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Dally-like protein sequesters multiple Wnt ligands in the *Drosophila* germarium

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Abstract

Cells in multicellular organisms rely on secreted ligands for development and morphogenesis. Several mechanisms modulate the availability and distribution of secreted ligands, determining their ability to signal locally and at long range from their source. One of these mechanisms is Dally-like protein (Dlp), a cell-surface glypican that exhibits biphasic functions in *Drosophila* wing discs, promoting Wg signaling at long-range from Wg source cells and inhibiting Wg signaling near source cells. In the germarium at the tip of the ovary, Dlp promotes long-range distribution of Wg from cap cells to follicle stem cells. However, the germarium also expresses other *Wnts* - *Wnt2, Wnt4,* and *Wnt6* - that function locally in escort cells to promote oogenesis. Whether and how local functions of these Wnts are regulated remains unknown. Here we show that the *dlp* overexpression phenotype is multifaceted and phenocopies multiple *Wnt* loss-of-function phenotypes. Each aspect of *dlp* overexpression phenotype is suppressed by co-expression of individual *Wnts,* and the suppression pattern exhibited by each Wnt suggests that Wnts have functional specificity in the germarium. Further, *dlp* knockdown phenocopies *Wnt* gain-of-function phenotypes. Together these data show that Dlp inhibits the functions of each Wnt. All four Wnts co-immunoprecipitate with Dlp in S2R+ cells, suggesting that in the germarium, Dlp sequesters Wnts to inhibit local paracrine Wnt signaling. Our results indicate that Dlp modulates the availability of multiple extracellular Wnts for local paracrine Wnt signaling in the germarium.

Keywords

Wnts; local paracrine signaling; heparan sulfate proteoglycan; oogenesis; stem cell niche

Introduction

Glypicans are cell surface heparan sulfate proteoglycans known to regulate the distribution of secreted ligands. In vertebrates and in flies, glypicans regulate ligands such as Wg

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Competing interests

The authors declare no competing financial interests.

(Wingless), Hh (Hedgehog), and Dpp (Decapentaplegic) that form morphogen gradients and activate downstream target genes in a dose dependent manner (Baeg et al., 2001; Belenkaya et al., 2004; Desbordes and Sanson, 2003; Filmus et al., 2008; Franch-Marro et al., 2005; Fujise et al., 2003; Gallet et al., 2008; Han et al., 2004; Han et al., 2005; Kirkpatrick et al., 2004; Kreuger et al., 2004; Lecuit et al., 1996; Mullor et al., 1997; Nellen et al., 1996; Neumann and Cohen, 1997; Nybakken and Perrimon, 2002; Strigini and Cohen, 1997; Zecca et al., 1996). The six glypicans found in vertebrates are grouped into two families, both represented by a single member in *Drosophila:* fly Dlp (Dally-like protein) represents GPC1, GPC2, GPC4, and GPC6 whereas fly Dally represents GPC3 and GPC5 (Filmus et al., 2008). Glypicans lack transmembrane domains, instead attaching to cell surfaces via GPI (Glycosylphosphatidylinositol) anchors such that the core protein and the GAG (Glycosaminoglycan) polysaccharide chains interact with secreted ligands (Khare and Baumgartner, 2000; Nakato et al., 1995; Yan et al., 2009). Specifically, with respect to Wg, studies in wing imaginal discs have shown that Dlp can restrict ligand availability near the source cells while simultaneously promoting availability of Wg to distant cells, whereas Dally acts as a Wg co-receptor (Baeg et al., 2004; Han et al., 2005; Yan et al., 2009). The spreading of Wg from source cells, while not necessary for wing patterning, is important for gut homeostasis in adult flies and for proper patterning of Malpighian tubules in *Drosophila* embryos (Alexandre et al., 2014; Beaven and Denholm, 2018; Tian et al., 2019).

Secreted ligands such as Wg and Hh have long-range functions in the germarium of the ovary, as both are expressed in terminal filament cells and cap cells (also known as apical cells) (Fig. 1 A) and activate signaling in distant follicle stem cells (about 50 μm away from apical cells) to promote their maintenance, proliferation, and differentiation (Dai et al., 2017; Forbes et al., 1996a; Forbes et al., 1996b; Reilein et al., 2017; Song and Xie, 2003; Vied et al., 2012; Wang and Page-McCaw, 2014; Zhang and Kalderon, 2001). Extending or truncating signaling ranges of Wg and Hh from cap cells affects follicle stem cell number and proliferation (Song and Xie, 2003; Wang and Page-McCaw, 2014; Zhang and Kalderon, 2001). Two studies shed light on how signaling ranges of secreted ligands are achieved in the germarium: 1) Cap cells extend cytoplasmic projections, known as cytonemes, to deliver Hh from cap cells to escort cells to activate Hh signaling in escort cells (Rojas-Rios et al., 2012). 2) Escort cell localized Dlp facilitates long-range Wg spreading from cap cells to follicle stem cells to activate sufficient Wg signaling in follicle stem cells (Wang and Page-McCaw, 2014).

In addition to activating long-range signaling in follicle stem cells, cap-cell derived Wg/Wnt6 and escort-cell derived Wnt2/Wnt4 activate local paracrine Wnt signaling in escort cells (Forbes et al., 1996b; Luo et al., 2015; Song and Xie, 2003; Waghmare and Page-McCaw, 2018; Wang et al., 2015), which form the differentiation niche to promote germline differentiation (Decotto and Spradling, 2005; Kirilly et al., 2011; Luo et al., 2015; Morris and Spradling, 2011; Upadhyay et al., 2016; Wang et al., 2015). Wg and Wnt6 from cap cells promote germline differentiation by inducing the expression of *thickveins (tkv),* a type I Dpp receptor, in escort cells (Luo et al., 2015; Penton et al., 1994). The escort-cell localized Tkv receptors act as decoy to sequester cap-cell derived Dpp, an inhibitor of germline differentiation (Luo et al., 2015). Similarly, Wnt4 signaling in escort cells also prevents differentiating germline cells from receiving the Dpp signal by inhibiting *dpp*

expression in escort cells (Mottier-Pavie et al., 2016). Further, Wnt4 promotes germline differentiation by maintaining escort-cell cytoplasmic processes that intermingle with the developing germline by regulating gap-junction and adherens-junction proteins such as Innexin, E-Cadherin, and Armadillo (Upadhyay et al., 2016); the intermingling of escort-cell processes with the differentiating germline is crucial for germline differentiation and movement of germline cysts out of the germarium (Decotto and Spradling, 2005; Kirilly et al., 2011; Morris and Spradling, 2011). Lastly, Wnt2 and Wnt4 promote germline differentiation by promoting escort-cell survival and removal of reactive oxygen species from escort cells by inducing expression of *Glutathione S-transferases*; removal of reactive oxygen species from escort cells is crucial for germline differentiation (Wang et al., 2015). Thus, Wg, Wnt2, Wnt4, and Wnt6 function in escort cells to promote germline differentiation (Luo et al., 2015; Upadhyay et al., 2016; Wang et al., 2015), which can be monitored by the presence of intracellular organelles called spectrosomes and fusomes (Deng and Lin, 1997; Lin and Spradling, 1995). In undifferentiated germline cells such as the germline stem cells and their immediate progeny, the spectrosomes appear round (Deng and Lin, 1997), whereas in differentiating cysts, the spectrosomes appear branched and are called fusomes (Lin and Spradling, 1995) (Fig. 1 A). The spectrosomes and fusomes are enriched in the cytoskeletal protein Hts (Hu-li tai shao), which is often used as an indicator of germline differentiation status (Yue and Spradling, 1992).

In addition to promoting germline differentiation, Wnts are also required for germline stem cell maintenance. Wnt6 from cap cells activates local paracrine Wnt signaling in anterior escort cells to promote their survival and maintain germline stem cells, and loss of Wnt6 function results in loss of anterior escort cells and loss of germline stem cells (Wang and Page-McCaw, 2018). The loss of Wnt2 or Wnt4 function in escort cells also results in loss of germline stem cells (Wang and Page-McCaw, 2018).

These studies show that Wg, Wnt2, Wnt4, and Wnt6 function locally in the germarium, but it is unclear whether or how the local paracrine functions of these Wnts are regulated. Here we report that overexpression of *dlp* in escort cells inhibits the local paracrine functions of Wg, Wnt2, Wnt4, and Wnt6, and these functions can be restored by co-expression of Wnt ligands. Further, *dlp* loss-of-function in escort cells phenocopies *Wnt* gain-of-function phenotypes in escort cells. Importantly, Wg, Wnt2, Wnt4, and Wnt6 Dlp co-immunoprecipitate with Dlp. Our results provide evidence for a model where Dlp sequesters multiple secreted Wnts and acts as an important regulator of local Wnt signaling in the germarium.

Materials and methods

Drosophila husbandry, genetics, and stocks

Fly stocks were reared at 25°C on standard cornmeal, yeast, molasses medium. Fly crosses and their progeny were reared at 18°C until adult progeny eclosed to ensure $Gal80^{ts}$-mediated Gal4 inactivation during development. Young flies (<1 day old) were collected from the crosses, crossed with w^{1118} males, transferred to fresh vials supplemented with yeast paste and incubated at 29°C to allow $Gal80^{ts}$ inactivation and UAS-transgene

activation for 7 days. The flies were transferred to fresh vials supplemented with yeast paste every two days during the 7-day incubation at 29°C.

The following fly stocks used in this study are described in Flybase and were obtained from Bloomington *Drosophila* stock center: *TubP-Gal80^{ts}* (BL7017), *UAS-dlp* (BL9160), *UAS-wg* (BL5918), *UAS-Wnt2* (BL6961), *UAS-arm^{S10}* (BL4782), *UAS-p35* (BL5072), *UAS-fz2* (BL41793), *UAS-dlp^{RNAi}* (BL34089). A second *UAS-dlp^{RNAi}* (VDRC10299) was obtained from Vienna *Drosophila* Resource Center. *PBac{602.P.SVS-1}dlpCPTI000445* (115031) was obtained from Kyoto Stock Center (Lowe et al., 2014). Other stocks used in this study include *c587-Gal4* (from Daniela Drummond Barbosa) (Song et al., 2004), which drives strong expression of *UAS*-transgenes in escort cells and weak expression of *UAS*-transgenes in follicle stem cells, *PZ1444* (from Alan Spradling) (Xie and Spradling, 2000), *fz3-RFP* (from Ramanuj Dasgupta) (Olson et al., 2011; Wang and Page-McCaw, 2014), *UAS-hid* (from Julien Royet), *UAS-Wnt4* (from Nicholas Tolwinski) (Peradziryi et al., 2011), and *UAS-Wnt6* (from Aurelio Teleman) (Doumpas et al., 2013). The control germaria used in this study were isolated from females of the genotype *c587-Gal4/w^{1118}; PZ1444/+; TubP-Gal80^{ts}/+* (Fig. 1–6, Fig. S1 A, S2, S3 A, D), *c587- Gal4/w^{1118}; fz3-RFP/+; TubP-Gal80^{ts}/+* (Fig. S1 C), and *c587-Gal4/w^{1118};UAS-mCD8:GFP/+; TubP-Gal80^{ts}/+* (Fig. S3 G).

