Supporting Information

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SI Materials and Methods

Generation and Validation of ARF1-Specific Antibodies. The *arf1* ORF (amino acids 1–182) was cloned into NcoI–XhoI sites of pET23d expression vector. The recombinant protein was expressed in *Escherichia coli* and purified by Ni–NTA binding followed by electroelution. Polyclonal antibodies raised in rabbit, rat, and mouse were analyzed by Western blot. Both antisera and preimmune serum were treated with caprylic acid to purify IgG (1). The specificity of antisera was checked by antigen–antibody competition analysis. A total of 25 μg or 50 μg of recombinant protein was preincubated with the antibody and the blocked antibody was used to probe the Western blot containing antigen. The absence of bands in lanes 1 and 2 probed with blocked antibodies indicates specificity of the antibody for the immunogen (Fig. S1*A*). A 1:500 dilution of the caprylic-acid–purified polyclonal antiserum was used for immunostaining. Specificity of antisera was also checked by staining ARF1 knockdown lymph glands (*HmldeltaGAL4>UASArfRNAi*) with antibody. No staining signal was seen compared with controls.

To check specificity of the ARF1 band detected in coimmunoprecipitation assay, ARF1 dsRNA was transfected in S2 cells and Western blot analysis was performed. For dsRNA preparation ARF1 full-length cDNA (CG8385) was used as a template for PCR amplification with primers containing T7 promoter sequence binding site (TAATACGACTCACTATAGGG); forward primer 5′GTATCGGTGAGGCGAGAGAG3′ and reverse primer 5′ TCAGCCGCTCTTTCTATGGT3′. PCR product was verified by sequencing and used as template to produce dsRNA using Megascript RNAi kit (Ambion, Life Technologies). dsRNA (501 bp) was used to transfect *Drosophila* S2 cells (Invitrogen; R690-07) cultured in Schneider's medium (Invitrogen; 11720018) supplemented with 10% FBS (GIBCO; 10082139) and antibiotics at room temperature. A total of 1×10^6 cells per 35-mm dish were transfected with 3 μg or 5 μg dsRNA using Effectene reagent (Qiagen; 301425). Cells were harvested for Western blot analysis on day 6. Cells were pelleted, washed with PBS, and lysed with lysis buffer at 4 °C. Proteins were separated on 12% SDS/PAGE, blotted on nitrocellulose membrane, blocked with 5% BSA, then incubated in rabbit anti-ARF1 antibody or mouse anti–alpha-tubulin antibody (Sigma-Aldrich; T9026) overnight at 4 °C, followed by washing in PBS supplemented with 0.1% Tween 20, and incubation with appropriate HRP-conjugated secondary antibodies. Proteins were visualized using chemiluminiscent substrate (Thermo Scientific; 34077).

In Situ Proximity Ligation Assay. Third instar larval lymph glands were dissected, fixed in 4% PF, permeabilized with PBS containing 0.1% Triton X (PTX), incubated in blocking solution provided in the proximity ligation assay (PLA) kit, followed by incubation in primary antibodies (rabbit anti-Asrij, or rabbit anti-Asrij N terminus, or rabbit anti-Asrij C terminus and mouse anti-ARF1 antibody). Excess antibodies were washed with PTX and tissue incubated in PLA probes (Duolink In Situ PLA Probe Anti-Mouse Minus; DUO92004) and (Duolink In Situ PLA Probe Anti-Rabbit PLUS; DUO92002). Ligase (1 unit/μL) and polymerase (10 units/μL) were used as per the manufacturer's instructions (DUOlink In Situ Detection reagents red; DUO92008). Samples were mounted using DAPI glycerol and imaged on a LSM510 confocal microscope. PLA positive spots larger than 0.5 μm were counted manually using LSM510 processing software (Carl Zeiss).

Generation of *UASarf1* Transgenic Flies. ARF1 full-length cDNA was subcloned from pOT2 vector into pUAST vector using EcoRI and XhoI cloning sites. *pUAST ARF1* constructs were injected into embryos (Best Gene) according to standard protocols to obtain *UASarf1* transgenic flies.

Fly Stocks and *Drosophila* Genetics. Fly stocks were maintained according to standard rearing conditions. Canton-S was used as the wild-type reference strain. Respective UAS or GAL4 parent strains or w1118 (for *asrij* null mutant) were used as controls. Tissue-specific GAL4 promoter line was used to drive the expression of the responder *UAS* genes. Stocks used are as follows: *UASarf1RNAi*, *UASPvrRNAi* (Vienna Drosophila RNAi Center, Vienna); *tepGal4*, *UASPTEN*, *UASPI3K^{caax}/Cyo* (L. Waltzer, University of Toulouse, Toulouse, France); *UASPvr* (Denise Montell, University of California, Santa Barbara, CA); *UASgarzRNAi* line (E. Sztul, University of Alabama, Birmingham, AL); *UAS-GFP*, *UAS-gap69c* [Raghu Padinjat, National Center for Biological Sciences (NCBS), Bangalore, India]; *PxnGAL4* (NCBS Fly Facility); *Dome2XeGFPGAL4* (Utpal Banerjee, University of California, Los Angeles); *Pcol85-Gal4, UASmCD8-GFP* (Michele Crozatier, University of Toulouse); *e33cGAL4* (K. Anderson, Memorial Sloan-Kettering Cancer Center, New York); *gcmGal4/Cyo twi-lacZ* (Lucas Waltzer, University of Toulouse); *HmldeltaGAL4*, *HmldeltaGAL4-GFP Lsp2Gal4*, and *btlGal4* (Bloomington Stock Center); *UAS-rab5GFP*, *UAS-rab7GFP*, *UAS-rab11GFP*, and *UAS-lamp7GFP* (Marcos Gonzalez Gaitan, University of Geneva, Geneva); and *UASasrij8M* expression line and *asrij* null mutant (2). Genotype labels in graphs are: controls (C), knockdown (KD), and overexpression (OV).

Immunostaining. Larvae of appropriate stage were obtained from timed embryo collections and used for the dissection of lymph gland, fat bodies, trachea, or wing disk. The dissections were done in phosphate buffer saline (PBS), fixed in 4% paraformaldehyde (PF), followed by washes with PTX. The dissected preparations were then blocked with 1% normal goat serum followed by overnight primary antibody incubation at 4 °C. The primary antibody incubation was followed by washes with PTX and block. Appropriate Alexa-Fluor–conjugated secondary antibodies were used. All dilutions were made in PBS.

Hemocyte Counts and Statistical Analyses. Hemocytes were obtained by puncturing the larval integument, allowed to attach in PBS for 1 h, followed by immunostaining as described (2). Images were captured from 10 fields for each larva ($n = 5$) using an Olympus IX70 microscope. Hemocytes were counted manually for each larva. Statistical significance analysis was done using single factor ANOVA (analyses of variance) with Microsoft Excel. P values <0.01 were considered significant.

