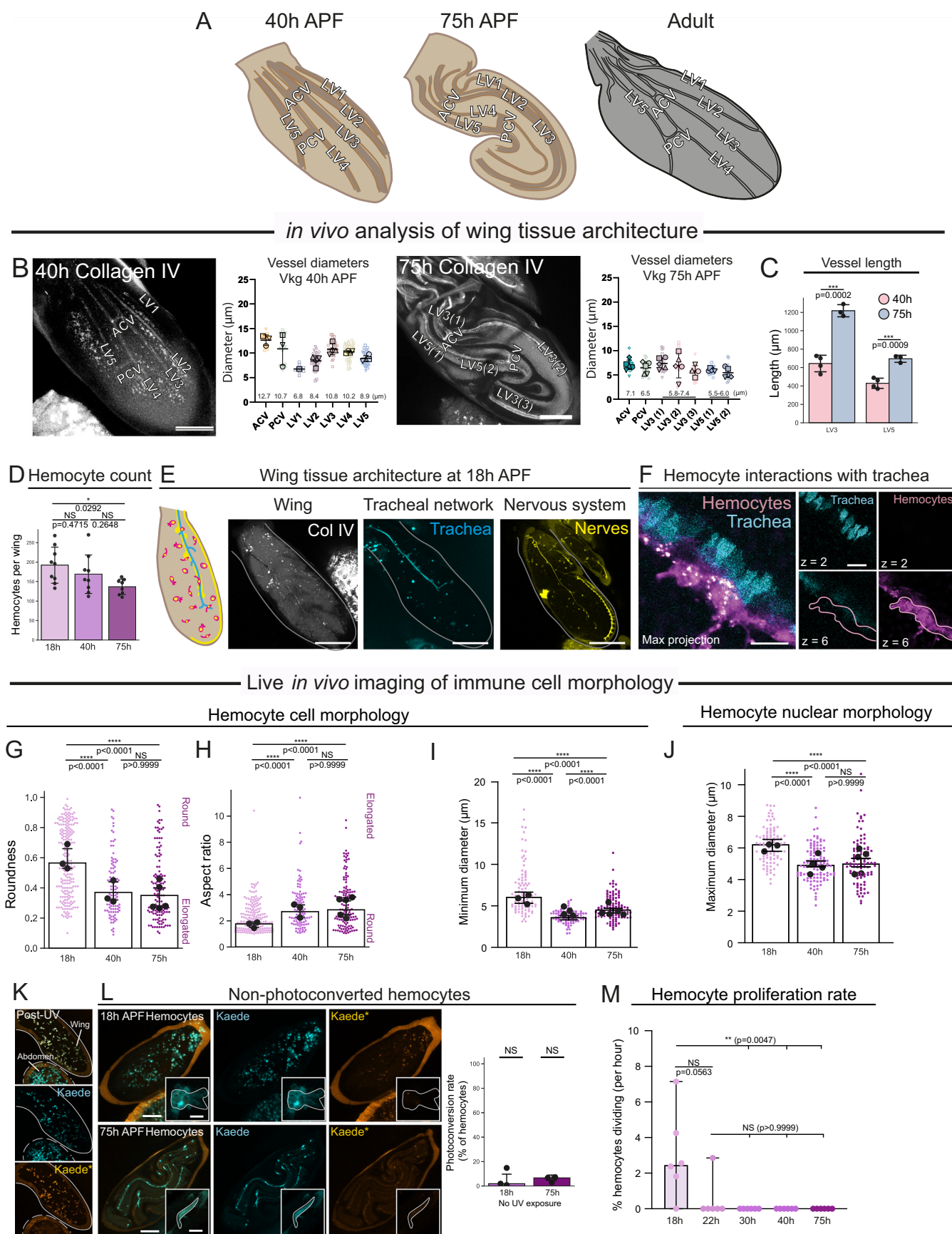


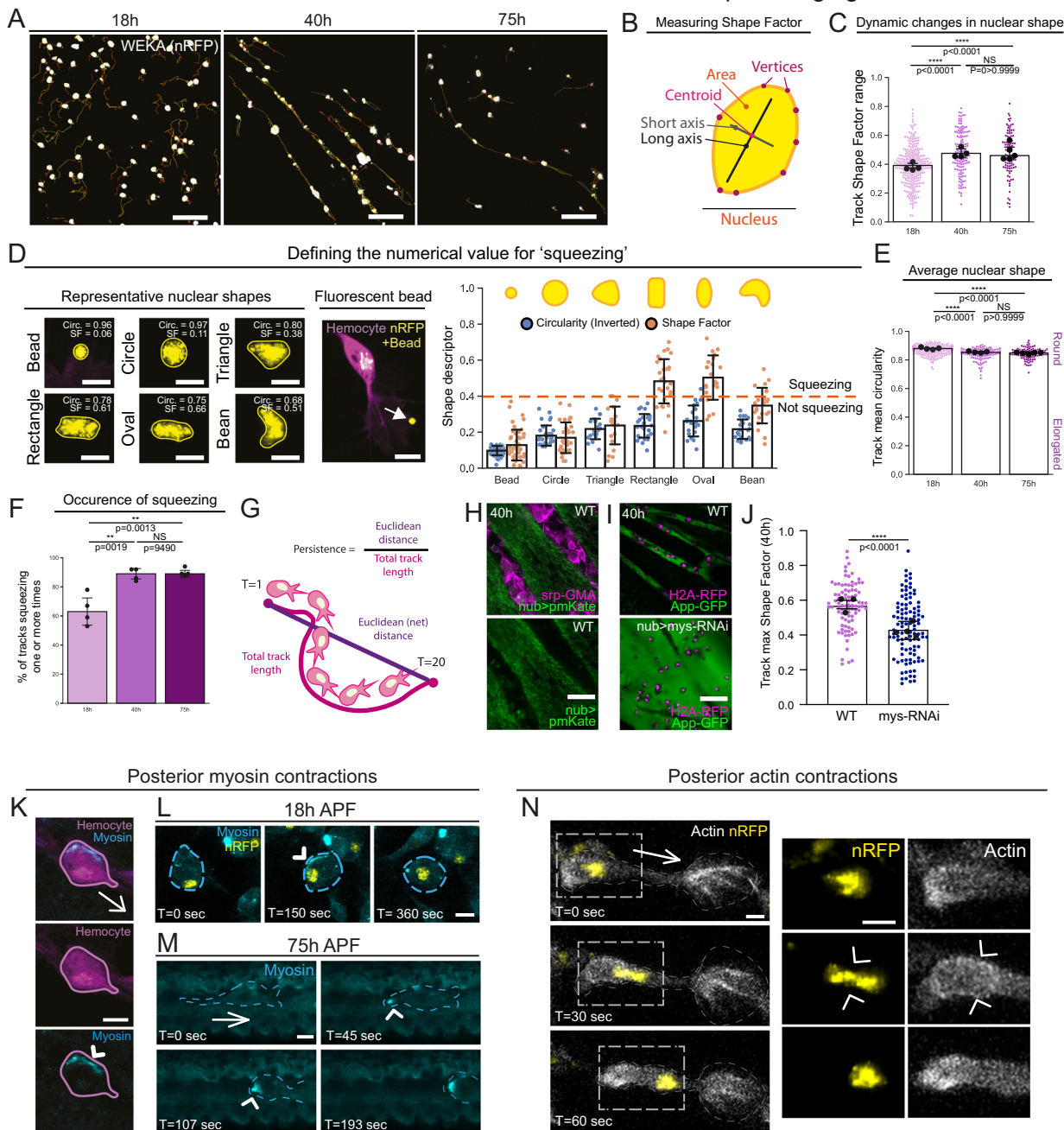
Expanded View Figures

Figure EV1. Migrating immune cells adapt upon transition into more confined vessel microenvironments in vivo.