Immunohistochemistry

Standard immunohistochemistry protocol was used to stain the ovaries (Wang and Page-McCaw, 2014). Ovaries were dissected in ice-cold PBS (phosphate buffered saline) and fixed in 4% paraformaldehyde (Ted Pella) in PBS for 20 minutes at room temperature. The samples were blocked in 5% normal goat serum in PBST (PBS plus 0.1% Triton X-100) for 1 hour at room temperature and incubated in primary antibodies overnight at 4°C. The next day, ovaries were incubated in secondary antibodies for 2 hours at room temperature in dark. Following secondary incubation, ovaries were incubated with DAPI (Invitrogen) in PBST at a final concentration of 1 μg/ml for 10 minutes at room temperature in dark. The samples were mounted in Vectashield (Vector Laboratories). The following primary antibodies used in this study were obtained from the Developmental Studies Hybridoma Bank (DSHB): mouse anti-Fas3 (7G10, 1:8), rat anti-Vasa (1:10), mouse anti-Hts (1B1, 1:5), mouse anti-LamC (LC28.26, 1:20), mouse anti-β-galactosidase (40–1a, 1:50), mouse anti-Dlp (13G8, 1:5), and mouse anti-Armadillo (N2 7A1, 1:15). Other primary antibodies used in this study include rabbit anti-Smad3 (Abcam, ab52903, EP823Y, phospho S523+S525, 1:200), rabbit anti-cleaved Caspase3 (Cell Signaling, 9661, 1:300), and rabbit anti-Zfh-1 (1:1000, from Ruth Lehmann). The following secondary antibodies were used from Jackson ImmunoResearch: Cy3-conjugated donkey anti-rat (712–165-150), FITC-conjugated donkey anti-rabbit (# 711–095-152), Cy3-conjugated goat anti-mouse IgG1 (115–165-205), FITC-conjugated goat anti-mouse IgG1 (#115–095-205), and FITC-conjugated goat anti-mouse IgG2a (# 115–095-206). All secondary antibodies were diluted 1:500.

Imaging, quantification, and statistics

All images were acquired using Zeiss Apotome ImagerM2 using either 40X oil or 63X oil-immersion objectives, and figures were made using Affinity Designer. Counts for germline

cysts, spectrosomes, escort cells, and germline stem cells were done by visualizing the samples directly under the microscope and adjusting the focal planes as required. Germline cysts posterior to 2a/2b boundary were identified based on FasIII labelled follicle cells, which surround the germline cysts, and germline cysts between the 2a/2b boundary and the first discernable egg chamber were counted for quantification. Spectrosomes were counted based on Hts staining, and germaria were binned into categorizes because getting an accurate spectrosome count was technically challenging. Germline stem cell counts shown in Fig. 1P were done based on spectrosome shape, position, and attachment to cap cells that were labelled with LamC1 staining. Germline stem cell counts shown in all other figures were done based on p-Mad staining and germaria were binned into categorizes described on the graphs. p-mad staining identifies both germline stem cells and undifferentiated cystoblasts, cell-types that share attributes of stemness (Xie and Spradling, 1998). Escort cells were counted based on *PZ1444* staining; cap cells were eliminated from counts based on their location and distinct morphology. For quantifying germline nuclear diameter, single optical sections from 4–5 samples of each genotype were chosen from 40X images. The optical section was chosen such that the largest nuclear diameter was apparent. Diameter of the largest germline nucleus (based on DAPI and Vasa staining) within the germarium was measured using ImageJ. Graphpad Prism was used to generate dot plots and Microsoft Excel was used to generate stacked bar graphs. For data represented as dot plots in Fig. 1 and Fig. 6 G (left), statistical analyses were done using unpaired two-tailed student's t-test. For all other dot plots that show comparisons of means across multiple genotypes, one-way ANOVA was performed and significant differences between genotypes was determined by Tukey's test. Mean and standard error of mean (s.e.m.) are represented on the dot plots. For data represented as stacked bar graphs, statistical analyses were done using chi-square test to determine differences in frequencies for each category within indicated genotypes. * indicates p value:0.01–0.05, ** indicates p value:0.0099–0.001, *** indicates p value<0.00099. 'n' on graph indicates sample size.

RNA isolation and RT-qPCR

c587-Gal4, PZ1444, Tub-Gal80^{ts} females were crossed to *w^{1118}, UAS-dlp* or *UAS- dlp +UAS-Wnt* males and incubated at 18°C until the F1 progeny eclosed. 1-day old females of the appropriate genotypes were identified based on markers on balancer chromosomes, crossed to *w^{1118}* males to stimulate oogenesis in fresh vials supplemented with yeast paste and transferred to 29°C for one day for UAS-transgene overexpression. Total RNA was isolated from 8–10 ovaries using RNeasy kit (Qiagen #74104) as per manufacturer's protocol. cDNA was generated using SuperScript™ IV VILO™ Master Mix (ThermosFisher Scientific 11766050) from 2 μg of RNA post DNAse treatment as per manufacturer's protocol. cDNA was diluted 1:5 for performing qPCR. All primers were tested for amplification specificity and efficiency; the primer efficiency ranged between 100%−114%. qPCR was performed using PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, A25742) as per manufacturer's protocol. PCR reactions with no template or no reverse transcriptase treatment were used as negative controls. Average C_t values were calculated from three technical replicates in all samples except in samples where we tried to detect *wg* transcripts in *c587* control or *dlp* overexpressing germaria. In these samples, we could obtain up to two technical replicates owing to technical challenges, which

most likely stem from low abundance of *wg* transcripts. ΔC_t was calculated by subtracting C_t value for housekeeping gene, *rp49* (Ribosomal protein 49), from C_t values for *Wnts.* Fold change reported in Fig. S4 was calculated using the formula $2^{-\Delta Ct}$. The primers used in this study are:

wg forward primer: TCCATGTGGTGGGGCATTG

wg reverse primer: AACGTAGAGTGGATCGCTGG

Wnt2 forward primer: GGAAATAAGATTAGTGTCCAGCTTT

Wnt2 reverse primer: GCGGCACATATTTCGCTGG

Wnt4 forward primer: GACGCAAGAAACCCCAACAA

Wnt4 reverse primer: CACTGCCGATCCTTGGTGAC

Wnt6 forward primer: AGTTTCAATTCCGCAACCGC

Wnt6 reverse primer: TCGGGAATCGCGCATTAAGA

rp49 forward primer: TAGCCCAACCTGCTTCAAGA

rp49 reverse primer: ATATCGATCCGACTGGTGGC

Plasmids

The artificial exon sequence encoding SVS tag was PCR amplified from genomic DNA isolated from homozygous *SVS-dlp* (*PBac{602.P.SVS-1}dlp[CPTI000445*] flies (Lowe et al., 2014) and cloned into pCR2.1 vector using TOPO/TA cloning kit (Thermo Fisher Scientific). Gibson Assembly (New England BioLabs Inc.) was used to introduce SVS sequence from pCR2.1 vector into *pOT2 dlp* plasmid between coding exons 1 and 2; the *SVS-dlp* sequence from pOT2 vector was subcloned into *pUAST* vector. *SVS-dlp* sequence in *UAS-SVS-dlp* was verified by DNA sequencing. *pAc5.1-wg-3XHA, pAc5.1-Wnt2–3XHA,* and *pAc5.1-Wnt6–3XHA* overexpression plasmids were obtained from K. Basler (Herr and Basler, 2012), and *UAS-Wnt4– 3XFLAG* plasmid was obtained from M. Buszczak (Mottier-Pavie et al., 2016).

S2R+ cell culture, transfection, and immunohistochemistry

S2R+ cells were obtained from DGRC *(Drosophila* Genomics Resources Center) and maintained at 25°C in complete Schneider's medium (Gibco), which is supplemented with 10% heat inactivated fetal bovine serum (Biowest) and 100 U/ml penicillin/streptomycin (Gibco). $2.5X10^5$ S2R+ cells per well were plated in 24-well plates one day prior to performing transient transfections. Cells were transfected with *pAc5.1-* and/or *UAS-*overexpression and *Metallothionein-Gal4* plasmids using $CaCl_2$ method (Wang and Page-McCaw, 2014). For cells transfected with two or more plasmids, equal amount of each plasmids was used, and the total amount of DNA transfected for each condition was 6 μg. Protein expression was induced by incubating transfected cells in complete Schneider's medium supplemented with $CuSO_4$ at a final concentration of 700 μM for 4 days at 18°C

(Wang and Page-McCaw, 2014). To maintain identical experimental conditions, *pAc5.1-wg-3XHA,pAc5.1-Wnt2–3XHA,* or*pAc5.1-Wnt6– 3XHA* transfected cells were also incubated in $CuSO_4$ + complete Schneider's medium even though protein expression from these plasmids does not rely on $CuSO_4$ treatment. The cells were either harvested for immunohistochemistry, co-immunoprecipitation, or western blot analysis.

For staining S2R+ cells, 50 μl of transfected cells were allowed to attach to 12-well multi-test slide (MP Biomedicals). The cells were fixed on the glass slide with 4% PFA in PBS for 10 minutes at room temperature. Fixed cells were washed 3X5 minutes with 1X PBS to maintain non-permeabilizing conditions and subsequently incubated with primary and secondary antibodies for 1 hour at room temperature; 3X5 minute washes with PBS were done post primary and secondary incubations as well. The antibodies were diluted in 5% NGS made with PBS. The S2R+ cells were stained with rabbit anti-GFP (AbCam,6556, 1:50) primary antibody and FITC- conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, 711–095-152, 1:200) secondary antibody and mounted in DAPI containing Vectashield (Vector Laboratories).

Co-immunoprecipitation assay and western blots

Co-immunoprecipitation assays were performed by lysing the cells in lysis buffer (2mM Tris HCl, 150mM NaCl, 1% NP-40 substitute, 1mM EDTA, 5% glycerol, pH:7.4) with protease inhibitor (Halt™ Protease inhibitor single use cocktail, Thermo Scientific). The crude lysate was centrifuged 13,000 r.p.m for 5 minutes at 4°C to obtain a clarified lysate that was incubated with GFP-Trap Magnetic Agarose beads (Chromotek) for 30 minutes at 4°C. The beads were washed with lysis buffer 3X30 minutes post incubation with the lysates. The protein complexes were eluted in 1X reducing sample buffer by heating the beads at 90°C for 5 minutes. The eluted samples were run on SDS-PAGE gels and western blots were performed.

For western blots, S2R+ cell pellets were harvested by centrifugation. The pellets were washed in 1X PBS and lysed in either in 1× NuPage LDS sample buffer (Invitrogen) (for non-reducing gels) or in 1× NuPage LDS sample + 5% β-Mercaptoethanol (for reducing gels). The cell lysates were denatured by heating at 90°C for 2 minutes and run on Tris-glycine (10% Mini-PROTEAN TGX) precast gels (Bio-Rad Laboratories). Separated proteins were transferred onto Hybond-C Extra nitrocellulose membranes (GE Healthcare) using wet-transfer protocol. The membranes were incubated in primary antibodies overnight at 4°C and incubated in secondary antibodies for 2 hours at room temperature in dark. The membranes were washed 3X10 minutes with 0.1% PBT (1XPBS+ 0.1% Tween-20) following each incubation and once with PBS prior to scanning. The membranes were scanned using Odyssey Infrared Imaging System (LI-COR Biosciences). Primary antibodies used for western blots in this study include rabbit anti-GFP (Torrey Pines Biolabs; 1:2500), rat anti-HA (3F10, Roche, 1:1000), and mouse anti-FLAG M2 (F3165; Sigma-Aldrich). Secondary antibodies (1:5000) used in this study include: donkey anti-rabbit IgG conjugated to IRDye 680, and goat anti-rat IgG or goat anti-mouse IgG conjugated to IRDye 800CW (LI-COR Biosciences).

Results

Overexpression of *dlp* in escort cells phenocopies *Wnt* loss-of-function in the germarium

dlp loss-of-function analyses in the germarium have shown that Dlp in escort cells enhances long-range Wg signaling to follicle stem cells (Wang and Page-McCaw, 2014), and Dlp in follicle stem cells negatively regulates stem cell competitiveness for niche occupancy in part by promoting Wg signaling in FSCs (Su et al., 2018). In this study, we overexpressed *dlp* in the germarium as a complementary approach to analyzing its function. In the wing imaginal discs, *dlp* restricts local paracrine Wg signaling (Yan et al., 2009); in the germarium, four Wnt ligands activate signaling locally in escort cells. Because of these observations, we asked if Dlp restricts local paracrine functions of Wnt ligands in the germarium. We used *c587-Gal4* driver, which drives expression strongly in escort cells (Decotto and Spradling, 2005) and weakly in follicle stem cells (Fig. 1 A'). Because *c587-Gal4* predominantly drives expression in escort cells, we analyze our data in the context of escort cells, although we cannot exclude the participation of follicle stem cells. Overexpression of *dlp* caused dramatic disruption of germarium structure and function (Fig. 1). We characterized three aspects of the *dlp* overexpression phenotype, which we present here in order of most to least obvious: 1) germline development, 2) escort-cell number, and 3) germline stem cell maintenance.