Imaging and Analysis. For colocalization analysis, single optical z-sections were analyzed using the Histo option in the LSM510 examiner software and the colocalization graphs were plotted. Number of images analyzed ($n = 15$). Images were processed uniformly for brightness and contrast using Adobe Photoshop CS3. White line in figures indicates primary lymph gland lobe boundary. Nuclei were stained with DAPI (blue).

Antibodies Used. Rabbit, rat, and mouse polyclonal antibodies were raised against recombinant ARF1 protein (see above). Other antibodies used were: chick or rabbit anti-GFP (Molecular Probes), mouse anti-Hemese, mouse anti-P1 antibody, mouse

anti-C4 antibody and mouse anti-L1 antibody (kind gift from Istvan Ando, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary), rabbit anti-GM130 antibody (kind gift from Martin Iowe, University of Manchester, Manchester, UK), guinea pig anti-Hrs (kind gift from P. Rorth, Institute of Molecular and Cell Biology, Singapore), mouse anti-Antennapedia, mouse anti-NICD antibody, mouse anticyclin A, mouse anti- BrdU (Developmental Studies Hybridoma Bank, Iowa City, Iowa), rabbit anti-H3P antibody (Abcam), rabbit anti-Asrij N terminus, rabbit anti-Asrij C terminus, mouse anti-ProPO antibody (Bioneeds), and rabbit anti-Asrij (2). Secondary antibodies were coupled to Alexa-Fluor 488 or Alexa-Fluor 568 (Molecular Probes).

Analysis of Sessile Hemocytes. Body wall samples were prepared for analysis of sessile hemocytes; larval cuticles were cut open from the dorsal side, along the longitudinal axis, cuticles were flattened, and secured with fine pins. All of the internal organs—fat body, trachea, lymph gland, and the digestive tract—were removed. The body wall samples were fixed, permeabilized, and immunostained with appropriate primary and secondary antibodies and DAPI as described (2). Images were captured using Zeiss LSM510 and LSM700 confocal microscopes. Low magnification body wall images were stitched together for representation. Posterior regions of the body wall preparations were imaged at higher magnification.

BrdU Assay. Lymph glands from third instar larvae were dissected for BrdU assay, incubated in 75 μg/mL BrdU (kind gift from Tejas Gupte, inStem, Bangalore, India) for 1 h, washed with PTX, fixed in 4% PF, followed by washes with PTX. Dissected preparations were incubated in 2 M HCl for 30 min, then washed with PTX, blocked with 1% normal goat serum, followed by incubation in mouse anti-BrdU antibody at 4 °C overnight followed by washes and incubation in appropriate Alexa-Fluor–conjugated secondary antibody. DAPI was used to mark the nucleus. Images were captured using Zeiss LSM510 confocal microscope. BrdU positive cells were counted manually using LSM510 processing software (Carl Zeiss).

SI Results

Immunolocalization of ARF1 in third instar larval lymph gland cells using specific antibodies (Fig. S1 *C* and *D*) showed expression in the PSC marked by Antennapedia (Antp), medullary zone (MZ) marked by tepGFP (thio-ester protein promoter Gal4 tagged with GFP), and cortical zone (CZ) marked by P1 (Fig. S2 *A–F*). Other *Drosophila* tissues such as fat body, trachea, and wing discs also express ARF1 (Fig. S2 *K–M*). All of the differentiated blood cell types in the larval hemolymph express ARF1 namely P1-expressing plasmatocytes, crystal cells marked by C4, and lamellocytes marked by L1 (Fig. S2 *G–I*). ARF1 is also expressed in adult plasmatocytes (Fig. 2*J*). In circulating larval hemocytes, ARF1 is endosomal and colocalizes with early endosomal marker Rab5, late endosomal marker Rab7, Golgi marker GM130, and lysosomal marker Lamp7. ARF1 shows very low colocalization with the recycling endosomal marker Rab11 (Fig. S3 *A–E*).

1. Harlow E, Lane DP (1988) *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).

2. Kulkarni V, Khadilkar RJ, Magadi SS, Inamdar MS (2011) Asrij maintains the stem cell niche and controls differentiation during Drosophila lymph gland hematopoiesis. *PLoS ONE* 6(11):e27667.