(A) Schematics of wing vessels, labelling lateral veins (LV1-5) and anterior and posterior cross veins (ACV/PCV) at 40 h APF, 75 h APF and adults. (B) Raw data for Fig. 1G; maximum projection of Vkg-GFP pupal wings (grey) at 40 h and 75 h APF. Super-plots depict vessel widths, where solid shapes depict mean vessel diameters and hollow shapes depict individual vessel measurements. (C) Length of LV3 and LV5 vessels at 40 h APF and 75 h APF. (D) Hemocytes per wing at 18 h, 40 h and 75 h APF. (E) Schematic of tracheal and nervous system in 18 h APF wings, with imaging of Collagen IV (grey), trachea (cyan) or nerves (yellow). Wings outlined in grey. (F) Maximum projection of hemocytes (magenta) and trachea (cyan) in a 75 h APF vessel (left), single z-slices illustrate tracheal location above hemocytes (outlined in magenta). (G-J) Hemocyte cytoplasmic roundness (G), aspect ratio (H), minimum diameter (I, cell body) and maximum nuclear diameter (J). Scatter plots show small dots (hemocytes) and large black dots (wing medians), bar chart shows median and 95% CI. (K) Photoconversion of wing hemocytes: UV-exposed Kaede-expressing hemocytes captured with 488 nm (Kaede) and 561 nm (photoconverted, Kaede*). Wing outlined in solid line, abdomen in dashed line. (L) Non-UV exposed hemocytes (Kaede) at 18 h and 75 h APF. Insets depict representative hemocytes. Bar chart shows % of photoconverted hemocytes at 18 h and 75 h APF with median and 95% CI. (M) Hemocyte proliferation rate (quantified as percentage of hemocytes per pupal wing dividing per hour) with median and 95% CI. Genotypes used were *Vkg-GFP* (B-C, E), *Srp > nRFP* (D), *Btl > GFP* (E), *Elav>Tom;Simu-GFP* (E), *Btl > GFP;Srp-mChe* (F), *Srp > nRFP; Crq > GFP* (G-J, M) and *Srp>Kaede* (K, L). Data information: Scale bars represent 100 μ m (B, L), 200 μ m (E), 10 μ m (F) or 15 μ m (L, insets). Error bars show mean $\pm$ SD of wings (B-D). *N* = 3 wings (L) for 18 h and 75 h APF. *N* = 4 and 5 (B) and *N* = 4 and 3 (C) wings for 40 h and 75 h APF. *N* = 9, 9 and 8 (D), *N* = 3, 3 and 5 (G, H) wings for 18 h, 40 h and 75 h APF, respectively. *N* = 3, 4 and 5 (I, J) wings for 18 h, 40 h and 75 h APF, respectively. *N* = 6, 6, 6, 6 and 6 (M) wings for 18 h, 22 h, 30 h, 40 h and 75 h APF, respectively. *N* = 372 and 169 hemocytes for 18 h and 75 h APF (L). *N* = 216, 102 and 136 hemocytes (G, H) and *N* = 93, 83 and 111 hemocytes (I) and *N* = 95, 110 and 98 hemocytes (J) for 18 h, 40 h and 75 h APF, respectively. Unpaired t test (C), Ordinary one-way ANOVA with Turkey's multiple comparisons test (E), One-way ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons test (G-J and M) or one sample Wilcoxon Signed Rank Test (L) was used to calculate significance was used to calculate significance. Source data are available online for this figure.