In *dlp* overexpressing germaria, we observed accumulation of multiple germline cysts with enlarged nuclei. To asses germline development, we investigated changes in FasIII stained germline cysts posterior to the 2a/2b boundary; FasIII labels follicle cells surrounding the germline cysts (Fig. 1 B–D). In control germaria, only one cyst was observed in region 2b, whereas in *dlp* overexpressing germaria, on average four cysts were observed, often misshaped and varied in size. To investigate changes in germline nuclear size in *dlp* overexpressing germaria, we used anti-Vasa staining (Fig. 1 E–G) to label the germline cells and measured the nuclear diameter. On average, the largest germline nucleus within the *dlp* overexpressing germarium was three times the diameter of control (Fig. 1 E–G).

To further characterize germline defects in *dlp* overexpressing germaria, we assessed the germline differentiation status in regions 1 and 2a of the germarium by examining the number and distribution of round spectrosomes and branched fusomes. Typically, 3–5 spectrosomes are located anteriorly labeling germline stem cells and cystoblasts, and several fusomes are located posteriorly within regions 1 and 2a labeling differentiating cysts (Deng and Lin, 1997; Lin and Spradling, 1995) (Fig. 1 H, J). Anti-Hts staining showed that overexpression of *dlp* caused accumulation of round spectrosomes (Fig. 1 I, J), a hallmark of loss of differentiation in the germarium (Kirilly et al., 2011). Interestingly, germline differentiation is promoted by Wg, Wnt2, Wnt4, and Wnt6 in the germarium (Luo et al., 2015; Upadhyay et al., 2016; Wang et al., 2015), and loss-of-function of these *Wnts* is phenocopied by *dlp* overexpression, suggesting that Dlp inhibits Wg, Wnt2, Wnt4, and Wnt6.

Loss of germline differentiation may be related to changes in escort cells because the developing germline depends on escort cells for differentiation (Eliazer et al., 2014; Hamada- Kawaguchi et al., 2014; Jin et al., 2013; Kirilly et al., 2011; Luo et al., 2015; Ma et

al., 2014; Mottier-Pavie et al., 2016; Mukai et al., 2011; Rangan et al., 2011; Upadhyay et al., 2016; Wang et al., 2015; Wang et al., 2011; Xuan et al., 2013). To examine escort cells, we counted cells expressing *PZ1444,* which is a *lacZ* containing enhancer trap that reliably labels both escort cells and cap cells in the germarium (Fig. 1 K). These two cell types can be distinguished by differences in their location and morphology (Xie and Spradling, 2000). The number of *PZ1444*- positive escort cells was decreased in *dlp* overexpressing germaria, with strong *PZ1444* staining detected only in the cap cells (Fig. 1 L–M). We confirmed these results using two additional markers of escort cells, Zfh1 and *fz3-RFP* (Maimon et al., 2014; Upadhyay et al., 2016; Wang and Page-McCaw, 2014), which confirmed a decrease in escort cells in *dlp* overexpressing germaria (Fig. S1). We next asked if the decrease in *PZ1444* - positive escort cells in *dlp* overexpressing germaria was caused by escort-cell death. We used anti-cleaved Caspase3 (Casp3*) staining to detect dying cells in the germarium (Castanieto et al., 2014). As expected, we did not detect significant Casp3* activity in the control germaria (Fig. S2A), whereas we did detect Casp3* when we overexpressed *hid,* a pro-apoptotic gene (Haining et al., 1999), as a positive control. Surprisingly, despite a strong decrease in *PZ1444*- positive escort-cell number, we did not detect significant Casp3* activity in *dlp* overexpressing germaria (Fig. S2C), although it is possible that we missed the window when Casp3* was present in dying cells. As an independent test, we co-expressed *p35* in *dlp* overexpressing germaria to inhibit cell death (Hay et al., 1994). We found that co-expression of *p35* partially restored both the number of *PZ1444*-positive escort cells and germline differentiation (Fig. S2 D–K). These results suggest that although cell death partially accounts for the apparent decrease in number of PZ1444-positive escort cells, it does not entirely explain the apparent decrease in number of PZ1444-positive escort cells in *dlp* overexpressing germaria.

We next asked if the apparent decrease in PZ1444-positive escort cells could be caused by loss of *PZ1444* expression in escort cells rather than loss of escort cells themselves. To ask if escort cells are present in *dlp* overexpressing germaria, we used anti-Dlp or anti-Armadillo antibodies as pan-somatic cell markers (Fig. S3 A–F‘). Both antibodies detected escort cells in regions 1 and 2a of control germaria and did not detect escort cells when *hid* was overexpressed, indicating that dying escort cells are lost from the germarium (Fig. S3 A–F‘). In the *dlp* overexpressing germaria, both antibodies detected PZ1444-negative escort cells surrounding the germline cells, distinguished by anti-Vasa staining (Fig. S3 D”, E”, F”). Further, labelling of escort-cell membranes using mCD8::GFP showed that cytoplasmic processes intermingle with developing germline in control germaria but are lost in *dlp* overexpressing germaria (Fig. S3 G, H). Taken together, our results suggest that although *dlp* overexpression in escort cells leads to some cell death, it predominantly leads to loss of escort cell-specific gene expression and morphology, which we refer to as loss of escort-cell identity. Interestingly, loss of escort cells and loss of escort-cell morphology result from *Wnt2* and *Wnt4* loss-of-function (Upadhyay et al., 2018; Wang et al., 2015), suggesting that Dlp inhibits Wnt2 and Wnt4.

We next asked if maintenance of the germline stem cells was affected in *dlp* overexpressing germaria. The germline stem cell niche, comprised of anterior escort cells and cap cells, secretes Dpp ligand to promote Dpp signaling in the germline stem cells to maintain their stemness (Song et al., 2004; Wang and Page-McCaw, 2018; Xie and Spradling, 1998, 2000).

The germline stem cells and cystoblasts can be labelled by the transcriptional co-activator of the Dpp signaling pathway, p-Mad (Song et al., 2004), and the germline stem cells can be identified by the position of their spectrosomes. In well-fed control flies, 2–4 germline stem cells were detected (Fig. 1 N, P). We observed a decrease in the number germline stem cells in the *dlp* overexpressing germaria (Fig. 1 O, P) indicting they were being lost from their niche. This decrease was not suppressed by co-expression of *p35* (Fig. S2 G–I, L). Interestingly, loss of germline stem cells in the germarium has been previously attributed to *Wnt2, Wnt4,* and *Wnt6* loss-of-function in the germarium (Wang and Page-McCaw, 2018), suggesting that Dlp inhibits Wnt2, Wnt4, and Wnt6.

In summary, most aspects of the *dlp* overexpression phenotype in germaria have been previously attributed to loss of Wg, Wnt2, Wnt4, and/or Wnt6 signaling in the escort cells (Sahai-Hernandez and Nystul, 2013; Upadhyay et al., 2016; Wang et al., 2015; Wang and Page- McCaw, 2018). This pattern suggests that *dlp* overexpression in escort cells disrupts functions of multiple Wnt ligands in the germarium.

To determine if the multifaceted phenotype of *dlp* overexpression was related to the inhibition of Wnt ligands, we asked if co-expression of wg, *Wnt2, Wnt4* or *Wnt6* with *dlp* in escort cells could suppress the *dlp* overexpression phenotype. Remarkably, all aspects were suppressed by co-expression of *Wnts.* We discuss these data below, first focusing on known Wnt functions followed by novel Wnt functions.

Wg, Wnt4, or to a lesser extend Wnt2, suppresses the loss of germline differentiation in *dlp* overexpressing germaria

Considering the germline differentiation phenotype in *dlp* overexpressing germaria, we noted that cap-cell derived Wg and Wnt6 and escort-cell derived Wnt2 and Wnt4 activate Wnt signaling in escort cells to promote germline differentiation (Luo et al., 2015; Wang et al., 2015). Co-expression of *wg* or *Wnt4* in *dlp* overexpressing germaria strongly suppressed the loss of germline differentiation (Fig. 2 A, B, C, E, I), whereas co-expression of *Wnt2* partially suppressed the loss of germline differentiation (Fig. 2 D, I). In contrast, co-expression of *Wnt6* had no effect (Fig. 2 F, I). Thus, our results suggest that *dlp* overexpression in escort cells inhibits Wg, Wnt2, and Wnt4 local paracrine functions causing loss of germline differentiation. Notably, it has been reported that *Wnt4* is a strong regulator of germline differentiation whereas *Wnt2* acts redundantly with *Wnt4* (Wang et al., 2015). Our results are consistent with these findings, as *Wnt4* is a stronger suppressor of *dlp* overexpression phenotype than *Wnt2.* We note that although accumulation of spectrosome is a proxy for loss of germline differentiation, spectrosome accumulation may also indicate failure of cystoblasts to undergo mitotic divisions. Thus it is possible that the suppression of spectrosome accumulation by co-expression of wg, *Wnt4,* or *Wnt2* a may indicate that Wnt signaling promotes mitotic divisions in cystoblasts.

Next, we tested if canonical Wnt signaling downstream of Wg, Wnt2 or Wnt4 was required in escort cells to promote proper germline differentiation. The canonical Wnt signaling pathway acts through its transcriptional co-activator, Armadillo/β-Catenin (van de Wetering et al., 1997). Previous studies have established that expression of constitutively active *armadillo, armadillo*^{S10} (*arm*^{S10}), causes hyperactivation of the canonical Wnt signaling

pathway without affecting its function at the adherens junctions (Cox et al., 1996; Pai et al., 1997; Somorjai and Martinez-Arias, 2008). Co-expression of *arm*^{S10} in *dlp* overexpressing germaria partially suppressed the loss of differentiation (Fig. 2 G, I), suggesting that both canonical and non-canonical Wnt pathways are involved downstream of Wnt ligands to promote germline differentiation. We also tested if co-expression of Wnt pathway receptor, *fz2* (Bhanot et al., 1999) could suppress the inhibitory effects of Dlp on Wnt ligands. Surprisingly, we found that co-expression of *fz2* in *dlp* overexpressing germaria enhanced the loss of germline differentiation (Fig. 2 H, I) indicating that mere overexpression of *fz2* receptor is not sufficient to activate Wnt signaling in escort cells likely because it is not the rate-limiting component for signaling. Indeed, our results suggest that overexpression of *fz2* acts like a dominant negative, enhancing the *dlp* overexpression phenotype, which could be explained if the excess Fz2 were disconnected from downstream signaling.

Wg or Wnt4 suppresses the loss of escort-cell survival and identity in *dlp* overexpressing germaria

Considering the escort cell phenotypes in *dlp* overexpressing germaria, we noted that *Wnt4* is required for escort-cell survival, morphology, and germline differentiation (Upadhyay et al., 2016; Wang et al., 2015). Co-expression of *Wnt4* suppressed the loss of escort-cell survival and identity caused by *dlp* overexpression (Fig. 3 A, B, E I). We expected this result because *Wnt4* co-expression with *dlp* strongly suppressed the loss of germline differentiation (Fig. 2). Interestingly, co-expression of *wg* in *dlp* overexpressing germaria also suppressed the loss of escort-cell survival and identity and further increased the number of escort cells (Fig. 3 C, I). In contrast, co-expression of *Wnt2, Wnt6, arm*^{S10}, or *fz2* in *dlp* overexpressing germaria did not restore *PZ1444* expression (Fig. 3 D, F–I). These results show that *dlp* overexpression in escort cells indeed inhibits Wnt4 function causing loss of escort-cell survival and identity and suggest that Wg can substitute for Wnt4 in escort cells when expressed ectopically. Further, the maintenance of escort cells appears to be independent of the canonical Wnt signaling pathway downstream of Wg or Wnt4 because it is not suppressed by *arm*^{S10}.

