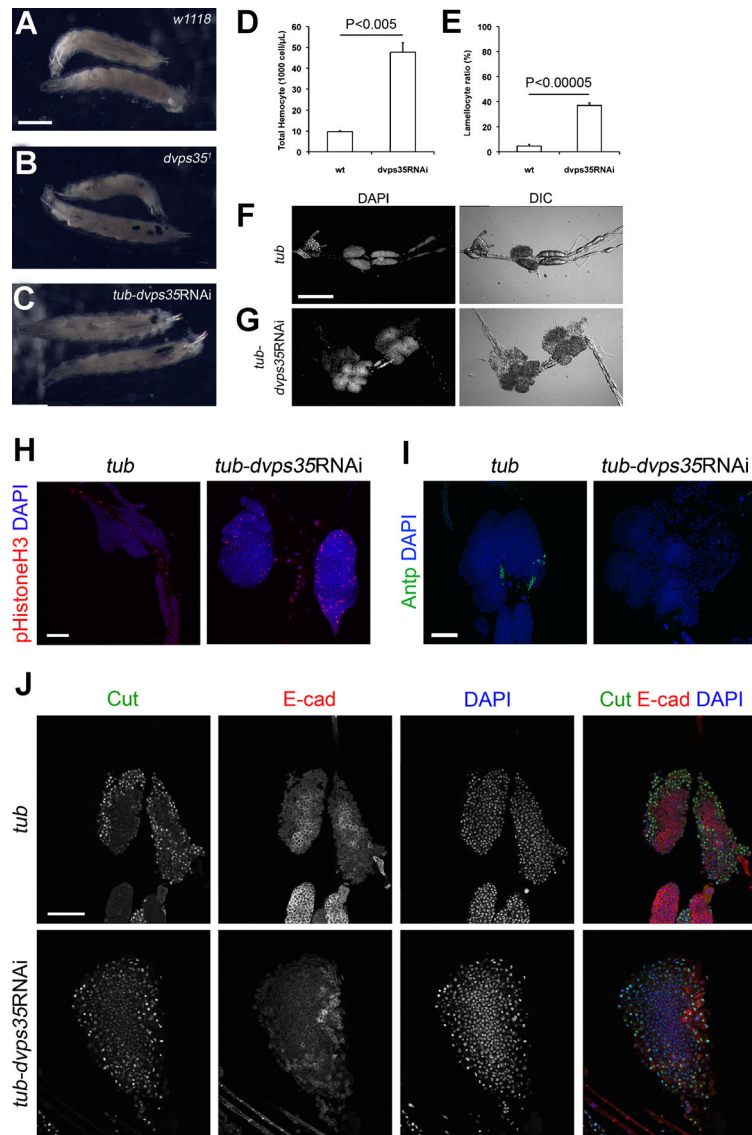
Fig. 1. Loss of retromer activity activates the cellular immune response in *Drosophila* prior to infections. Third instar larvae of (A) wild type, (B) *dvps35* mutants, and (C) *tubGal4-dvps35*RNAi. Scale bar, 1 mm. D: Total hemocyte count of wild type and *tubGal4-dvps35*RNAi third instar larvae. E: Lamellocyte ratio in total hemocytes of wild type and *tubGal4-dvps35*RNAi third instar larvae. Lymph glands of (F) *tub-Gal4* and (G) *tub-Gal4-dvps35*RNAi third instar larvae. Anterior, left. Scale bar, 200 μm. H: phospho-Histone H3 and DAPI staining in lymph glands of *tub-Gal4* and *tub-dvps35*RNAi third instar larvae. I: Antp and DAPI staining in lymph glands of *tub-Gal4* and *tubGal4-dvps35*RNAi third instar larvae. J: Cut, DE-cadherin, and DAPI staining in lymph glands of *tub-Gal4* and *tubGal4-dvps35*RNAi third instar larvae. Anterior to the top in all images in H–J. Scale bar in H–J, 50 μm.