Quantification of nuclear behaviour from 4D time-lapse imaging



in vivo analysis of phagosomal effects

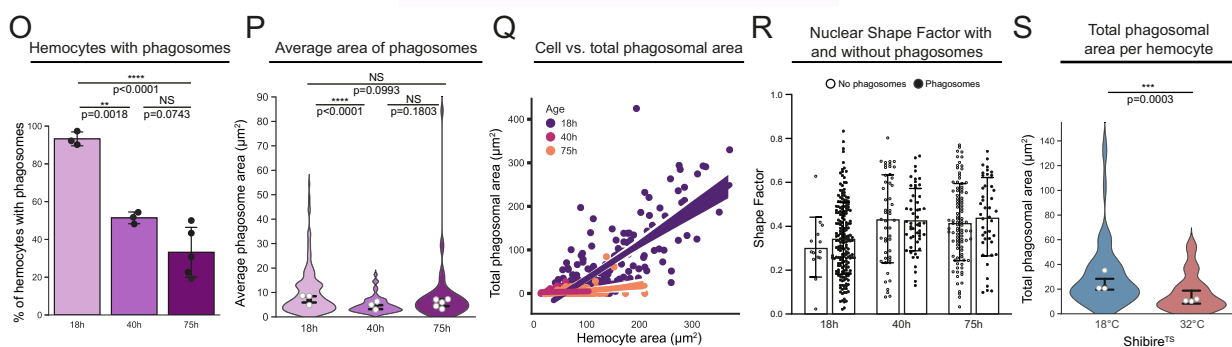
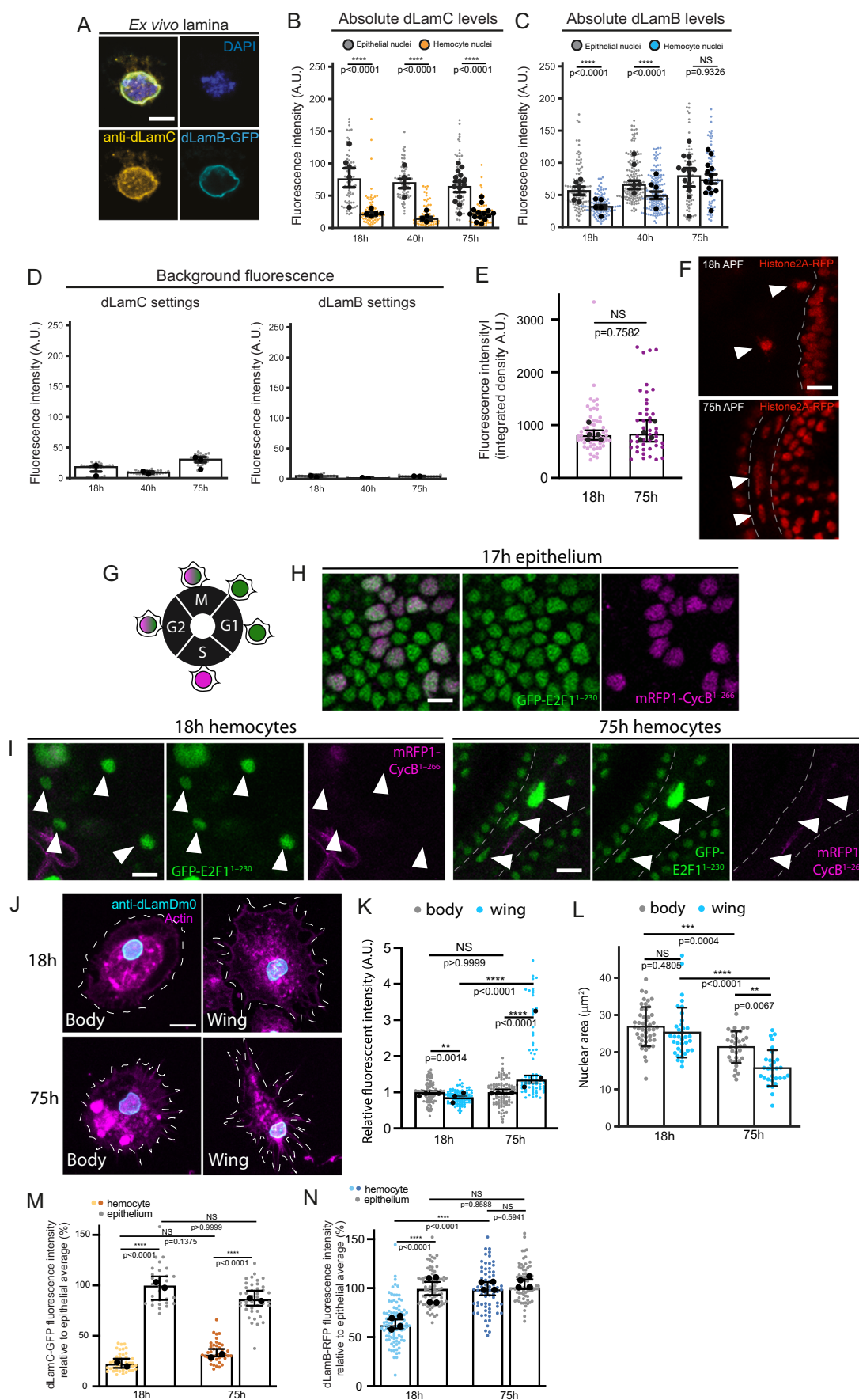


Figure EV2. Dynamic nuclear shape changes during immune cell migration in vessels in vivo.