Wnt6, or to a lesser extent Wnt2 or Wnt4, suppresses the loss of germline stem cells in *dlp* overexpressing germaria

Considering the germline stem cell loss in *dlp* overexpressing germaria, we noted that germline stem cell maintenance in the germarium relies on cap-cell derived Wnt6 and escort-cell derived Wnt2 and Wnt4 (Luo et al., 2015; Wang and Page-McCaw, 2018). Consistent with these findings, we find that co-expression of *Wnt6* with *dlp* in escort cells strongly suppressed the loss of germline stem cells, and co-expression of *Wnt2* or *Wnt4* partially suppressed the loss of germline stem cells caused by *dlp* overexpression (Fig. 4 A, B, D, E, F, I). In contrast, co-expression of *wg* with *dlp* had no effect on germline stem cell loss (Fig. 4 C, I). These results suggest that in addition to inhibiting Wnt2 and Wnt4 functions, *dlp* overexpression also inhibits Wnt6 function in the germarium. The co-expression of *arm*^{S10} in *dlp* overexpressing germaria did not suppress the loss of germline stem cells suggesting that non-canonical Wnt signaling downstream of Wnt ligands is required for germline stem cell maintenance (Fig. 4 G, I). As observed for escort cells, co-

expression of *fz2* in *dlp* overexpressing germaria enhanced the loss of germline stem cell phenotype (Fig. 4 H, I).

Wnt6 suppresses the accumulation of multiple germline cysts and nuclear size in *dlp* overexpressing germaria

Considering the accumulation of excess germline cysts in the *dlp* overexpressing germaria, we noted that cyst accumulation has been reported when *wg* was knocked down in germaria, and these cysts contain germline cells with enlarged but fragmented nuclei (Sahai-Hernandez and Nystul, 2013; Song and Xie, 2003). In contrast, we observe that the increase in germline nuclear diameter in these cysts occurs without nuclear fragmentation, which has not been reported as a *Wnt* loss-of-function phenotype to our knowledge. We tested whether each phenotype was suppressible by co-expression of each *Wnt.* The accumulation of multiple germline cysts caused by *dlp* overexpression was suppressed by co-expression of *Wnt6* but not by wg, *Wnt2, Wnt4,* or arm^{S10} (Fig. 5 A–H). Similarly, the increase in germline nuclear size caused by *dlp* overexpression was suppressed by *Wnt6* (Fig. 5 I, J, N, P) but not by wg, *Wnt2, Wnt4,* or arm^{S10} (Fig. 5 K, L, M, O, P). The suppression of both phenotypes by co-expression of *Wnt6* suggests that the accumulation of germline cysts and increase in germline nuclear diameter may be aspects of the same phenotype in *dlp* overexpressing germaria. Our results uncover potential novel functions of Wnt6, which were precluded in previous *Wnt6* loss-of-function analyses possibly because of functional redundancy with unknown factors that may also be regulated by Dlp (see Discussion).

Suppression by different Wnts is not related to their expression levels

We observed that different aspects of the *dlp* overexpression phenotype were suppressed by specific Wnts (summarized in Table 1). However, it was possible the suppression by different Wnts reflected different levels of overexpression because the *UAS-Wnt* constructs were inserted in different regions of the genome. We performed RT-qPCR on whole ovaries to determine levels of *Wnt* transcripts in control and *Wnt* overexpressing conditions. *Wnt* overexpression was restricted to one day post eclosion by use of $Gal80^{ts}$ to inhibit *c587-Gal4.* When overexpressed, *wg, Wnt2,* and *Wnt6* transcript levels were similar, within a twofold range; *Wnt4* transcript level was about twenty-fold lower (Fig. S4). Despite being expressed at comparable levels, *wg, Wnt2,* and *Wnt6* suppressed different aspects of the *dlp* overexpression phenotype (Table 1). Similarly, even though *Wnt4* was expressed at a much lower level, it did suppress some aspects of the *dlp* overexpression phenotype (loss of germline differentiation, loss of escort-cells, and loss of germline stem cells, Table 1). Although transcript levels and extracellular protein levels many not correlate precisely, these data suggest that Wnt levels do not determine their functions. Unexpectedly, we found evidence that *Wnt4* transcription was subject to homeostatic feedback regulation. Specifically, we observed when *c587>dlp* inhibited Wnt signaling, endogenous *Wnt4* transcript levels were increased sevenfold; yet when we experimentally increased *Wnt4* levels by overexpressing it in the *c587>dlp* background, we were only able to achieve a 1.6-fold increase over the *c587>dlp* levels alone (Fig. S4). Our data suggest that different Wnts have specific functions not explained by differences in expression levels.

Loss of *dlp* in escort cells phenocopies *Wnt* gain-of-function phenotypes in germarium causing increase in escort-cell number and loss of germline stem cells

Our results so far suggest that overexpression of *dlp* in escort cells inhibits the functions of all Wnt ligands in the escort cells. To determine if endogenous Dlp also inhibits Wnt ligands in escort cells, we asked if the loss of *dlp* in escort cells resulted in increased Wg, Wnt2, Wnt4, and/or Wnt6 function. We used two independent *UÂS-dlp*^{RNAi} lines to test the effect of *dlp* knockdown in escort cells and compared the phenotypes with that of *wg, Wnt2, Wnt4,* or *Wnt6* overexpression (Fig. 6). Knockdown of *dlp* or overexpression of *wg* or *Wnt6* ligands in escort cells caused an increase in escort cell number compared to control germaria (Fig. 6 A–G). Knockdown of *dlp* in escort cells also resulted in loss of germline stem cells similar to that observed in wg, *Wnt2,* and *Wnt6* overexpressing germaria (Fig. 6 H–K, M, O). Overexpression of *Wnt4* did not cause significant loss of germline stem cells (Fig. 6 L, O) or any other phenotype that we could discern, possibly reflecting its lower level of overexpression as noted above. The increase in escort-cell number in *dlp* knockdown or *Wnt* overexpressing germaria did not affect germline differentiation, and the germaria appeared similar to controls (Fig. 6 H–M, N). These results indicate that knockdown of *dlp* in escort cells phenocopies *Wnt* overexpression and indicate that endogenous Dlp inhibits Wnt functions in the germarium.

Wg, Wnt2, Wnt4, and Wnt6 co-immunoprecipitate with Dlp in S2R+ cells

Our experiments show that Dlp can functionally inhibit Wnts in the germarium. Previous studies have shown that Wg co-immunoprecipitates with Dlp when co-expressed in S2R+ cells (Yan et al., 2009), so we asked if Wnt2, Wnt4, and Wnt6 could also co-immunoprecipitate with Dlp. Previous analysis of Dlp in cell culture (Kreuger et al., 2004; Wang and Page-McCaw, 2014) has made use of a GFP-Dlp fusion protein with an N-terminal GFP similar to that on a *UAS-GFP-dlp* transgene expressed in flies (Marois et al., 2006). However, overexpression of this *UAS-GFP-dlp* transgene did not phenocopy the wild-type *UAS-dlp* overexpression phenotypes that we observed in the germarium (data not shown), and this lack of function *in vivo* suggested that the cell-culture GFP-Dlp construct is not fully functional either. Therefore, we generated a functional tagged Dlp construct for expression in S2R+ cells based on a homozygous viable *dlp* protein-trap fly line *(PBac{602.P.SVS-1}dlp*^{CPTI000445}*),* which expresses a *SVS* (*StrepII- Venus -StrepII*)-tagged *dlp* from the endogenous locus (Fig. S5 A) (Lowe et al., 2014). We verified that *UAS-SVS-dlp* was expressed in S2R+ cells (Fig. S5 B, C) and could be detected at the cell-surface under non-permeabilizing conditions (Fig. S5 B). We also verified that SVS-Dlp overexpressed in S2R+ cells migrated on SDS-PAGE gels as expected, under both reducing and non-reducing conditions (Fig. S5 C). Anti-GFP immunoblots show that the anti-GFP beads reliably pulled down SVS-Dlp. We overexpressed *SVS-dlp* with *wg-3XHA, Wnt2–3XHA, Wnt4– 3XFLAG,* or *Wnt6–3XHA,* which have been previously confirmed to be functional (Herr and Basler, 2012), to test if Wnt ligands were co-immunoprecipitated with SVS-Dlp (Fig. 7). Consistent with previous findings, Wg-3XHA co-precipitated with SVS-Dlp (Fig. 7 A) (Yan et al., 2009). Similarly, we found that Wnt2–3XHA, Wnt4–3XFLAG, and Wnt6–3XHA (Fig. 7 B–D) also co-precipitated with SVS-Dlp. Although we could not completely eliminate the low-level non-specific binding of Wg-3XHA, Wnt2–3XHA or Wnt4–3XFLAG to the anti-GFP beads, there was a dramatic enhancement of the signal in

the presence of SVS-Dlp suggesting that Dlp physically interacts with multiple Wnt ligands in S2R+ cells. Thus, together our genetic experiments and co-immunoprecipitation assays suggest that Dlp sequesters multiple Wnt ligands, restricting their ability to function in the germarium (Fig. 8).

Discussion

The availability of secreted ligands in extracellular space is important for determining the activation of signaling pathways in target cells. Several mechanisms exist in multicellular organisms to regulate the optimal levels of secreted ligands in the vicinity of the target cells (Takada et al., 2017). The best understood mechanism *in vivo* is Dlp-mediated regulation of Wg in the wing disc, where Dlp acts as a Wg exchange factor, facilitating long-range signaling to cells where Wg level is low and restricting local paracrine signaling to cells where Wg level is high (Yan et al., 2009). Similarly, Dlp in escort cells promotes long-range spreading of Wg from cap cells to follicle stem cells (Wang and Page-McCaw, 2014). In this study, we show that *dlp* overexpression in escort cells results in a dramatic phenotype (Fig. 1) that can be attributed to restricting local paracrine signaling of multiple Wnt ligands. Four Wnts are expressed in wild-type germaria: *Wnt2* and *Wnt4* are expressed in escort cells, *wg* and *Wnt6* are expressed in cap cells, and all four Wnts activate local paracrine Wnt signaling in escort cells (Luo et al., 2015; Wang et al., 2015). The Dlp overexpression phenotype appears to be a cumulative phenotype, arising from simultaneous loss of many *Wnt* functions, as summarized in Table 1. First, *dlp* overexpression in escort cells causes loss of germline differentiation, previously reported as *wg, Wnt2, Wnt4,* or *Wnt6* loss-of-function phenotypes (Hamada-Kawaguchi et al., 2014; Luo et al., 2015; Upadhyay et al., 2016; Wang et al., 2015); further, germline differentiation is restored by co-expression of wg, *Wnt2,* or *Wnt4* (Fig. 2). Second, *dlp* overexpression causes loss of escort-cell survival, identity, and morphology (Fig. S1, S2, S3), as evidenced by partial suppression by *p35* and changes in gene expression; previously it was reported that *Wnt4* loss-of-function causes a loss of escort-cell survival and morphology (Upadhyay et al., 2016; Wang et al., 2015).

Further, escort-cell number and identity is restored by co-expression of *wg* or *Wnt4* (Fig. 3). Third, *dlp* overexpression causes loss of germline stem cells, previously reported as *Wnt2, Wnt4,* or *Wnt6* loss-of-function phenotypes (Wang and Page-McCaw, 2018); further, germline stem cell number is restored by co-expression of *Wnt2, Wnt4,* or *Wnt6* (Fig. 4). In loss of function experiments, *dlp* knockdown in escort cells phenocopies the *Wnt* gain-of-function phenotypes of increased escort-cell number and loss of germline stem cells (Fig. 6). These results indicate that Dlp inhibits local paracrine signaling by Wg, Wnt2, Wnt4, and Wnt6. This inhibition is likely mediated by physical interaction between Dlp and each Wnt ligand, as Dlp co- immunoprecipitates with all four Wnt ligands in S2R+ cells (Fig. 7 and Fig. 8). Thus, Dlp appears to bind Wnt ligands generally, and we identify a mechanism by which Dlp modulates the local functions of of Wg, Wnt2, Wnt4, and Wnt6.