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; Luo et al., 1995; Qiu et al., 1998; Minakhina and Steward, 2006; Lemaitre and Hoffmann, 2007). In *tubGal4-dvps35*RNAi larvae, the total hemocyte count and the ratio of lamellocytes were significantly enhanced compared to the wild type (Fig. 1D,E). *tubGal4-dvps35*RNAi larvae also displayed lymph gland hypertrophy when compared to the control *tubGal4* larvae (Fig. 1F,G). Phospho-histone H3 staining suggested a higher proliferation rate in lymph glands in *tubGal4-dvps35*RNAi larvae than that in control (Fig. 1H). We also observed an expansion of the cortical zone, a reduction of the medullary zone and an absence of the posterior signalling center (Fig. 1I,J), 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* (*IM1*) were highly expressed in whole larvae of *dvps35* mutants (Fig. 2A). Furthermore, *Drs* and
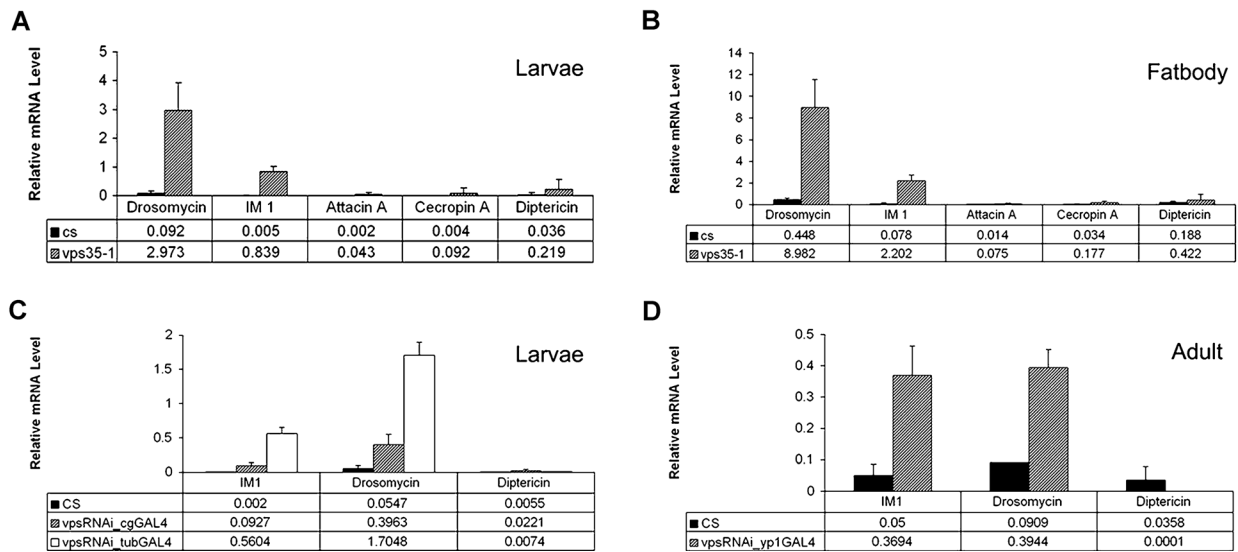
Fig. 2. Loss of retromer activity activates the humoral response regulated by Toll. mRNA expression level assayed by q-PCR of Toll or IMD pathway-regulated antimicrobial peptide genes in (A) whole larvae or (B) larval fat body of wild type and *dvps35* mutants. CS, Canton-S. *Drosomycin* and *IM1* are Toll-regulated genes. *AttacinA*, *CecropinA*, and *Diptericin* are IMD-regulated genes. *Ribosomal protein 49* (*rp49*) serves as internal control. Expression level of Toll or IMD pathway-regulated antimicrobial peptide genes in (C) larvae or (D) adult of wild type, *cgGal4-dvps35*RNAi, *tubGal4-dvps35*RNAi and *yp1Gal4-dvps35*RNAi.