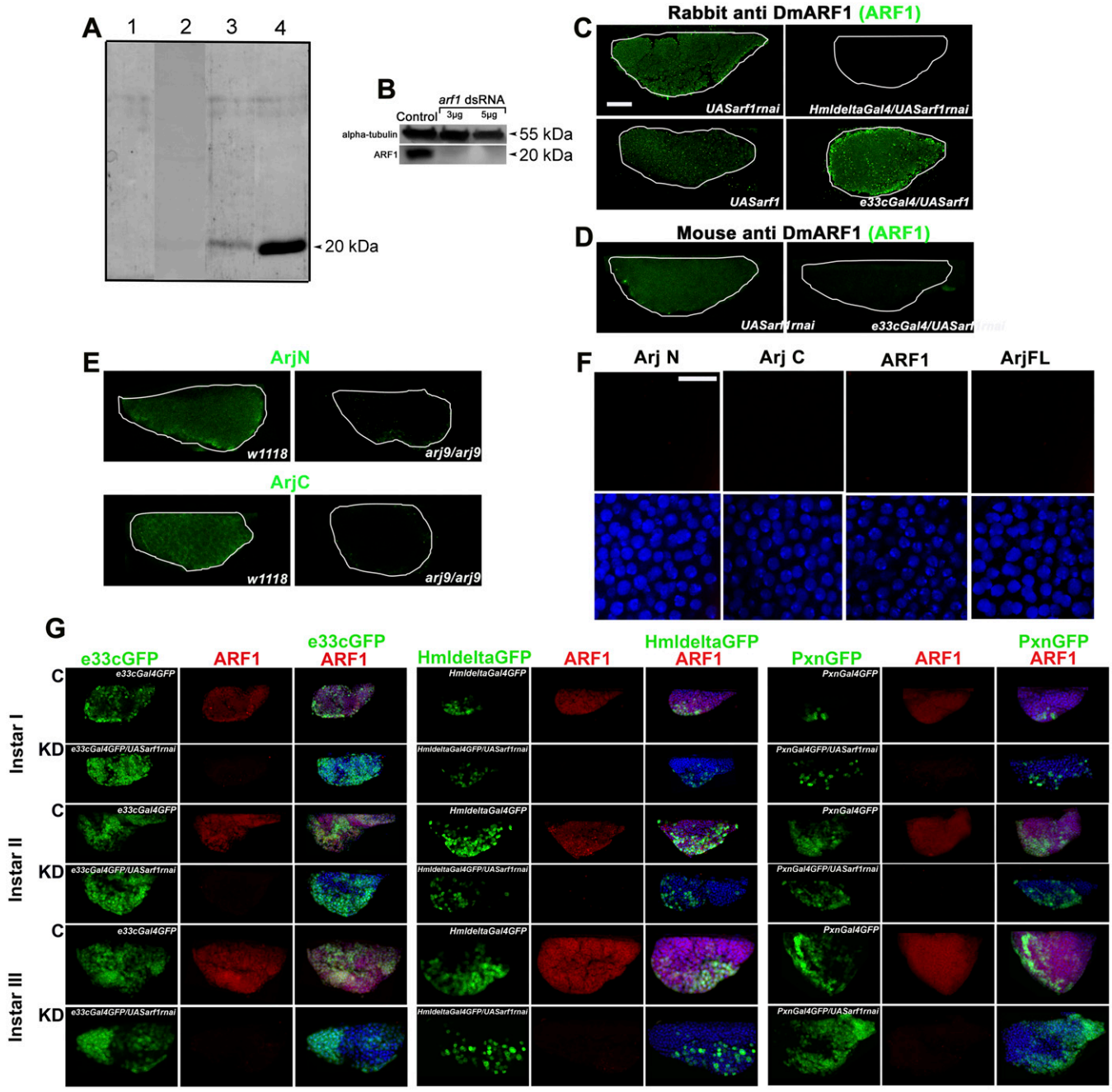

Fig. S1. Validation of anti-ARF1 antibodies and arjN and arjC antibodies (related to Fig. 1). (*A*) Antigen–antibody competition assay for validating specificity of ARF1 antibody. Lane 1 (25 μg) or lane 2 (50 μg) of antigen preincubated with rabbit anti-ARF1 antibody. Lane 3 (25 μg) or lane 4 (50 μg) of antigen probed with rabbit anti-ARF1 antibody. (*B*) S2 cell lysates transfected with 3 μg or 5 μg *arf1* dsRNA probed with rabbit anti-ARF1 and mouse anti–alpha-tubulin antibody. (*C* and *D*) ARF1 knockdown lymph glands do not show labeling with ARF1 antibody (green). (*E*) *asrij* null mutant does not show staining with ArjN and ArjC antibodies (green). (*F*) Wild-type lymph gland samples probed with single antibody controls for in situ proximity ligation assay. (*G*) *e33cgal4GFP*, *HmldeltaGal4GFP*, and *PxnGal4GFP* (green) mediated ARF1 knockdown validation using ARF1 (red) antibody. Genotypes are as indicated. Nuclei are stained with DAPI (blue). [Scale bar, 20 μm (*C*–*E* and *G*) and 5 μm (*F*).]

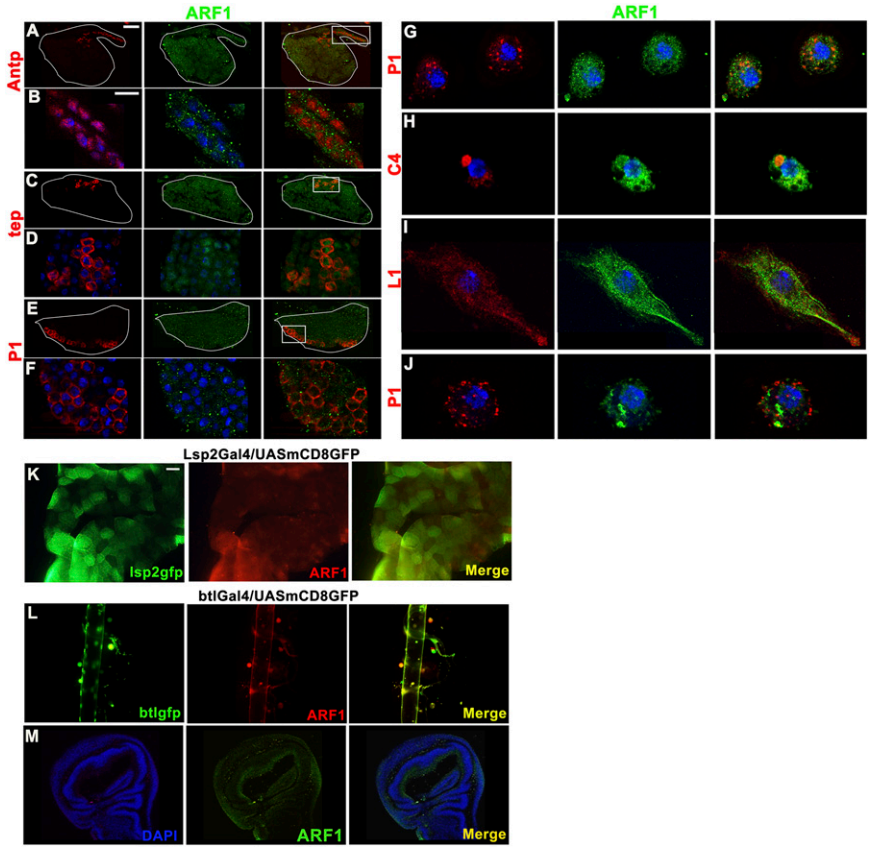

Fig. S2. ARF1 is expressed in the *Drosophila* hematopoietic system and other tissues (related to Fig. 1). (*A–J*) Immunostaining for ARF1 expression (green) in (*A–F*) primary lobe of third instar larval lymph gland, (*G–I*) circulating larval hemocytes, and (*J*) adult hemocytes. Samples were costained for antibodies against lineage markers (red) to identify (*A*) Antp+ PSC, (*C*) tep4+ MZ, (*E*) P1+ CZ, (*G* and *J*) P1+ circulating plasmatocytes, (*H*) C4+ crystal cells, and (*I*) L1+ lamellocytes. High magnification images of the boxed regions in (*A*, *C*, and *E*) showing ARF1 coexpression in the different zones is shown (*B*, *D*, and *F*). High magnification image (*D*) of the boxed region (*C*) has been flipped by 90°. *Right* panels are merged images. (*K–M*) Expression of ARF1 (red) in fat body marked by lsp2GAL4mCD8GFP (*K*), trachea by btlGAL4mCD8GFP (*L*), and wing disk (*M*). White line indicates primary lymph gland lobe boundary. Nuclei were stained with DAPI (blue). [Scale bar, 50 μm (*K–M*), 20 μm (*A*, *C*, and *E*), and 5 μm (*B*, *D*, *F*, and *G–J*).]