The *Wnt* loss-of-function phenotypes caused by *dlp* overexpression are suppressed by respective *Wnt* co-expression. Importantly, every Wnt is able to suppress one or more phenotypes (Figs. 2–4, Table 1), indicating that all *Wnts* are expressed at sufficient levels to offset the effects of *dlp* expression. Moreover, no one Wnt suppresses all the *dlp*

overexpression phenotypes even though most *Wnts* are expressed at comparable levels, suggesting that Wnts have specific functions independent of their expression levels (Fig. S4).

An exception to the expected pattern of suppression is the loss of germline differentiation caused by *dlp* overexpression, which is strongly suppressed by *wg* but not *Wnt6* (Fig. 2) even though previous loss-of-function analyses indicate that Wnt6 is a stronger promoter of germline differentiation than Wg (Luo et al., 2015). In the previous study, *wg* knockdown may have been incomplete, as our efforts to knockdown *wg* in cap cells have not significantly altered Wg antibody staining around cap cells (unpublished); thus, the role of Wg in germline differentiation may have been previously underestimated (Fig. 2). Further, in the previous study, cap-cell specific knockdown of *Wnt6* caused such a small increase in spectrosome number that we would have interpreted this phenotype as comparable to control. Hence, we propose that Wg and not Wnt6 is a strong regulator of germline differentiation.

We observed an accumulation of germline cysts in *dlp* overexpressing germaria suggesting that germline cysts fail to exit the germaria (Fig. 5). The accumulated cysts were of variable shape and size, and they contained germline cells with increased nuclear size (Fig. 5). Interestingly, both phenotypes were suppressed by co-expression of *Wnt6* (Fig. 5) even though *Wnt6* loss-of-function is not reported to cause accumulation of germline cysts or increase in germline nuclear size. The suppression of both of these phenotypes by *Wnt6* suggests that these phenotypes may be related. One possibility is that Wnt6 regulates budding of germline cysts from the germaria. In the absence of Wnt6 signaling caused by *dlp* overexpression, germline cells continue to develop resulting in increased germline nuclear size, typical of nurse cells later in development, despite failing to exit the germaria. Alternatively, Wnt6 signaling might regulate cell-cycle progression in the developing germline, and loss of Wnt6 signaling in *dlp* overexpressing germaria results in accumulation of germline cells with increased nuclear size, a hallmark of endocycling cells (Edgar and Orr-Weaver, 2001). In this scenario, the increase in germline nuclear size prevents the exit of cysts from the germaria, resulting in their accumulation. These potential roles of Wnt6 signaling may have eluded previous *Wnt6* loss-of- function studies because of functional redundancy with other factors and are uncovered only when these factors are also inhibited by *dlp* overexpression. Although previous studies have shown that Wg signaling is important for cyst development posterior to 2a/2b boundary in the germarium. (Sahai-Hernandez and Nystul, 2013; Song and Xie, 2003; Wang and Page-McCaw, 2014), *wg* co-expression does not suppress the accumulation of germline cysts in *dlp* overexpressing germaria. What might account for this discrepancy? It is plausible that different phenotypes are sensitive to different Wnt thresholds. We speculate that although loss of Wg function caused by *dlp* overexpression is sufficient to cause loss of germline differentiation and escort-cell identity (Fig. 2 C and I, Fig. 3 C and I), it is not sufficient to cause the accumulation of multiple cysts. Indeed, in previous studies, multiple cysts accumulate only when *wg* is knocked down in adult escort cells for a prolonged duration, suggesting that this phenotype is exhibited only when Wg function is strongly compromised. We argue that *wg* co-expression is unable to suppress the accumulation of multiple germline cysts because this phenotype is not caused by *wg* loss-of-function in *dlp* overexpressing germaria. It is important to note that

because *c587-Gal4* drives weak expression of *UAS-dlp* in follicle stem cells, the accumulation of cysts in *dlp* overexpressing germaria may be attributed to *Wnt* loss-of-function in follicle stem cells that give rise to follicle cells surrounding the germline cysts. Further studies are required to understand how cyst accumulation and nuclear size are related and the role of Dlp-Wnt signaling module in regulating these aspects of oogenesis.

Previously we found that the maintenance of ovarian germline stem cells depends on Wnt2, Wnt4, and Wnt6, as loss of any of these *Wnts* results in loss of germline stem cells (Wang and Page-McCaw, 2018). In this study, we find that overexpression of wg, *Wnt2,* or *Wnt6* also results in loss of germline stem cells (Fig. 6). Similarly, both loss and gain of *dlp* causes loss of germline stem cells (Fig. 4 and Fig. 6); interestingly, Dlp has been shown to be required for male germline stem cell maintenance (Hayashi et al., 2009). Thus, germline stem cell maintenance is sensitive to increased and decreased Wnt levels, and *dlp* modulates the amount of Wnts available for signaling in the anterior escort cells of the germline stem cell niche.

Escort cells form the differentiation niche (Decotto and Spradling, 2005; Kirilly et al., 2011; Morris and Spradling, 2011), and perturbing escort cells causes loss of germline differentiation (Eliazer et al., 2014; Hamada-Kawaguchi et al., 2014; Jin et al., 2013; Kirilly et al., 2011; Luo et al., 2015; Ma et al., 2014; Mottier-Pavie et al., 2016; Mukai et al., 2011; Rangan et al., 2011; Upadhyay et al., 2016; Wang et al., 2015; Wang et al., 2011; Xuan et al., 2013). These studies have shown that germline differentiation depends on escort-cell survival and escort-cell cytoplasmic processes, and these functions are regulated by Wnt signaling. However, because these studies relied on escort-cell identity markers to label escort cells, they may have underestimated the number of escort cells present when Wnt signaling is lost, as our data indicate that escort cells can be present even when escort-cell identity is lost (Fig. S2 and S3). We find that germline differentiation is separable from escort-cell survival and identity, because they are each suppressed by different sets of Wnt ligands. Our suppression results suggest that 1) a canonical Wg/Wnt2/Wnt4-Armadillo signaling axis in escort cells regulates germline differentiation (Fig. 2), and 2) an armadillo-independent Wg/Wnt4 signaling axis promotes escort-cell survival and identity, as indicated by *PZ1444, fz3-RFP,* and *zfh-1* expression (Fig. S1, Fig. 3). These two signaling axes are illuminated when *dlp* and arm^{S10} are overexpressed — germline differentiation is restored even though *PZ1444* escort-cell labeling is absent. This result suggests that the germline cells can differentiate normally even when es-cortcell identity is lost. Furthermore, overexpression data suggest there is a third Wg/Wnt6- Armadillo signaling axis in escort cells that regulates escort-cell number, as overexpression of *wg, Wnt6* or arm^{S10} increases the number of PZ1444-labelled escort cells (Fig. 3 C, Fig. 6 C, F, G) (Wang et al., 2015). Thus, we find that three independent Wnt signaling axes function in escort cells to regulate different escort-cell properties.

dlp overexpression studies have relevance to human cancers (Kaur and Cummings, 2019). Dlp is an ortholog of vertebrate Glypicans 1/2/4/6, which constitute one of the two sub-families of glypicans (Filmus et al., 2008). Studies based on cancer cell lines and murine models show that certain cancer types such as pancreatic, ovarian, and breast cancers upregulate glypicans, which can promote proliferative, metastatic, and angiogenic potential

of cancer cells (Kaur and Cummings, 2019). Of note, Glypican 4 is highly upregulated in pancreatic cancer cells and potentiates the Wnt/β-catenin pathway (Cao et al., 2018). In breast cancer cells, upregulated Glypican 6 inhibits the canonical Wnt/β-catenin pathway and induces non-canonical Wnt5A signaling (Yiu et al., 2011). Our results suggest that these glypicans may modulate Wnt signaling by sequestering Wnt ligands.

Our results suggest a model where multiple Wnts - Wg, Wnt2, Wnt4, and Wnt6 - are sequestered by Dlp resulting in inhibition of local paracrine Wnt functions. Reciprocally, loss of *dlp* in escort cells increases local Wnt signaling (this study) and simultaneously decreases long- range Wg signaling (Wang and Page-McCaw, 2014). How can Dlp sequester Wnts but also promote long-range Wg spreading? We envision that cell-surface Dlp molecules on escort cells act like pinball flippers keeping Wnts mobile in the extracellular space by the continual ligand binding and release. This mechanism allows both long-range spreading of Wg from cap cells to follicle cells as well as local Wnt signaling in paracrine fashion in escort cells, typical for Wg, Wnt2, Wnt4, and Wnt6 signaling (Luo et al., 2015; Wang et al., 2015; Wang and Page-McCaw, 2018). This mechanism explains why loss of *dlp* results in increased ligand availability for local signaling and simultaneously decreased Wg spreading from cap cells to follicle stem cells. In contrast, when Dlp is overexpressed, the pool of extracellular Wnts available for signaling is decreased because Wnt sequestration is increased, which inhibits local Wnt signaling and presumably long-range Wg signaling. This inhibition of local Wnt signaling in escort cells compromises escort-cell identity and function. We conclude that Dlp binds to multiple extracellular Wnts and modulates the levels of extracellular Wnts available for signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Dally-like protein (Dlp) modulates local paracrine Wnt signaling in the germarium
- Wg, Wnt2, Wnt4, and Wnt6 are functionally inhibited by Dlp
- Wg, Wnt2, Wnt4, and Wnt6 have functional specificity in the germarium
- Wg, Wnt2, Wnt4, and Wnt6 co-immunoprecipitate with Dlp