IM1 were greatly induced in the dissected larval fat bodies of *dvps35* mutant larvae (Fig. 2B). Consistent with null mutants, induction of *Drs* and *IM1* was also observed in whole larvae, knocking down *dvps35* by RNAi driven by *cgGal4* (fat body and hemocyte expression) or *tub*Gal4 (ubiquitous expression) (Fig. 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 *IM1* expression after *dvps35*RNAi (Fig. 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 *IM1* 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 *IM1* was highly activated in the absence of *dvps35* function, the expression of *AttacinA*, *CecropinA*, and *Diptericin* was not significantly changed (Fig. 2A–D). 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 (Fig. 1) (Qiu et al., 1998; Huang et al., 2005).

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 (Fig. 3A). Similarly, DIF was also localized in the nuclei of the fat bodies of *dvps35* mutants (Fig. 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-dvps35*RNAi larvae, knocking down *stat* (*tubGal4-stat*RNAi-*dvps35*RNAi), which encodes a transcriptional factor in the JAK/STAT pathway, neither rescued the generation of melanotic aggregates (Fig. 4A), nor suppressed the overexpression of Toll-regulated antimicrobial peptide genes (*Drs* and *IM1*, Fig. 4B–C). As controls, the knock down of *vps35* and *stat* was highly efficient (Fig. 4D–E). 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, supporting 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
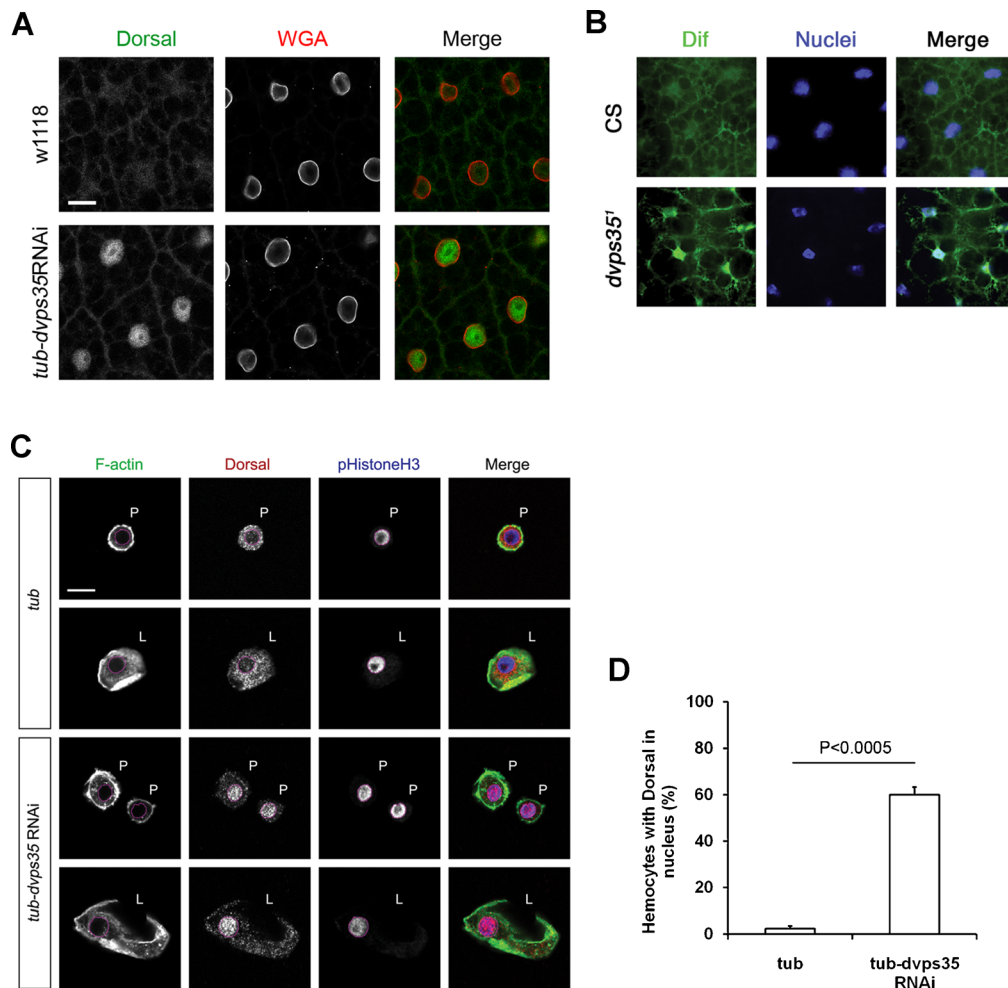
Fig. 3. Loss of retromer activity activates the Toll but not IMD pathway. A: Staining of Dorsal and nuclear membrane (WGA) in the fat body of wild type (*w1118*) and *tubGal4-dvps35*RNAi third instar larvae. Scale bar, 20 μm. B: Staining of DIF and DAPI in the adult fat body of wild type (CS) and *dvps35* mutant. CS, Canton-S. C: Staining of F-actin, Dorsal and phospho-Histone H3 in hemocytes of *tubGal4* and *tubGal4-dvps35*RNAi third instar larvae. P, plasmatocytes; L, lamellocytes; red dotted circles, nuclei. Scale bar, 10 μm. D: Ratio of hemocytes with Dorsal in the nuclei in *tubGal4* and *tubGal4-dvps35*RNAi third instar larvae.