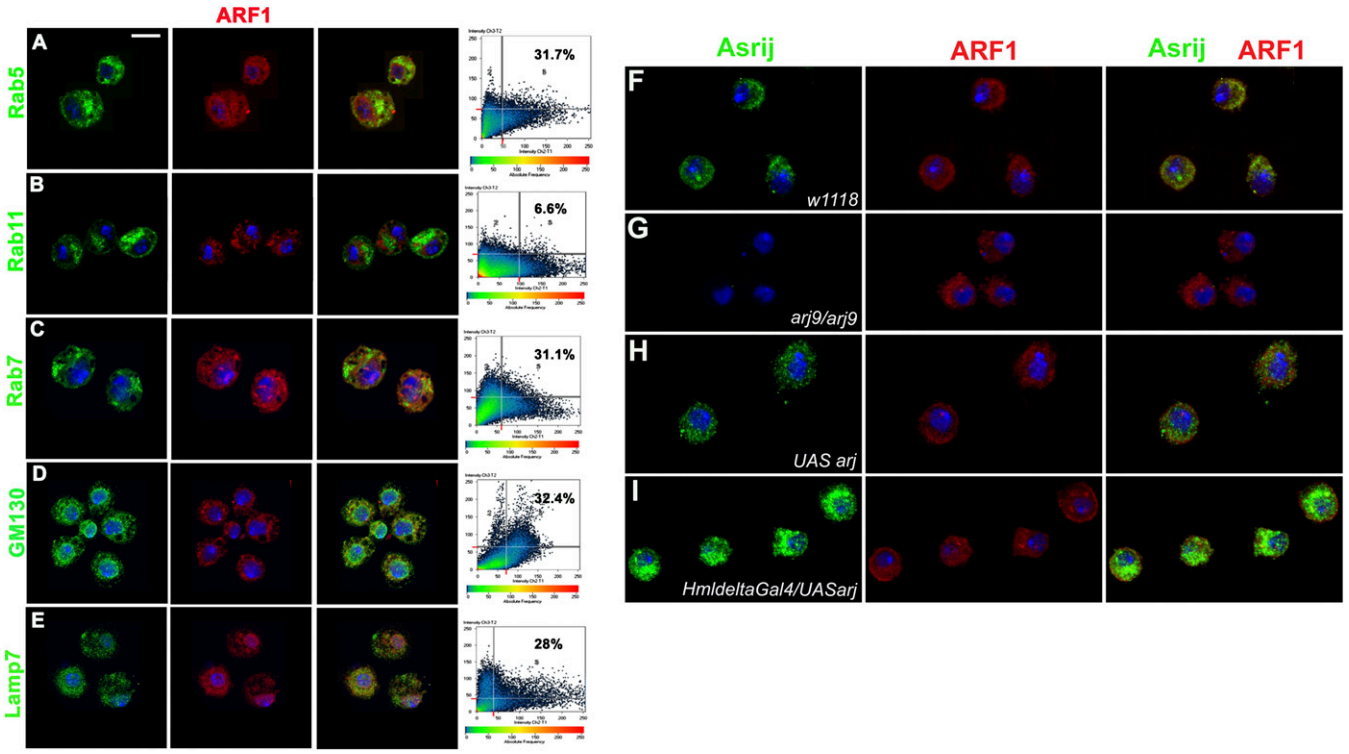

Fig. S3. Subcellular localization of ARF1 and expression in Asrij perturbed conditions (related to Fig. 1). Subcellular localization of ARF1. Immunofluroscence analysis of hemocytes stained for ARF1 expression (red, *Center*) and other subcellular markers (green, far *Left*) Rab5GFP (*A*), Rab11GFP (*B*), Rab7GFP (*C*), GM130 (*D*), and Lamp 7GFP (*E*). (*F–I*) ARF1 expression is not affected in Asrij perturbed condition. Immunofluorescence analysis of hemocytes stained for Asrij (green, far *Left*) and ARF1 (red, *Center*). Nuclei are stained with DAPI (blue). Far *Right* panels of the set are merged images. *w1118* parental controls (*F*), *asrij* null mutants (*G*), Asrij overexpression parental control (*H*), and Asrij overexpression progeny (*I*). Genotypes are as indicated. Nuclei are stained with DAPI (blue). Far *Right* panels of the set are merged images. [Scale bar, 5 μm (*A–I*).]