(A) Example hemocyte nuclear migration tracks after processing with the MIA plugin from 18 h, 40 h and 75 h APF. (B) A custom shape measurement 'Shape Factor' used to measure nuclear elongation. Nuclear boundaries inferred from an input shape (WEKA segmented probability map of the nucleus) were used to create a polygon that closely approximates the nuclear shape. The vertices and the integral of the polygon area were used to infer the long and the short axis, then used to calculate a Shape Factor value between 0 (round) and 1 (elongated) (Olenik et al, 2023). (C) The range of Shape Factor values from a 20 min imaging period at 18 h, 40 h and 75 h APF. (D) Representative nuclei for the different shape categories. Image shows a hemocyte (cytoplasm in magenta, nucleus in yellow) and a fluorescent bead (arrow) in a 18 h APF pupal wing. Bar chart shows circularity and Shape Factor (SF) measurements for fluorescent beads and nuclei of different shapes, where small dots are individual nuclei. Dotted line shows the threshold set for squeezing ($SF = 0.4$); $N = > 20$ nuclei per category. (E) The average nuclear circularity measured from the same dataset of nuclear tracks as 2D. (F) Percentage of tracks (over 20 min imaging period) with at least one time-point having nuclear $SF \geq 0.4$. (G) Schematic showing how persistence, Euclidean (net) distance and total track length are measured. (H-J) Nub-Gal4 drives epithelial-specific gene expression (H); Nub-Gal4 mediated expression of mys-RNAi inhibits vessel formation (I) and reduces hemocyte nuclear deformation in 40 h APF vessels (J, bar chart with median and 95% CI). (K) 40 h APF hemocyte (cytoplasm in magenta, Myosin II in cyan) with rear localised Myosin II (arrowhead); arrow indicates the direction of migration. (L) 18 h APF hemocyte with a Myosin II flash (arrowhead) (nucleus in yellow, Myosin II in cyan). (M) 75 h APF hemocyte with rear Myosin II contraction (arrowhead) (Myosin II in cyan); related multi-channel nRFP and Myosin II images shown in Fig. 2K. (N) Actin networks around hemocyte nucleus (white arrowheads) during squeezing (actin in grey, nucleus in yellow); hemocyte outlined in grey. (O) Percentage of hemocytes with at least one phagosome at 18 h, 40 h or 75 h APF. (P) The average phagosome area per hemocyte, in those with phagosomes. (Q) Correlation between cell area and total phagosomal area. Regression fit for each age is plotted with 95% CI. (R) Nuclear Shape Factor of hemocytes with or without phagosomes at 18 h, 40 h or 75 h APF. (S) Total phagosomal area in individual *Shibire^{ts1}* hemocytes (for those with phagosomes) as measured from individual z-stacks. Cyan dashed lines mark hemocytes, and arrows indicate migration direction. Genotypes used were: *Srp > nRFP* (A, C-F), *Srp > nRFP; Crq > GFP* (D), *Nub > palm-mKate; srp-GMA* (H), *Nub > mys-RNAi; Srp-H2AmCherry, App-GFP* (I, J), *Nub-Gal4; Srp-H2AmCherry, App-GFP* (I, J), *Srp-mCherry/Sqh^{AX3}; sqh-Sqh-GFP* (K), *Srp > nRFP/Sqh^{AX3}; sqh-Sqh-GFP* (L, M), *Srp > nRFP; Srp-GMA* (N), and *Srp > GFP, SrpH2AmCherry/UAS-Shi^{TS}* (O-S). Data information: Scale bars represent 50 μ m (A), 5 μ m (D, K-N), 20 μ m (H, I). $N = 4, 4$ and 5 (C, E), $N = 4$ (F), $N = 3$ and 4 (J), $N = 3, 3$ and 5 (O-R), $N = 3$ (S) wings for 18 h, 40 h and 75 h APF, respectively. $N = 90$ and $N = 105$ hemocytes (J), $N = 286, 139$ and 102 tracks (C, E), $N = 206, 62$ and 55 hemocytes, and $1308, 79$ and 77 phagosomes measured (O) and $N = 216, 102$ and 136 hemocytes (P-R) for 18 h, 40 h and 75 h APF, respectively. $N = 95$ and 32 hemocytes and $N = 618$ and 146 phagosomes for 18 °C and 32 °C, respectively (S). Scatter plots show small dots (individual nuclear tracks) and large black dots (wing medians), and bar chart shows median and 95% CI (C, E). Bar chart shows mean $\pm$ SD (D, F, L, O, R). Violin plots depict hemocytes, with wing medians in white dots, median and 95% CI shown in black (P, S). One-way ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons test (C, E, P), Ordinary one-way ANOVA with Turkey's multiple comparisons test (F, O), or Mann-Whitney U test (J, S) was used to calculate significance. Source data are available online for this figure.