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 (*Tl*[r3], *Tl*[RxA], *Spz*[2], and *Spz*[rm7]) and two independent lines of Spz RNAi to reduce the activity of these two genes. In *tubGal4-dvps35*RNAi larvae, reducing the activity of Toll (*Tlr3/TlRxA*) efficiently suppressed the induction of Toll-regulated antimicrobial peptide genes (Fig. 5A,B). Similarly, reducing the activity of Spz (*Spz*[2]/*Spz*[rm7], and Spz RNAi) also gave rise to an efficient suppression in *tubGal4-dvps35*RNAi larvae (Fig. 5C–H). It is note worthy that the knock down of *dvps35* caused a modest increase of Spz transcript (Fig. 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-dvps35*RNAi larvae, knocking down SPE by two independent RNAi lines (driven by *tub-Gal4*) did not suppress the induction of Toll-regulated antimicrobial peptide genes (Fig. 5I–L). We noted that again there was an enhanced expression of SPE in *tubGal4-dvps35*RNAi (compared to *tubGal4*, Fig. 5K), suggesting a positive feedback regulation of the SPE gene after activation of Toll (Lemaitre et al., 1996; Jang et al., 2006). 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
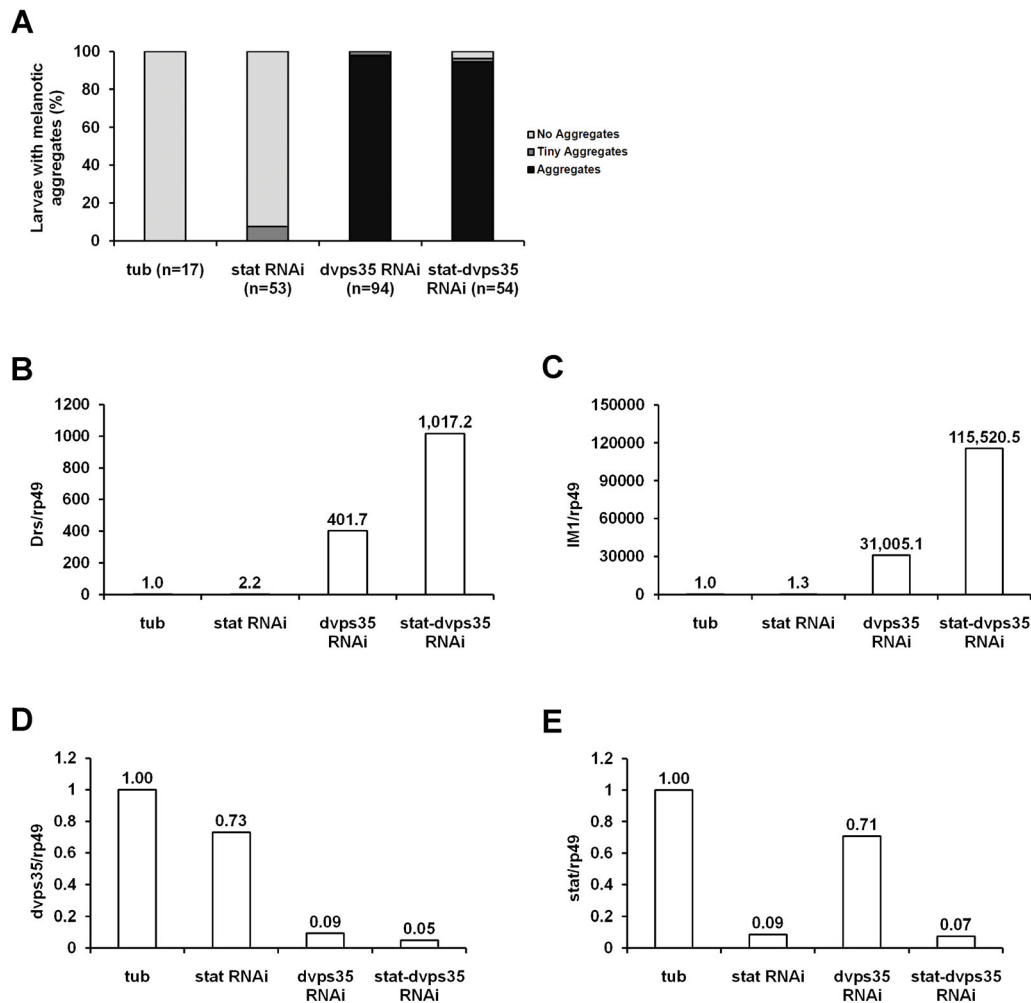