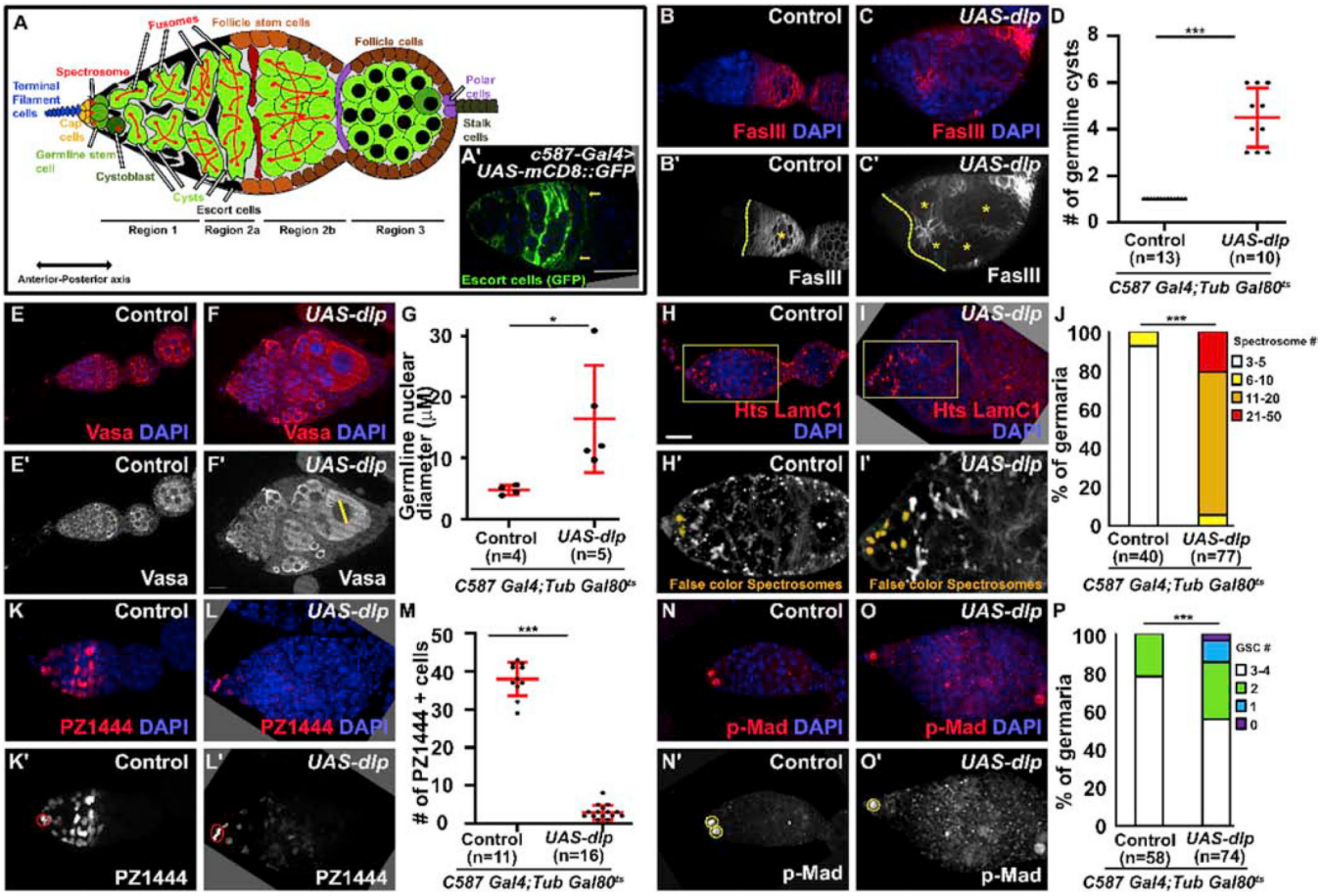

Figure 1: *dlp* overexpression in escort cells disrupts germarium structure and function.

A) Somatic and germline cell types in *Drosophila* germarium are shown. **A')** Expression domain of *c587-Gal4* is shown. Yellow arrows in A' show weak expression in follicle stem cells. **B-D**) *dlp* overexpression caused an increase in germline cysts. Germline cysts posterior to 2a/2b boundary were counted based on FasIII staining in control (B, B') and *dlp* overexpressing (C, C') germaria. Germline cysts per germarium are quantified in D. Yellow dotted lines indicate 2a/2b boundary and yellow asterisks show germline cysts in B' and C'. **E-G**) *dlp* overexpression caused an increase in germline nuclear size. Germline cells were identified based on Vasa staining in control (E, E') and *dlp* overexpressing (F, F') germaria. Nuclear diameter (yellow line) is quantified in G. **(H-J)** *dlp* overexpression caused a loss of germline differentiation. Spectrosomes (yellow false color in H' and I') were identified based on Hts staining in control (H, H') and *dlp* (I, I') overexpressing germaria. Spectrosome number per germarium is quantified in J. LamC1 labels nuclear membranes of cap cells in H, I. Yellow boxes in H and I indicate regions that have been magnified in H' and I'. **K-M**) *dlp* overexpression caused a loss of PZ1444-positive escort cells. *PZ1444* enhancer trap stains escort cells and cap cells in control (K, K') and *dlp* overexpressing (L, L') germaria. Red dotted circles in K', L' outline cap cells. PZ1444-positive escort-cell number is quantified in M. **N-P**) *dlp* overexpression caused a loss of germline stem cells. Germline stem cells are outlined in yellow in control (N, N') and *dlp* overexpressing (O, O') germaria. Germline stem cells are quantified in P. All experiments were performed using

c587-Gal4 and *TubP-Gal80^{ts}* to express transgenes in escort cells for seven days post eclosion. Nuclei in all samples were stained with DAPI (blue). Scale bars: 20 μm. Bar in A' applies to A' only. Bar in F' applies to E and F only. Bar in H applies to B, H, K, and N.

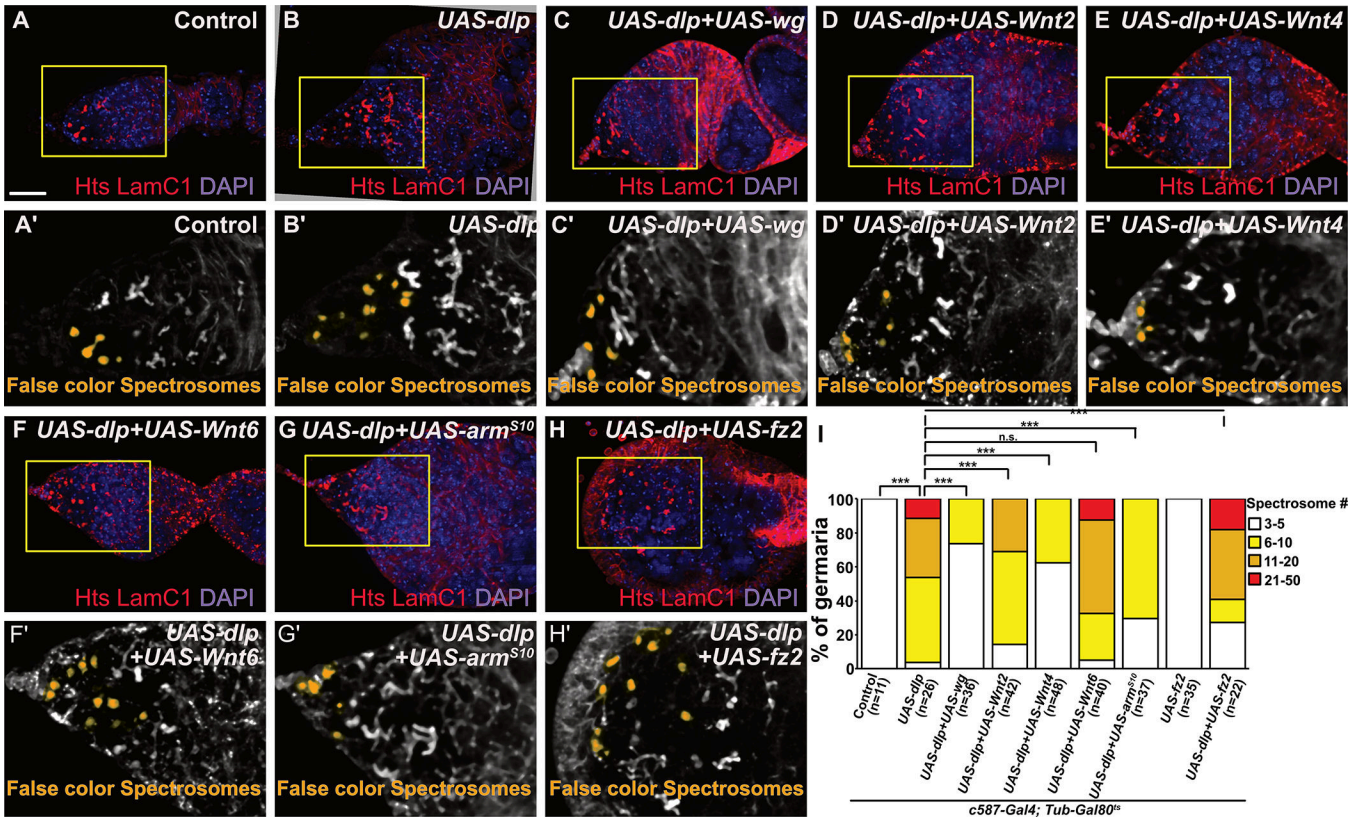

Figure 2: Loss of germline differentiation caused by *dlp* overexpression is suppressed by co-expression of *wg* or *Wnt4*.

A-H) Germaria were stained with anti-LamC1 and anti-Hts, which label cap-cell nuclear membranes and spectrosomes (yellow false color in A'-H') respectively. Compared to control germaria (A-A'), *dlp* overexpressing germaria showed accumulation of round spectrosomes (B, B'). Spectrosome accumulation caused by *dlp* overexpression was strongly suppressed by *wg* (C-C'), *Wnt2* (D-D'), or *Wnt4* (E-E') but not by *Wnt6* (F-F'). Spectrosome accumulation caused by *dlp* overexpression was partially suppressed by arm^{S10} (G-G') but enhanced by *fz2* (H-H') co-expression. Yellow boxes in A-H indicate regions magnified in A'-H'. **I)** Quantification of spectrosomes for indicated genotypes is shown. All experiments were performed using *c587- Gal4* and $TubP\text{-}Gal80^{ts}$ to express transgenes in escort cells for seven days post eclosion. Nuclei in all samples were stained with DAPI (blue). Scale bar: 20 μm.

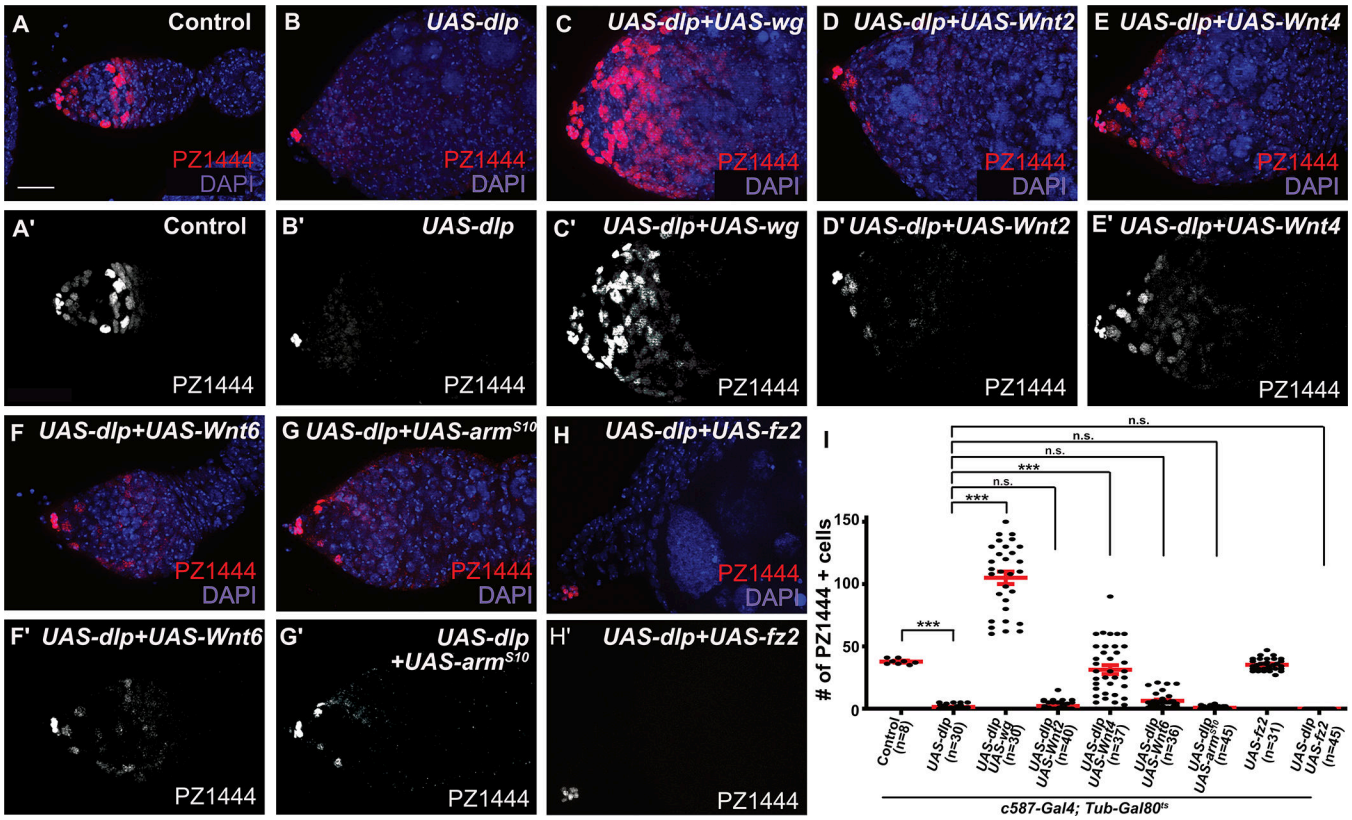

Figure 3: Loss of escort-cell survival and identity caused by *dlp* overexpression is suppressed by co-expression of *wg* or *Wnt4.*

A-H) *PZ1444* identified escort cells in control (A-A'), *dlp* (B-B'), *dlp+wg* (C-C'), *dlp +Wnt2* (D-D'), *dlp+Wnt4* (E-E'), *dlp+Wnt6* (F-F'), *dlp+arm*^{S10} (G-G'), and *dlp+fz2* (H-H') overexpressing germaria. **I)** Quantification of PZ1444-positive cells for indicated genotypes show that loss of *PZ1444* expression caused by *dlp* overexpression is strongly suppressed by *wg* or *Wnt4.* All experiments were performed using *c587-Gal4* and *TubP-Gal80*^{ts} to express transgenes in escort cells for seven days post eclosion. Nuclei in all samples were stained with DAPI (blue). Scale bar: 20 μm.