◀ **Figure EV3. Modulation of immune cell nuclear lamina composition upon transition to wing vessels.**

(A) Ex vivo hemocyte nucleus with dLamB-GFP (cyan) stained with DAPI (blue) and anti-dLamC-Alexa488 (yellow). (B, C) Raw anti-dLamC (B) or dLamB (C) fluorescence intensity of hemocyte and epithelial nuclei at 18 h, 40 h and 75 h APF. (D) Background fluorescence levels of hemocytes treated without primary antibody, imaged with anti-dLamC or dLamB settings. (E, F) Raw Histone2A-RFP fluorescence intensity of hemocyte nuclei (arrowheads) at 18 h and 75 h APF. (G-I) Nuclear Fucci labelling of cell cycle stages in epithelium (H) and hemocytes (arrowheads, I). (J-L) Ex vivo anti-dLamB (cyan) and Phalloidin (magenta) staining of wing or body hemocytes (J) with quantification of anti-dLamB fluorescence, displayed relative to that of body hemocytes (K) and hemocyte nuclear area (L). (M, N) Quantification of in vivo endogenous dLamC-GFP (M) or dLamB-RFP (N) fluorescence in hemocytes and adjacent epithelial cells, displayed relative to 18 h APF epithelial average. Genotypes used were: *Srp > nRFP; UAS-dLamB-GFP* (A), and *Srp > GFP, SrpH2AmChe* (B-D and J-L), *H2A-RFP* (E, F), *UAS-Fucci²[GFP-E2F1, mRFP1-CycB]* (H, I), *dLamC-GFP* (M) and *dLamB-tagRFP* (N). Data information: Scale bars represent 5 μ m (A, J) or 15 μ m (F, H, I). Scatter plots with small dots (hemocytes) and large black dots (wing section medians), bar chart shows median and 95% CI. *N* = 5, 4 and 14 (B), *N* = 5, 5 and 13 (C), or *N* = 2 wing sections (D) from >8 wings (B, C) or 2 wings (D) (from separate pupae) stained per age (all stains done in one session), and *N* = 74, 67 and 72 nuclei (B), *N* = 106, 151 and 84 nuclei (C), *N* = 30, 27 and 26 nuclei (D, dLamC) or *N* = 19, 33 and 35 nuclei (D, dLamB) measured for 18 h, 40 h and 75 h APF, respectively; *N* = 60 and 46 nuclei (E), *N* = 117 and *N* = 103 body hemocyte and *N* = 88 and *N* = 75 wing hemocyte nuclei (K), *N* = 48 and *N* = 34 body hemocyte and *N* = 36 and *N* = 30 wing hemocyte nuclei (L), *N* = 41 and *N* = 40 hemocyte nuclei and *N* = 30 and 40 epithelial nuclei (M) and *N* = 97 and *N* = 76 hemocyte nuclei and *N* = 86 and 77 epithelial nuclei (N) measured for 18 h and 75 h APF, respectively. Mann-Whitney U test was used to calculate significance for (B-E) and One-way ANOVA (Kruskal-Wallis) with Dunn's multiple comparisons test (K-N). Source data are available online for this figure.