Fig. 4. The JAK-STAT pathway is not essential for the generation of melanotic aggregates or the activation of the Toll pathway in the absence of dVps35 activity. A: Ratio of third instar larvae with melanotic aggregates in *tubGal4*, *tubGal4-stat*RNAi, *tubGal4-dvps35*RNAi, and *tubGal4-stat*RNAi-*dvps35*RNAi. Expression level of (B) *Drs*, (C) *IM1*, (D) *dvps35*, and (E) *stat* in third instar larvae of *tubGal4*, *tubGal4-stat*RNAi, *tubGal4-dvps35*RNAi, and *tubGal4-stat*RNAi-*dvps35*RNAi.

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 (Fig. 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 (Fig. 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 kDa C-terminal fragment of 106 amino acids that binds to Toll (Weber et al., 2003; Hu et al., 2004; Lemaitre and Hoffmann, 2007). Nonetheless, multiple processed forms with sizes smaller than the full-length 37 kDa precursor but

larger than the 16 kDa final product were previously observed during both early embryogenesis and infections correlating with the activation of the Toll pathway (Morisato and Anderson, 1994; Levashina et al., 1999). Here we observed a similar cleavage pattern of Spz during infections and in *tubGal4-dvps35*RNAi. In larvae with Gram[+] bacterial infections, which activate the Toll pathway, Spz was mainly cleaved into two bands migrating around 30 kDa (indicated by the bracket in Fig. 6C). The putative active form of Spz (16 kDa, indicated by an [*] in Fig. 