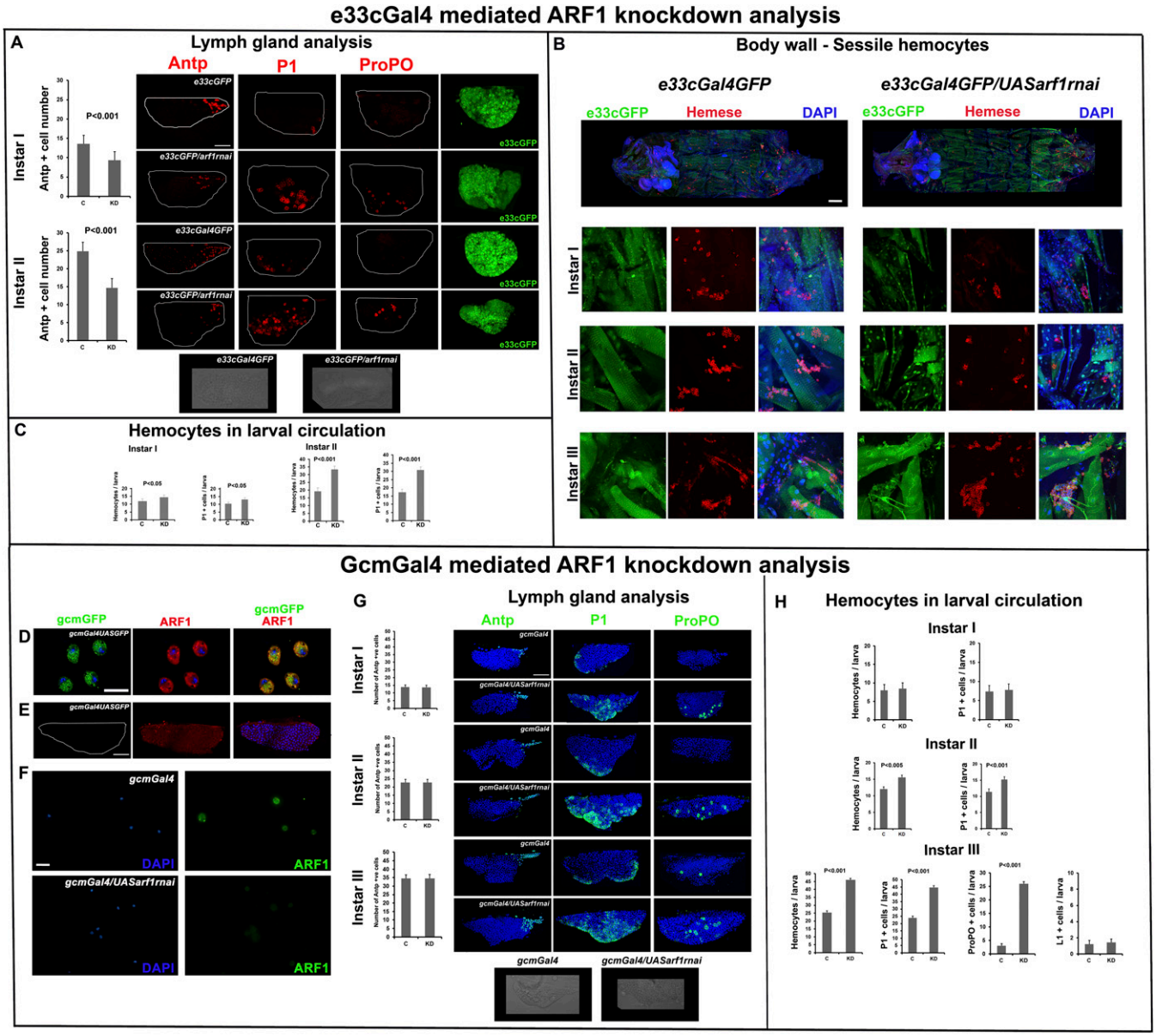

Fig. S4. *arf1* knockdown using e33cGal4 or gcmGal4 affects hemocyte homeostasis (related to Fig. 2). Knockdown of *arf1* using (*A*–*C*) *e33cGal4GFP* coexpressing *GFP* (green) and (*D*–*H*) *gcmGAL4* analyzed at larval instars I, II, and III. (*A*) *arf1* knockdown in lymph gland using *e33cGal4* shows a moderate decrease in PSC size marked by Antp (red), (also shown in the boxed *Inset*) and increased plasmatocyte and crystal cell differentiation marked by P1 and ProPO (red), respectively. Graph shows Antp+ cell count per lymph gland. Phase images (*Bottom* of the panel) of *arf1* knockdown and control show no sign of lymph gland disintegration. (*B*) Body wall of *arf1* knockdown larvae stained for Hemese (red) and GFP (green). No difference is seen in hemese+ or GFP+ sessile hemocytes compared with controls. Individual low magnification images were stitched to create a montage of the entire larva, represented at the *Top* of each panel. Panels below show magnified view of representative regions of the body wall. (*C*) Analysis of hemocytes in circulation: Graphs represent total hemocyte count (DAPI+ cells) and differential count for hemocytes expressing P1 or ProPO or L1 as indicated. (*D* and *E*) Validation of *gcmGal4* mediated *UAS-GFP* transgene expression. Note lack of expression in the lymph gland. (*F*) *arf1* knockdown with gcmGAL4 shows lack of ARF1 (green) expression in hemocytes (*G*), no change in the PSC size marked by Antp (green), and increased plasmatocyte and crystal cell differentiation marked by P1 and ProPO (green), respectively. Graph shows Antp+ cell count per lymph gland. Phase images (*Bottom* of the panel) of *arf1* knockdown and control show no sign of lymph gland disintegration. (*H*) Analysis of hemocytes in circulation: Graphs represent total hemocyte count (DAPI+ cells) and differential count for hemocytes expressing P1 or ProPO or L1 as indicated. *P* values are as indicated above the graph. Nuclei are marked with DAPI (blue). Number of larvae analyzed are shown ($n = 10$) for lymph gland analysis and ($n = 5$) for circulating and sessile hemocyte analysis. [Scale bar, 50 μm (body wall montages), 20 μm (*A*, *B*, and *E*–*G*), and 5 μm (*D*).]

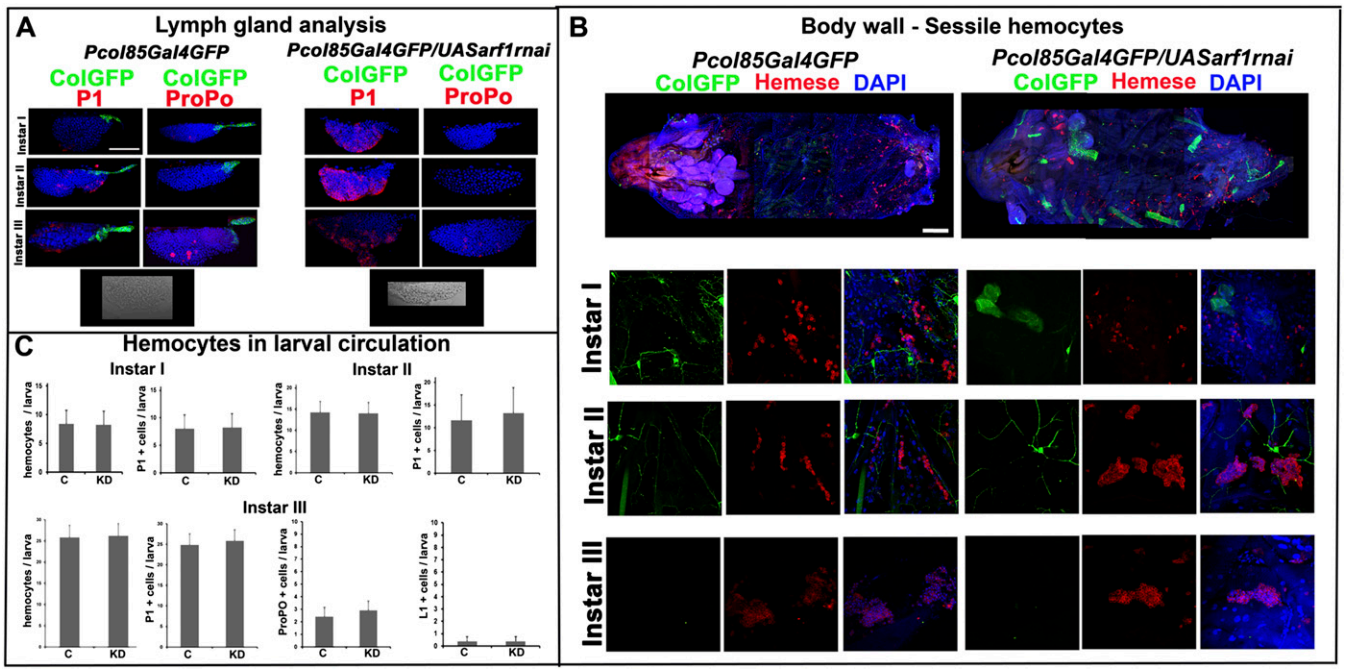

Fig. S5. ARF1 plays a cell autonomous role in PSC maintenance (related to Fig. 2). PSC-specific knockdown of *arf1* using *Pcol85Gal4GFP* coexpressing *GFP* (green) analyzed at larval instars I, II, and III. (*A*) Lymph gland primary lobe staining shows increased plasmatocyte and crystal cell differentiation marked by P1 and ProPO (red), respectively, compared with *Pcol85Gal4GFP* control. Phase images (*Bottom* of the panel) of *arf1* knockdown and control show no sign of lymph gland disintegration. (*B*) Body wall of *arf1* knockdown larvae stained for hemese (red) and PcolGFP (green) show no difference in hemese-expressing cells compared with controls. No GFP expression is detected in sessile hemocytes. Individual low magnification images were stitched to create a montage of the entire larva, represented at the *Top* of each panel. Panels below show magnified view of representative regions of the body wall. (C) Analysis of hemocytes in circulation: Graphs represent total hemocyte count (DAPI+ cells) and differential count for hemocytes expressing P1 or ProPO or L1 as indicated. Nuclei are marked with DAPI (blue). Number of larvae analyzed are shown ($n = 10$) for lymph gland analysis and ($n = 5$) for circulating and sessile hemocyte analysis. [Scale bar, 50 μm (body wall montages) and 20 μm (*A* and *B*).]

