Figures and figure supplements

Plasma membrane overgrowth causes fibrotic collagen accumulation and immune activation in *Drosophila* adipocytes

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Figure 1. Endocytic defects cause Collagen accumulation in Drosophila adipocytes. (A) Schematic depiction of Collagen IV production, secretion and incorporation into basement membranes. (B) *shibire* knock-down (*BM-40-SPARC*<sup>shii</sup>) and dominant negative *shibire*<sup>K44A</sup> (*BM-40-SPARC*<sup>shiDN</sup>) cause Vkg-GFP accumulation in third instar larva adipocytes (marked with RFP). (C) Confocal images of third instar larva adipocytes stained with anti-Dynamin antibody. Staining is absent upon *shi* knock-down and increased by *shiK44A* expression. Nuclei stained with DAPI. (D) Localization of Vkg-GFP and Cg25C (anti-Cg25C staining) in wild type and *BM-40-SPARC*<sup>shii</sup> adipocytes. Collagen IV accumulates in the periphery of *shii* adipocytes. (E) Vkg accumulation in...
BM-40-SPARC>Rab5i and >Rab5DN adipocytes. (F) Vkg accumulation in BM-40-SPARC>Chci and >Chci<sub>DN</sub> adipocytes. (G) Presence of Vkg-GFP is reduced in discs from BM-40-SPARC>shi<sub>i</sub>, >shi<sub>i</sub>DN, >Rab5i and >Rab5DN larvae. Vkg-GFP decrease quantified in graph. n ≥ 5 for each genotype. Differences with wild type are in all cases significant (Mann–Whitney tests, **p < 0.01). (H) Elongation of the ventral nerve cord (VNC) in BM-40-SPARC>shi<sub>i</sub>DN and >Rab5DN.

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**Figure 1**—figure supplement 1. (A) Confocal images of adipocytes from shi<sub>1</sub> and shi<sub>2</sub> thermosensitive mutants. Shifting larvae to restrictive temperature for 3 hr causes mild pericellular accumulation of Collagen IV (Vkg-GFP in green), myr-RFP membrane marker in red. (B) Western blots of hemolymph probed with an anti-Cg25C antibody (1:5000). Hemolymph was collected by turning 10 larvae inside-out inside 100 μl of PBS. 10 μl of 2-Mercaptoethanol-reduced sample (equivalent to the blood of 1 larva) were loaded per genotype. We bled wild type larvae (w<sup>1118</sup>) and larvae where vkg or Cg25C were knocked down in adipocytes (Cg<sup>><sub>vkgi</sub></sup>+tub-GAL80<sub>ts</sub> and Cg<sup>><sub>Cg25Ci</sub></sup>+tub-GAL80<sub>ts</sub> respectively). For vkg and Cg25C knock-down, and in order to circumvent embryonic/L1 lethality, temporary inhibition of GAL4-driven knock-down was achieved with thermosensitive GAL4 inhibitor tub-GAL80<sub>ts</sub> (larvae were grown at 18°C to prevent knock-down, transferred to 30°C to initiate knock-down in L1/L2 stage and bled 3 days later in L3 stage). Note that knock-down of Vkg increases Cg25C signal, expected as monomeric Cg25C cannot be incorporated into BMs in the absence of Viking (Pastor-Pareja and Xu, 2011). (C) Pencellular Vkg accumulation in adipocytes from BM-40-SPARC>Hrs<sup>i</sup>, >RN-trei, >AP-2α<sup>i</sup> and >AP-2μ<sup>i</sup> larvae. (D) Western blots of hemolymph extracted from wild type (w<sup>1118</sup>), BM-40-SPARC>shi<sub>i</sub>, >cact<sub>i</sub> and >Tl10B<sub>ts</sub> larvae probed with an anti-GFP antibody (1:5000). The amount of blood loaded in each well is equivalent to 1 larva.