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 kDa range was also seen in *tubGal4-dvps35*RNAi larvae, with no detectable 16 kDa form of Spz. The very low or undetectable level of the 16 kDa form of Spz observed during infections or in *tubGal4-dvps35*RNAi is possibly due to a rapid degradation when in complex with Toll. The two bands of Spz at 30 kDa 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
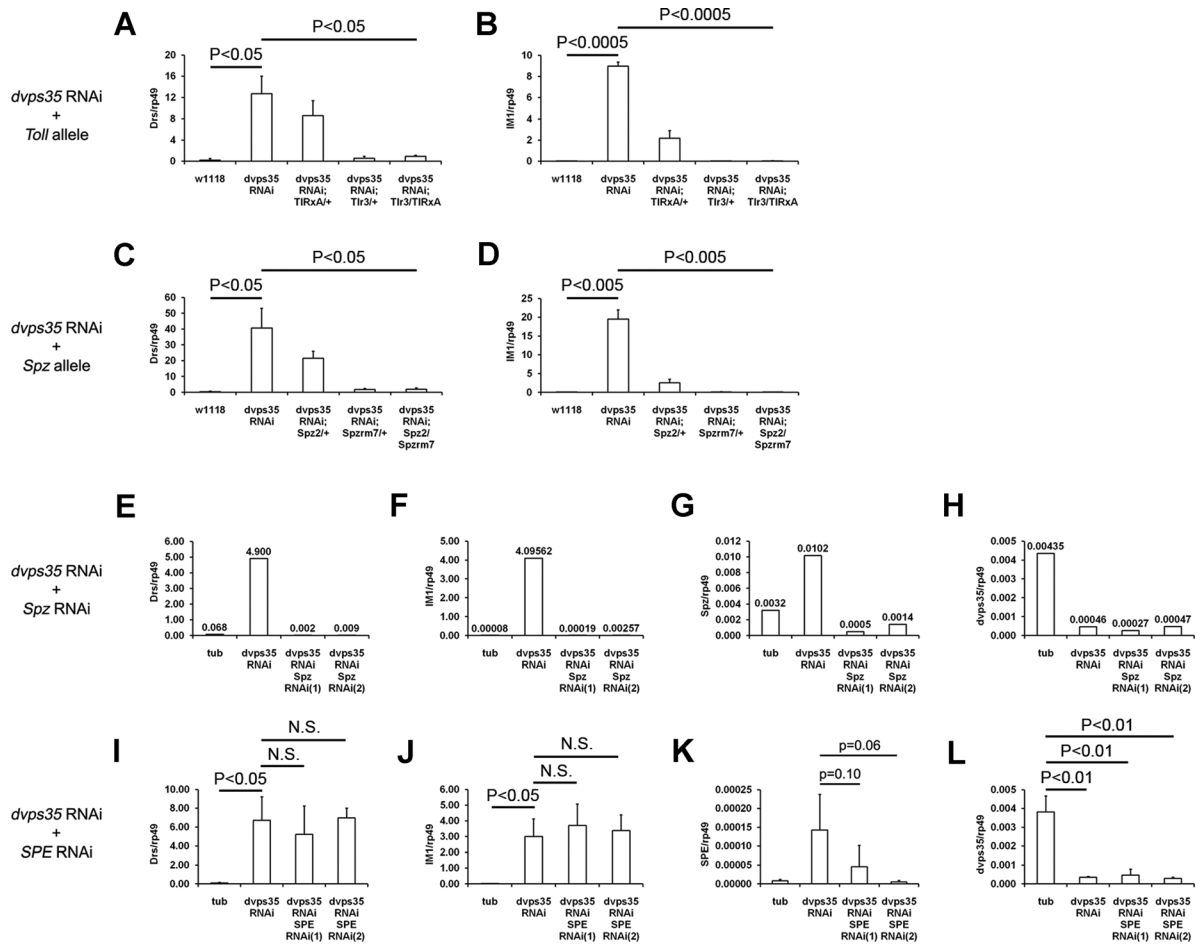
Fig. 5. Genetic epistasis analysis places the action of retromer at the level of Spz. Expression level of (A) *Drs* and (B) *IM1* in third instar larvae of wild type and *tubGal4-dvps35*RNAi with different combinations of *Toll* hypomorphic (*Tl³*) or null (*Tl^RxA*) alleles. Expression level of (C) *Drs* and (D) *IM1* in third instar larvae of wild type and *tubGal4-dvps35*RNAi with different combinations of *Spz* null alleles (*Spz²* and *Spz^rm7*). Expression level of (E) *Drs*, (F) *IM1*, (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). Expression level of (I) *Drs*, (J) *IM1*, (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).