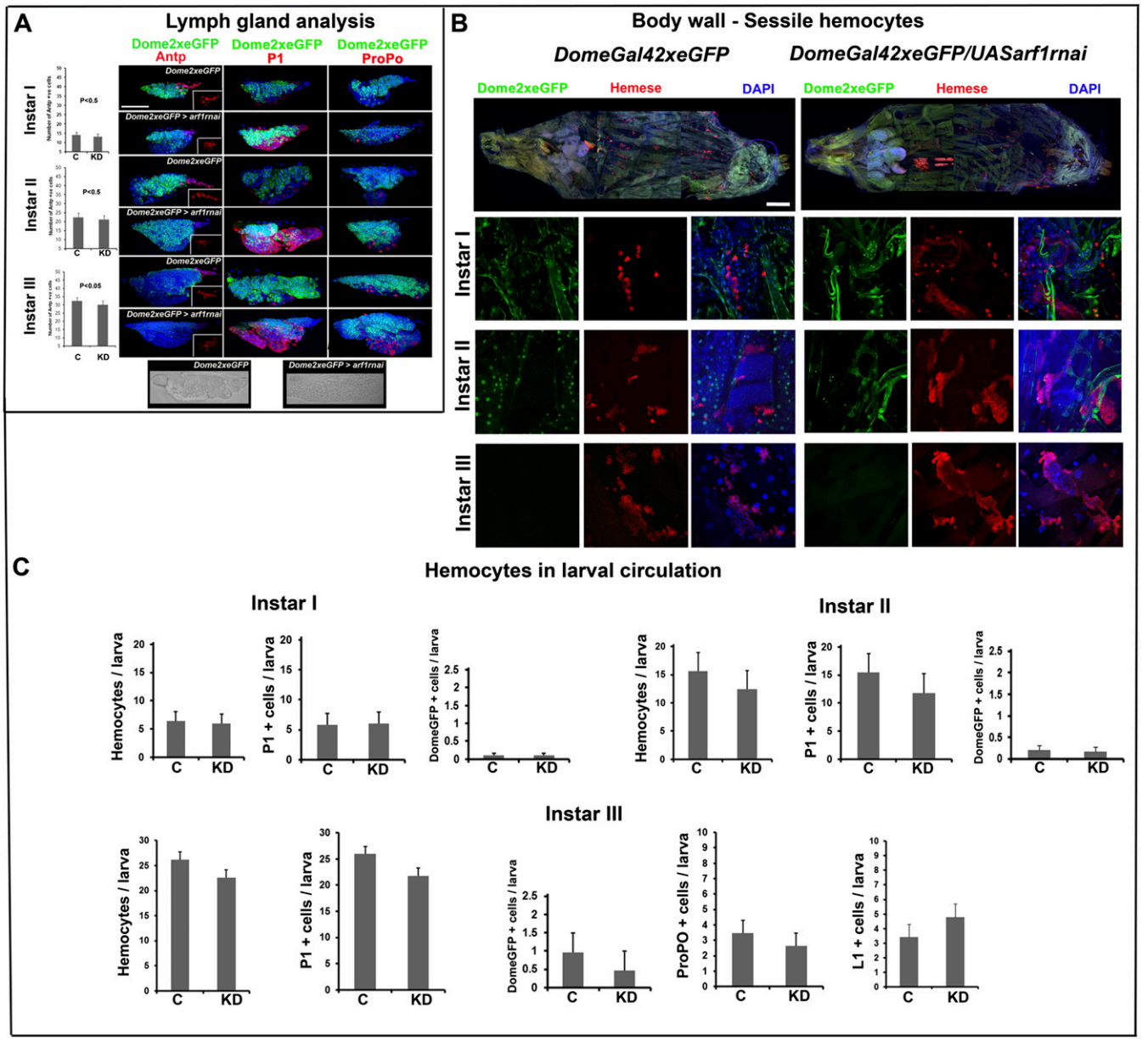

Fig. S6. MZ-specific ARF1 perturbation affects prohemocyte maintenance (related to Fig. 2). MZ-specific knockdown of *arf1* using *DomeGal4GFP* coexpressing *GFP* (green) analyzed at larval instars I, II, and III. (*A*) Lymph gland primary lobe staining in *DomeGal4GFP*-mediated *arf1* knockdown does not show any significant change in the PSC size marked by Antp (red), (also shown in the boxed *Inset*). Plasmatocyte and crystal cell differentiation marked by P1 and ProPO (red), respectively, is increased compared with *DomeGal4GFP* control. Phase images (*Bottom* of the panel) of *arf1* knockdown and control show no sign of lymph gland disintegration. (*B*) Body wall of *arf1* knockdown larvae stained for hemese (red) and DomeGFP (green) show no difference in hemese-expressing cells compared with controls. No GFP expression is detected in sessile hemocytes. Individual low magnification images were stitched to create a montage of the entire larva, represented at the *Top* of each panel. Panels below show magnified view of representative regions of the body wall. (C) Analysis of hemocytes in circulation: Graphs represent total hemocyte count (DAPI+ cells) and differential count for hemocytes expressing P1 or ProPO or L1 as indicated. *P* values are as indicated above the graph. Nuclei are marked with DAPI (blue). Number of larvae analyzed are shown (n = 10) for lymph gland analysis and (n = 5) for circulating and sessile hemocyte analysis. [Scale bar, 50 μm (body wall montages) and 20 μm (*A* and *B*).]