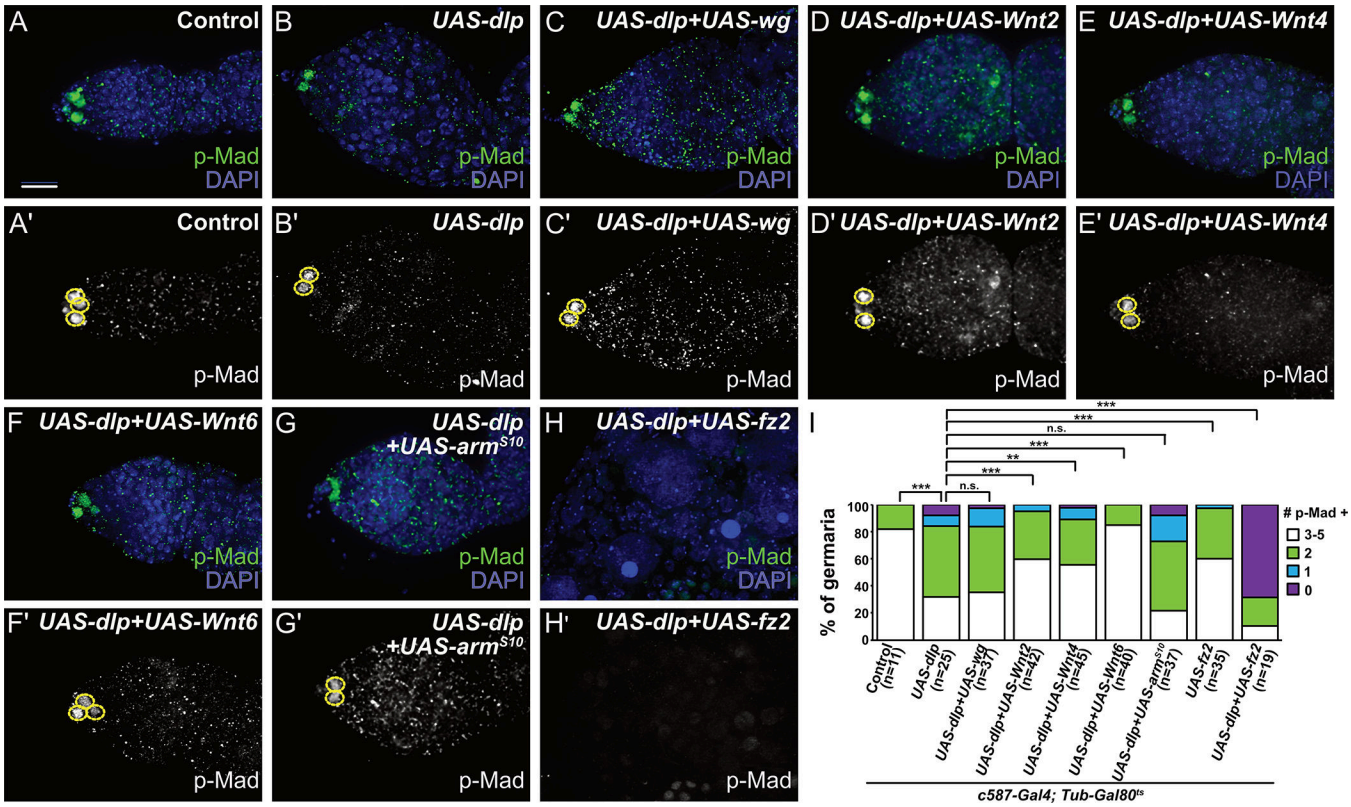

Figure 4: Loss of germline stem cells caused by *dlp* overexpression is suppressed by co-expression of *Wnt6.*

A-H) p-Mad staining labeled germline stem cells (yellow dotted circles in A'-G') in control (A-A'), *dlp* (B-B'), *dlp+wg* (C-C'), *dlp+Wnt2* (D-D'), *dlp+Wnt4* (E-E'), *dlp+Wnt6* (F-F'), *dlp+arm*^{S10} (G-G'), and *dlp+fz2* (H-H') overexpressing germaria. **I)** Quantification of p-Mad positive cells for indicated genotypes show that loss of germline stem cells caused by *dlp* overexpression is strongly suppressed by *Wnt6.* All experiments were performed using *c587- Gal4* and *TubP-Gal80*^{ts} to express transgenes in escort cells for seven days post eclosion. Nuclei in all samples were stained with DAPI (blue). Scale bar: 20 μm.

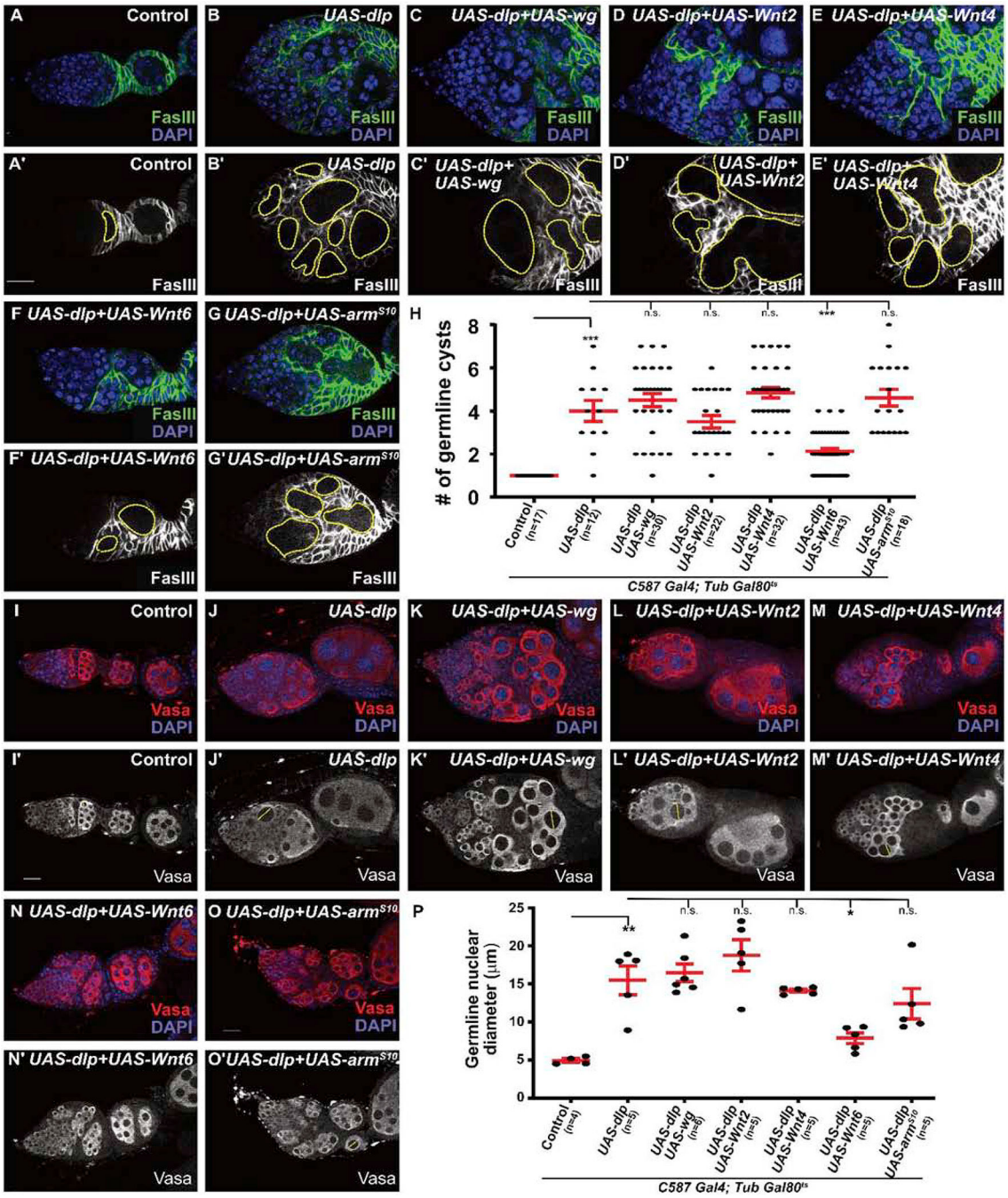

Figure 5: Accumulation of multiple germline cysts and increase in germline nuclear size caused by *dlp* overexpression is suppressed by co-expression of *Wnt6*.

A-G) FasIII staining of follicle cells from control (A-A'), *dlp* (B-B'), *dlp+wg* (C-C'), *dlp +Wnt2* (D-D'), *dlp+Wnt4* (E-E'), *dlp+Wnt6* (F-F'), and *dlp+arm^{S10}* (G-G') germaria show that the accumulation of multiple germline cysts caused by *dlp* overexpression is suppressed by *Wnt6*. Yellow dotted lines in A'-G' outline the germline cysts observed in single optical section. **H)** Quantification of the number of germline cysts for indicated genotypes is shown. **I-P)** Germline cells were labelled with Vasa. Compared to control germaria (I-I'), germline

nuclear size is increased in *dlp* overexpressing germaria (J-J'). The increase in germline nuclear size caused by *dlp* overexpression is not suppressed by *wg* (K-K'), *Wnt2* (L-L'), *Wnt4* (M-M'), or arm^{S10} (O-O'), but is suppressed by *Wnt6* (N-N'). Yellow line indicates the diameter of the largest germline nucleus in I'-O'. **H)** Quantification of nuclear diameter for indicated genotypes. All experiments were performed using *c587-Gal4* and $TubP\text{-}Gal80^{ts}$ to express transgenes in escort cells for seven days post eclosion. Nuclei in all samples were stained with DAPI (blue). Scale bar: 20 μm. Bar in A' applies to A-G. Bar in I' applies to I-O.