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 *dvps35* 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 (Fig. 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
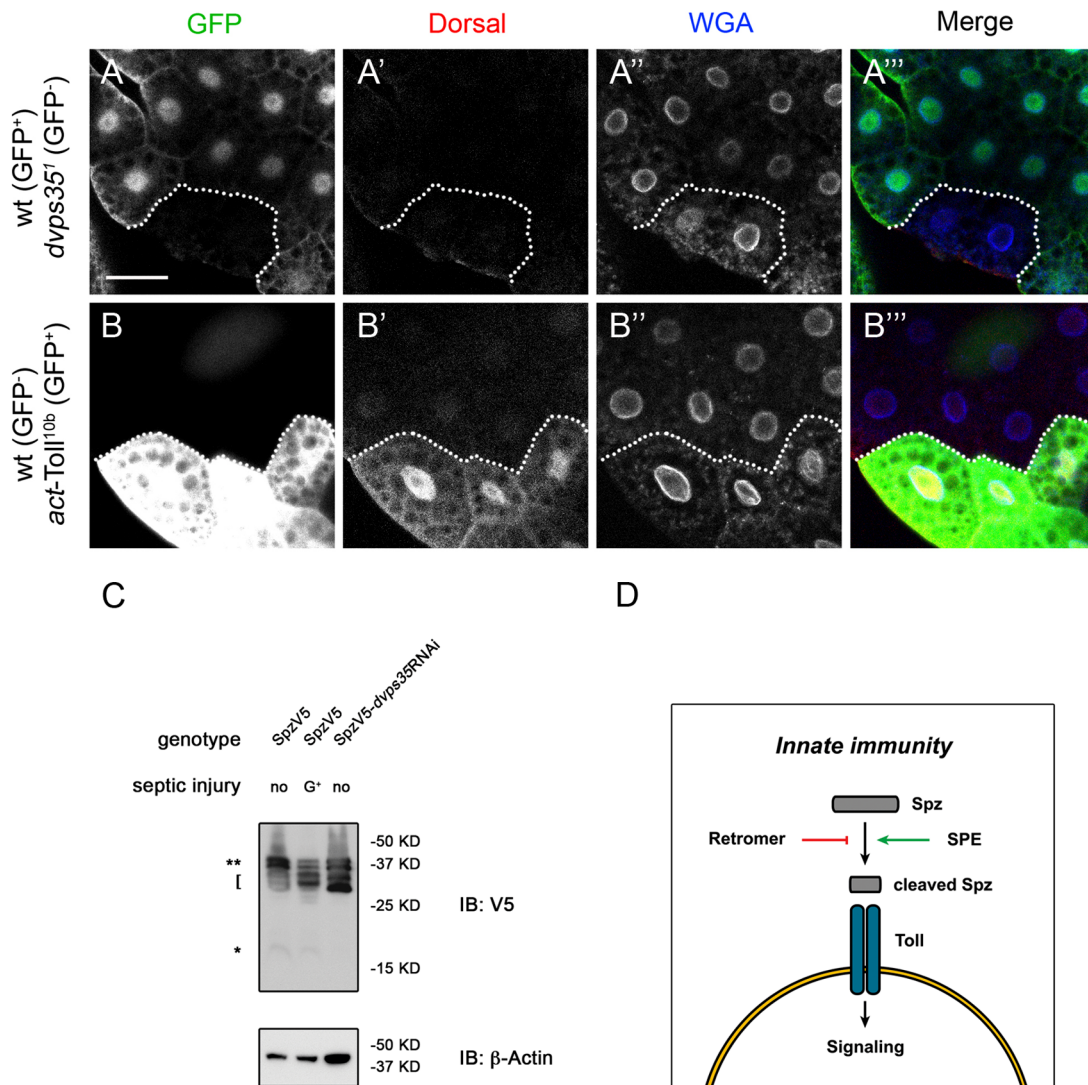
Fig. 6. Retromer regulates the maturation/processing of the ligand Spz. A–A''': Staining of GFP, Dorsal and nuclear membrane (WGA) in larval fat bodies containing mosaic clones of *dvps35* mutant cells. *dvps35* mutant cells are marked by absence of GFP. B–B''': Staining of GFP, Dorsal and nuclear membrane (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 μm. 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-*dvps35*RNAi (no infection). **Full length Spz. Bracket, two-band pattern around the 30 kDa range of cleaved Spz. *16 kDa C-terminal fragment of Spz. D: A model illustrating retromer suppresses the activation of Spz independently of SPE.

retromer-dependent Spz maturation is part of the activation mechanism of the Toll pathway during infections.

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