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Figure 1—figure supplement 2. UAS-Dcr2 expression (BM-40-SPARC-Gal4>UAS-Dcr2) does not affect Collagen IV localization (Vkg-GFP) in adipocytes compared to Vkg-GFP control larvae (+) and larvae expressing GAL4 but not Dcr2 (BM-40-SPARC-Gal4).
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Figure 2. Collagen accumulation in endocytosis-defective cells is pericellular and autonomous. (A) Vkg-GFP accumulation in a shi adipocyte (BM-40-SPARC>shi) expressing membrane marker myr-RFP. (B) shi adipocyte stained with phallloidin (F-actin). (C) shi adipocyte stained with cell-impermeable membrane dye FM4-64, labelling plasma membrane (PM) around accumulations. (D) shi adipocyte stained with fixable Texas-Red-coupled Dextran (70,000 MW), labelling PM around accumulations. (E) Antibody stainings of wild type and shi adipocytes performed without permeabilization (no detergent) in order to detect extracellular Collagen IV. In contrast to the accumulations in shi adipocytes, intracellular accumulations of Collagen IV in Tango1 adipocytes cannot be stained in the absence of permeabilization and are shown as a control. (F) Mosaic fat body (act-GAL4>shi flip-out clones, marked with RFP) showing Vkg-GFP accumulation in shi cells. Accumulation is suppressed by a GFP-targeting dsRNA (iGFPi). (G) Pericellular accumulation in mosaic shi fat body expressing Cg25C-RFP. Clones marked with GFP. (H) Localization of the endocytic marker Transferrin Receptor (Cg>TfR-GFP) in wild type adipocytes. (I) In shi adipocytes, TfR concentrates in PM pockets containing Collagen IV (anti-Cg25C). (J) In Rab11 adipocytes, TfR localizes to intracellular vesicles that completely fill the cytoplasm. No TfR is detected at the PM.

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Figure 2—figure supplement 1. (A) Confocal images showing the PM of shi' and T108 adipocytes stained with fixable Texas-Red-coupled Dextrans (3000 and 70,000 MW), labelling PM around Collagen IV (Vkg-GFP)
Figure 2—figure supplement 1. Continued

(B) Electron micrographs of the PM in BM-40-SPARC>shii adipocytes showing instances of connection (arrows) between the pericellular accumulations (asterisks) and the extracellular space showing that these pockets are not isolated cisternae, but part of a very intricate PM. (C) Confocal images of wing discs (posterior ventral hinge) showing localization of Cg25C-GFP and Cg25C-RFP in the basement membrane after expression in the fat body controlled by Cg-GAL4 (Cg> Cg25C-GFP and Cg> Cg25C-RFP respectively). Images of wild type (w1118) discs are shown as controls to exclude auto-fluorescence. Nuclei stained with DAPI (blue). (D) Images of live larvae expressing Cg25C-RFP and Cg25C-GFP in the fat body (Cg> Cg25C-GFP and Cg> Cg25C-RFP). Knock-down of PH4εEFB, required for Collagen IV trimerization, causes tagged Cg25C to accumulate in the blood (note strong fluorescent signal filling the body cavity).

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Figure 3. Pericellular Collagen trapping is due to PM overgrowth. (A) Confocal sections of adipocyte PM (myr-RFP marker). PM expansion is observed in BM-40-SPARC>shii, >Rab5 and >Chc adipocytes, whereas PM flattening occurs in >Rab11 adipocytes along with accumulation of intracellular vesicles. (B) Electron micrographs of adipocyte PM. Figure 3. continued on next page.
Figure 3. Continued

PM from control, BM-40-SPARC>shi', >Rab5', >Chc' and >Rab11' larvae. Internal cell volume indicated through yellow transparency. Asterisks mark pericellular deposits. (C) Quantification of PM depth and sinuosity (see Figure 3—figure supplement 2). Depth measurements obtained from confocal (n = 12) and electron (n = 10) micrographs. PM sinuosity is the ratio between the length of PM between two points on that membrane and the linear distance separating them (n ≥ 15). Differences with controls were significative as indicated (Mann-Whitney tests, **p < 0.01, ***p < 0.001). (D) Adipocytes from wild type larvae grown on lipid-depleted food, BM-40-SPARC>shi' larvae and BM-40-SPARC>shi' larvae grown on lipid-depleted food. Vkg-GFP accumulation and PM excess are both suppressed by lipid-depletion.

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Figure 3—figure supplement 1. (A) Confocal images of fat body dissected from first, second and third instar larvae. myr-RFP membrane marker in white. PM convolution is apparent in third instar larvae. (B) Confocal (BODIPY staining) and electron micrographs showing surface lipid droplets (arrows) surrounded by PM in larva 3 adipocytes. (C) Electron micrographs of the PM of adipocytes from control w1118, BM-40-SPARC>shii, >Chc and >Rab5 third instar fat body. Intracellular volume indicated with a transparent yellow layer in right panels. Pericellular deposits of Figure 3—figure supplement 1. continued on next page.
Figure 3—figure supplement 1. Continued

extracellular material marked by asterisks. (D) Electron micrographs of the PM of adipocytes from larvae grown in lipid-depleted medium.