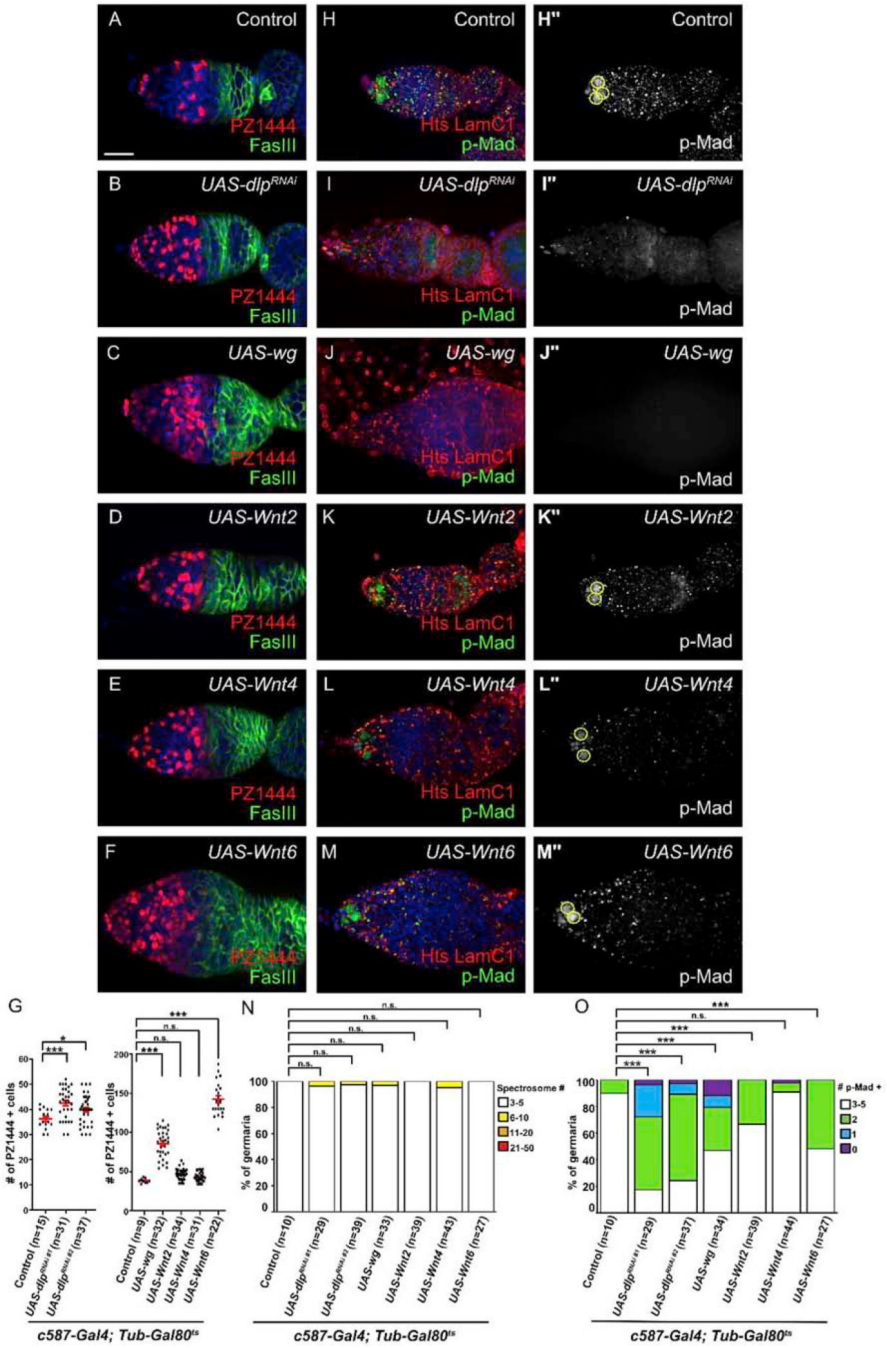

Figure 6: *dlp* loss-of-function in escort cells phenocopies *Wnt* gain-of-function phenotypes.

A-F) Escort cells were labelled using *PZ1444* (A-F) in control (A) $dlp^{RNAi\#1}$ (B), *wg* (C), *Wnt2* (D), *Wnt4* (E), and *Wnt6* (F) overexpressing germaria. **G)** Quantification of PZ1444-positive escort cells showed that knockdown of *dlp* or overexpression of *Wnt* ligands in escort cells causes a varying range of increase in PZ1444-positive escort-cell number. **H-M)** Spectrosomes were identified based on Hts staining (red) and germline stem cells (yellow dotted circles in H"- M") were identified based on p-Mad staining (green in H-M, gray scale in H'-M') in control (H-H'), $dlp^{RNAi\#1}$ (I-I'), *wg* (J-J'), *Wnt2* (K-K'), *Wnt4* (L-L'), and

Wnt6 (M-M') overexpressing germaria. **N)** Spectrosome counts for indicated genotypes show that neither knockdown of *dlp* nor overexpression of *Wnt* ligands in escort cells affects germline differentiation. **O)** Germline stem cell counts for indicated genotypes show that knockdown of *dlp* or overexpression of wg, *Wnt2* or *Wnt6* ligands in escort cells causes a varying range of loss of germline stem cells. All experiments were performed using *c587-Gal4* and *TubP-Gal80*^{ts} to express transgenes in escort cells for seven days post eclosion. Nuclei in all samples were stained with DAPI (blue). Scale bar: 20 μm.

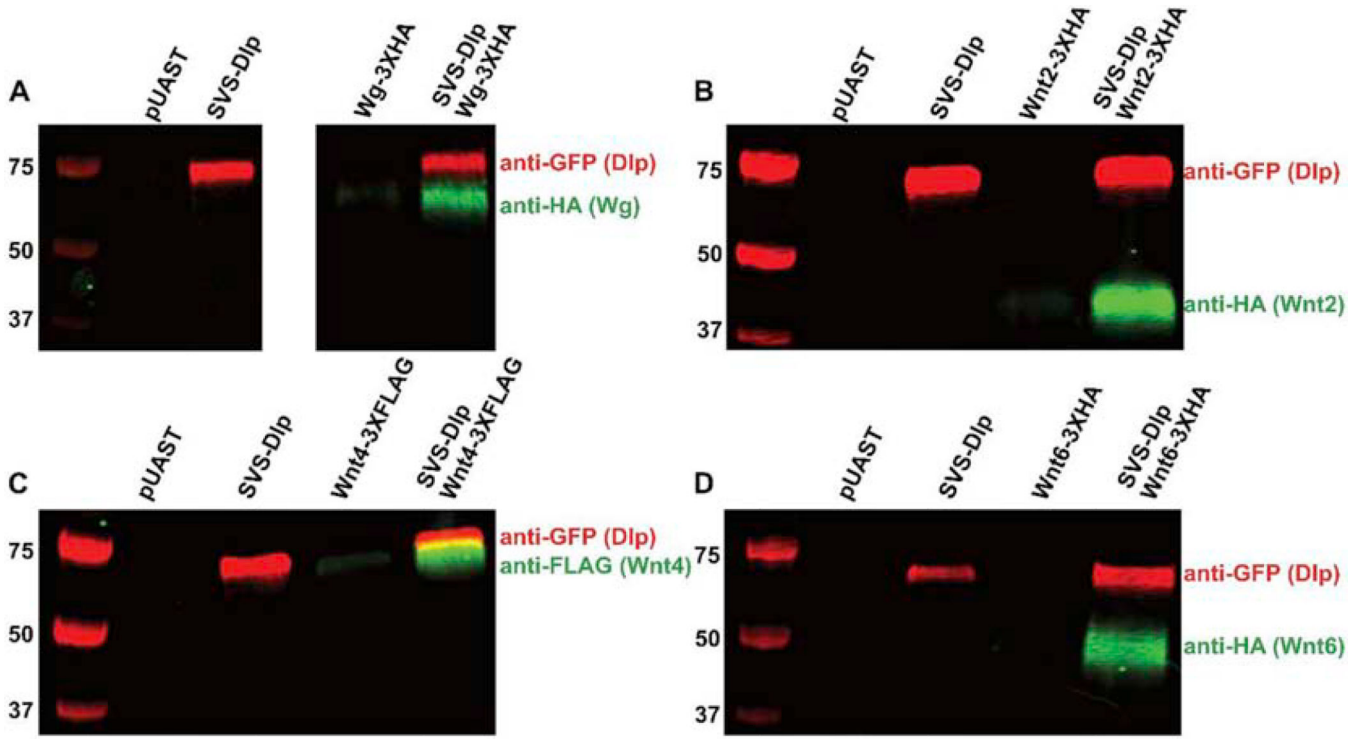

Figure 7: Wg, Wnt2, Wnt4, and Wnt6 co-immunoprecipitate with Dlp in S2R+ cells.

A-D) Immunoblots following co-immunoprecipitation with anti-GFP show that Wg (A), Wnt2 (B), Wnt4 (C), and Wnt6 (D) can bind SVS-Dlp in a complex when co-overexpressed with *SVS-dlp* in S2R+ cells. The western blots were performed under reducing conditions and probed with rabbit anti-GFP antibody (to detect the N terminal subunit of SVS-Dlp) in A-D and with Rat anti-HA (A-C) or mouse anti-FLAG (D) to detect tagged Wnt proteins. Data shown in (A) are from the same blot, and the intervening lane was excluded.

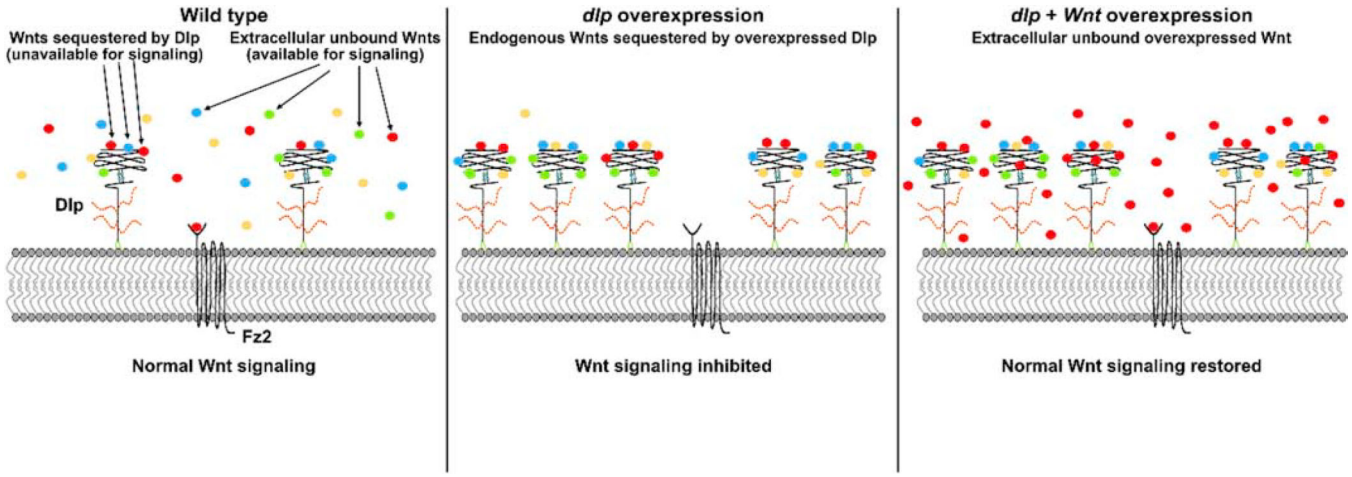

Figure 8: Model depicting mechanism by which Dlp modulates extracellular availability of secreted Wnts.

Left: In wild type germaria, a fraction of extracellular Wnt ligands (Wg, Wnt2, Wnt4, Wnt6) is sequestered by endogenous Dlp restricting local paracrine Wnt signaling. Center: Overexpression of Dlp sequesters more of the endogenous Wnts, inhibiting local paracrine Wnt signaling. Right: Wnt signaling is restored by co-expression of an individual Wnt.

Table 1.

dlp overexpression phenotypes are suppressed by co-expression of *Wnt* ligands.

***dlp* overexpression phenotypes**	**Reported *Wnt* loss-of-function phenotype**	**References**	***dlp* overexpression phenotype suppressed by co-expression of**
Loss of germline differentiation	*wg* and *Wnt6*	(Luo et al., 2015)	wg, *Wnt2*, *Wnt4*
	Wnt2, *Wnt4*	(Wang et al., 2015)	
Loss of escort-cell survival and identity	*Wnt2*, *Wnt4*[*]	(Upadhyay et al., 2016)	*wg*, *Wnt4*
Loss of germline stem cells	*Wnt2*, *Wnt4*, *Wnt6*[*]	(Wang and Page-McCaw, 2018)	*Wnt2*, *Wnt4*, *Wnt6*
Increase in germline nuclear diameter	-	-	*Wnt6*
Accumulation of multiple germline cysts	-	-	*Wnt6*

[*] No data reported for *wg* loss-of-function.