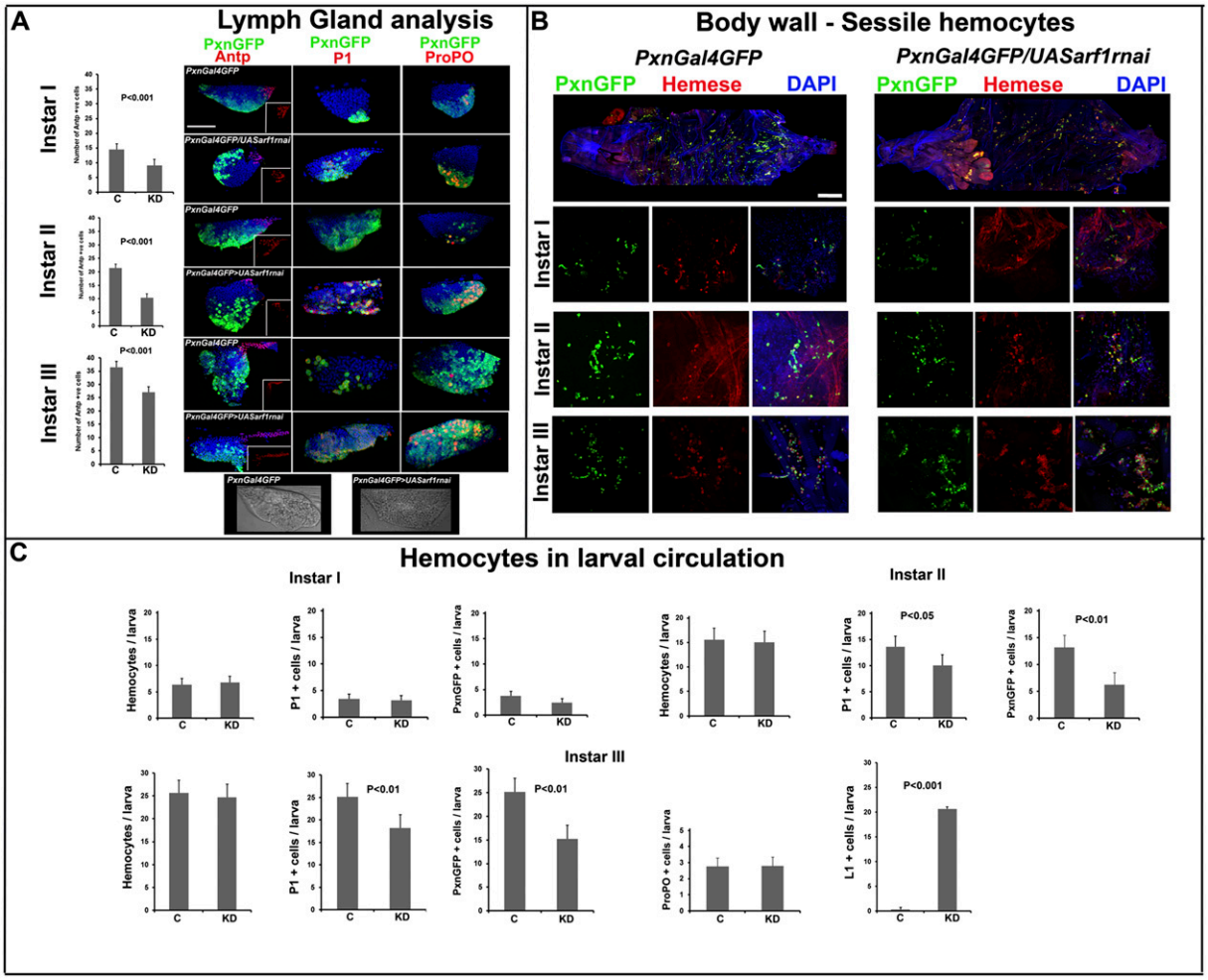

Fig. S7. CZ-expressed ARF1 plays a nonautonomous role in niche and MZ maintenance (related to Fig. 2). CZ-specific knockdown of *arf1* using *PxnGal4GFP* coexpressing *GFP* (green) analyzed at larval instars I, II, and III. (*A*) Lymph gland primary lobe staining in *PxnGal4GFP*- mediated *arf1* knockdown shows a moderate decrease in the PSC size marked by Antp (red) (also shown in the boxed *Inset*) with increased plasmatocyte and crystal cell differentiation marked by P1 and ProPO (red), respectively, compared with *PxnGal4GFP* control. Phase images (*Bottom* of the panel) of *arf1* knockdown and control show no sign of lymph gland disintegration. (*B*) Body wall of *arf1* knockdown larvae stained for hemese (red) and GFP (green). No difference is seen in hemese+ or GFP+ sessile hemocytes compared with controls. Individual low magnification images were stitched to create a montage of the entire larva, represented at the *Top* of each panel. Panels below show magnified view of representative regions of the body wall. (*C*) Analysis of hemocytes in circulation: Graphs represent total hemocyte count (DAPI+ cells) and differential count for hemocytes expressing P1 or ProPO or L1 as indicated. *P* values are as indicated above the graph. Nuclei are marked with DAPI (blue). Number of larvae analyzed are shown ($n = 10$) for lymph gland analysis and ($n = 5$) for circulating and sessile hemocyte analysis. [Scale bar, 50 μm (body wall montages) and 20 μm (*A* and *B*).]