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**Figure 3—figure supplement 2.** Schematic explanation of PM sinuosity and depth measurements performed in electron micrographs. See ‘Materials and methods’ section.

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Figure 4. Perlecan, like Collagen IV, originates in the fat body. (A) Schematic representation of the in vivo YFP interference strategy (iYFPi) to knock-down expression of YFP-trapped Perlecan (Trol-YFP) and ascertain its tissue of origin. Expression of a short hairpin RNA targets the YFP sequence in the YFP-trapped mRNA for degradation through RNAi. (B) Localization of Perlecan (Trol-YFP trap) in wing discs from trolCPT1-002049/Y flies. iYFPi in the fat body (BM-40-SPARC>iYFPi) eliminates expression of Trol-YFP in the wing disc and produces tissue hyperconstriction, a previously described trol loss-of-function phenotype (Pastor-Pareja and Xu, 2011). Phalloidin staining of F-actin in red to reveal disc deformation.

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Figure 5. Pericellular deposits in adipocytes are fibrotic. (A) Localization in wild type and BM-40-SPARC>shii adipocytes of Trol-YFP (trolCPTI-002049; see Figure 4). Perlecan accumulates in >shii adipocytes. (B) Perlecan accumulation in BM-40-SPARC>shii and >Rab5' adipocytes. (C) Perlecan accumulation in r4>shii adipocytes is suppressed by Collagen IV knock down. (D) Pericellular Nidogen accumulation (anti-Ndg staining) in r4>shii adipocytes is suppressed by Collagen IV knock down. (E) Pericellular Vkg accumulation in BM-40-SPARC>shii adipocytes is suppressed by knocking down prolyl-hydroxylase PH4αEFB. (F) BM-40-SPARC>shii adipocytes do not accumulate secretion marker secr-GFP. Intracellular secr-GFP retention in BM-40-SPARC>Rab1' is shown as a control.

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Figure 5—figure supplement 1. (A) PM of fat body adipocytes stained with anti-Trol antibody (magenta). Pericellular accumulation of Perlecan (Trol) in r4>shii adipocytes is suppressed by additionally knocking down expression of vkg and Cg25C Collagen IV chains. (B) Localization of 26-29-protease (26-29-pCA06735 GFP-trap) and Ferritin 1HCH (Fer1HCHG188 GFP-trap) in adipocytes from wild type L3 larvae, Cg>shii L3 larvae and Cg>sec23 L2 larvae. Note intracellular accumulation upon sec23 knock-down, which confirms that 26-29-p and Fer1HCH are indeed adipocyte-secreted. (C) Western blots of hemolymph extracted from wild type (w1118), Fer1HCHG188, Drs-GFP, BM-40-SPARC>secr-GFP and 26-29-pCA06735 larvae probed with anti-GFP antibody (1:5000). The amount of blood loaded in each well is equivalent to 2.5 larvae. Whereas Fer1HCH-GFP (expected molecular weight 50 kDa), Drs-GFP (34 kDa) and secr-GFP (27 kDa) are detected in the hemolymph as clear single bands, 26-29-p (87 kDa) seems to be processed. (D) Dorsal view of a live larva expressing secr-GFP in adipocytes (BM-40-SPARC>secr-GFP) showing accumulation of GFP in pericardial cells, a hemolymph filtering nephrocyte-like cell type.

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Figure 6. Fibrotic deposits and PM overgrowth upon Toll activation. (A) Pericellular Vkg deposits (Vkg-GFP) and PM overgrowth in BM-40-SPARC>cact adipocytes. (B) Vkg deposits and VNC elongation in BM-40-SPARC>Tll06 larvae. (C) Vkg deposits and PM overgrowth in adipocytes 1 day after infection with Micrococcus luteus. (D) Electron
Figure 6. Continued