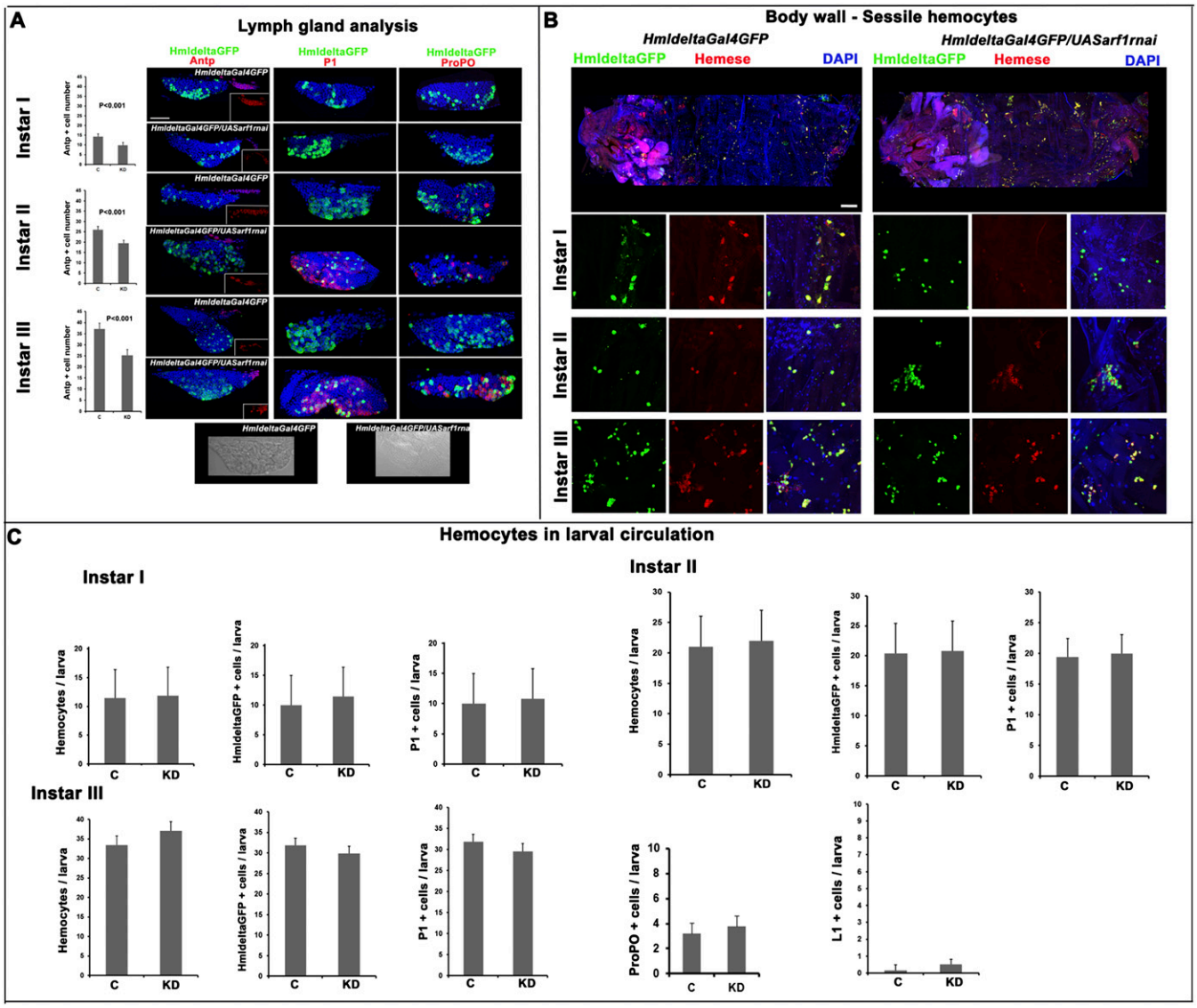

Fig. S8. *arf1* knockdown using HmldeltaGal4 affects hemocyte homeostasis (related to Fig. 2). Knockdown of *arf1* using *HmldeltaGal4GFP* coexpressing *GFP* (green) analyzed at larval instars I, II, and III. (*A*) Lymph gland primary lobe staining in *HmldeltaGal4GFP* mediated *arf1* knockdown shows a moderate decrease in the PSC size marked by Antp (red), also shown in the boxed *Inset*. Increased plasmatocyte and crystal cell differentiation marked by P1 and ProPO (red), respectively, are seen compared with *HmldeltaGal4GFP* control. Phase images (*Bottom*) of *arf1* knockdown and control show no sign of lymph gland disintegration. (*B*) Body wall of *arf1* knockdown larvae stained for hemese (red) and GFP (green). No difference is seen in hemese+ or GFP+ sessile hemocytes compared with controls. Individual low magnification images were stitched to create a montage of the entire larva, represented at the *Top* of each panel. Panels below show magnified view of representative regions of the body wall. (*C*) Analysis of hemocytes in circulation: Graphs represent total hemocyte count (DAPI+ cells) and differential count for hemocytes expressing P1 or ProPO or L1 as indicated. *P* values are as indicated above the graph. Nuclei are marked with DAPI (blue). Number of larvae analyzed are shown ($n = 10$) for lymph gland analysis and ($n = 5$) for circulating and sessile hemocyte analysis. [Scale bar, 50 μm (body wall montages) and 20 μm (*A* and *B*).]

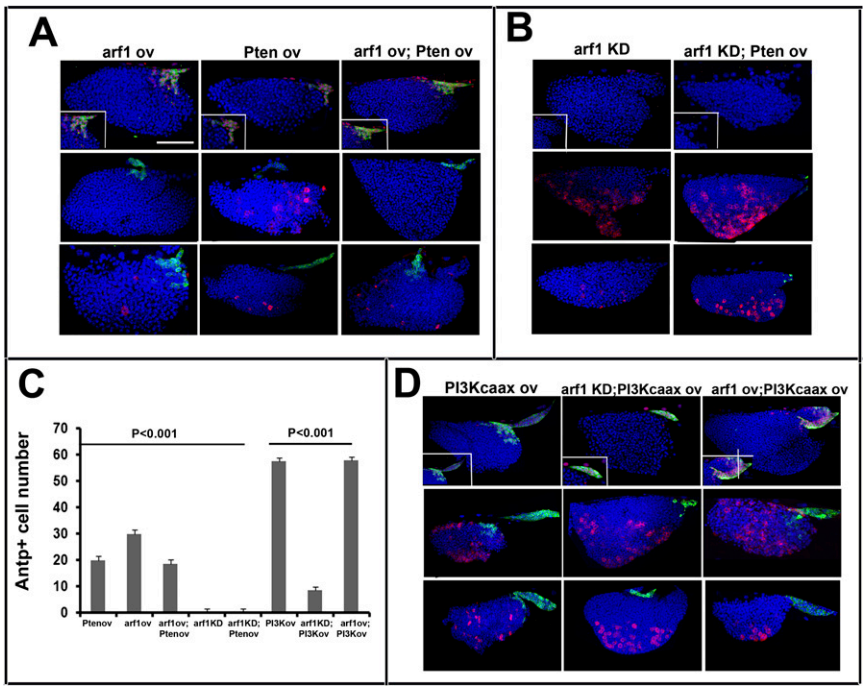

Fig. S9. ARF1-mediated regulation is required for optimal levels of Insulin signaling (related to Fig. 6). (*A* and *B*) PSC marked with Antp (red) is decreased in *arf1* overexpression in Pten overexpression background, whereas it is completely abolished in arf1 knockdown in Pten overexpression genetic background. There is increased plasmatocyte and crystal cell differentiation marked by P1 and ProPO (red), respectively, in *arf1* knockdown in Pten overexpression conditions, whereas differentiation is unaffected or suppressed in *arf1* overexpression in Pten overexpression conditions. (*C* and *D*) Graphical representation (*C*) and representative stained images (*D*) showing increased number of Antp (red) positive cells coexpressing PcolGal4GFP (green) observed in *arf1* overexpression in PI3Kcaax overexpression genetic background with increased differentiation marked by P1 and ProPO (red). Moderate restoration of the niche size marked by Antp (red) is seen in arf1 knockdown larvae in PI3Kcaax overexpression conditions accompanied by differentiation marked by P1 and ProPO (red), respectively. The niche has been shown in the *Inset* in the images that show Antp status in various genetic backgrounds. Nuclei are marked with DAPI (blue). Number of larvae analyzed: $n = 10$. [Scale bar, 20 μm (*A*, *B*, and *D*).]