Micrographs of BM-40-SPARC>cact and >T10B adipocytes. The arrow marks connection of the deposits to the extracellular space. (E) Measurements of PM depth and sinuosity in adipocytes of indicated genotypes. Depth measurements obtained from confocal (n ≥ 7) and electron micrographs (n ≥ 10). Sinuosity measured in electron micrographs (n ≥ 15). Differences with wild type or appropriate control as indicated were significant in all cases (Mann–Whitney tests, **p < 0.01, ***p < 0.001). (F) Pericellular Vkg accumulation in BM-40-SPARC>cact and >T10B adipocytes is suppressed by knocking down prolyl-hydroxylase PH4αEFB. (G) Secretion marker secr-GFP does not accumulate in BM-40-SPARC>cact or >T10B adipocytes. (H) Pericellular Perlecan deposits (Trol-YFP) in BM-40-SPARC>cact and >T10B adipocytes. (I) Induction of antimicrobial peptide Drosomycin (Drs-GFP) fills adipocyte cytoplasm in BM-40-SPARC>cact, >T10B and Micrococcus luteus-infected larvae. (J) Rab1 knock-down causes intracellular Drosomycin retention and suppresses PM overgrowth in BM-40-SPARC>T10B adipocytes.

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Figure 6—figure supplement 1. (A) Nuclear accumulation of the Toll downstream transcription factor Dorsal (anti-Dorsal staining) in BM-40-SPARC>cacti adipocytes. (B) Pericellular Cg25C accumulation in adipocytes of mutant cact4 over cact-uncovering deficiency Df(2L)r10. (C) Electron micrographs of the PM of BM-40-SPARC>cact and >Toll108 adipocytes. Asterisks mark pericellular deposits. Arrows mark visible connections of the deposits to the extracellular space.

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Figure 6—figure supplement 1. Continued

space. (D) Antibody stainings of cact' and Tl108 adipocytes performed without permeabilization (no detergent) in order to detect extracellular Collagen IV. (E) Knock-down of Dif decreases the expression of Toll target gene Drosomycin (Drs-GFP) activated by cact' and Tl108 (BM-40-SPARC>cact' and >Tl108 adipocytes). (F) Localization of endocytic marker TfR-GFP in wild type, Cg>sh and Cg>cact' adipocytes. Intracellular TfR vesicles are seen in cact' adipocytes, same as wild type. (G) Drosomycin-containing vesicles (Drs-GFP) in the cytoplasm of BM-40-SPARC>Tl108 adipocytes. (H) Pericellular retention of Collagen IV (Vkg-GFP) at the PM of adipocytes from larvae dissected 1 or 2 days after Micrococcus luteus infection.

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Figure 7. Immune response to fibrotic deposits. (A) Melanotic fat body in a *ppb>shibe* fly. (B) Melanized fat body from an *rp4>shibire* larva. Hemocytes (blood cells) encapsulate the tissue. (C) Knock-down of Collagen IV reduces fat body melanization in *rp4>shibire* larvae. Cultures maintained at 30˚C. (D) Percentage of larvae displaying signs of melanization in indicated genotypes. n ≥ 30 per genotype. Differences with *rp4>shibire* and >Tp{R} controls were significative (χ² tests, ***p < 0.001). Cultures maintained at 30˚C. (E) Induction of c-Jun N-terminal kinase (JNK) downstream *puckered* (*puc-GFP enhancer trap*) in BM-40-SPARC>*shibire*, >Tp{R} and >cacti adipocytes. (F) Induction of Matrix Metallo-

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Figure 7. Continued
Protease 1 (anti-Mmp1 staining) in BM-40-SPARC>shi/>, >T108 and >cact adipocytes. (G) Expression of JAK/STAT activity reporter 10XSTAT-GFP in BM-40-SPARC>shi/>, >T108 and >cact adipocytes. (H) Expression of JAK/STAT-activating ligands in wild type, BM-40-SPARC>sh/ and >T108 adipocytes assessed by real time RT-PCR. Error bars represent 95% confidence intervals. rp49 expression was used for normalization.
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Figure 8. PM overgrowth leads to adipocyte fibrosis. Schematic representation summarizing the genesis of fibrotic deposits caused by PM excess and the ensuing reaction by the immune system. Defective endocytosis or excess secretion induced by Toll activity cause PM overgrowth in fat body adipocytes, which leads to hyperconvoluted PM morphology and pericellular trapping of Collagen IV and other extracellular matrix (ECM) proteins in the cell cortex. Fibrotic Collagen IV deposits trigger an immune response, as evidenced by tissue melanization and activation of the JAK/STAT and JNK pathways.
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Figure 8—figure supplement 1. Knock-down of BM-40-SPARC (BM-40-SPARC>BM-40-SPARC) causes PM accumulation of Collagen IV without PM overgrowth. DOI: 10.7554/eLife.07187.018