Zoological Studies **51**(7): 1122-1138 (2012)

Zoological Studies

Functional Screening of Human Genes by Expression in *Drosophila* Identifies Enthoprotin/LqfR as a Regulator of Hematopoiesis

Yung-Heng Chang[1,2], Wei-Ru Li[3], and Y. Henry Sun[1,2,3,*]

[1]*Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan*

[2]*Institute of Molecular Biology, Academia Sinica, Nangang, Taipei 115, Taiwan*

[3]*Department of Life Sciences and Institute of Genomic Sciences, National Yang-Ming University, Taipei 112, Taiwan*

(Accepted April 30, 2012)

Yung-Heng Chang, Wei-Ru Li, and Y. Henry Sun (2012) Functional screening of human genes by expression in *Drosophila* identifies enthoprotin/LqfR as a regulator of hematopoiesis. *Zoological Studies* **51**(7): 1122-1138. We expressed 83 human genes, whose expression is cell cycle dependent and changed in hepatoma, in *Drosophila* as a screen for conserved functions. We identified that overexpression of human enthoprotin and 2 novel isoforms of its fly homolog, Liquid facet Related (LqfR), induced melanotic mass formation. We showed that the effect was due to overproliferation of a hemocyte progenitor in the lymph gland, which is the primary hematopoietic organ in the larval stage. We further showed that LqfR acts in distinct regions in the lymph gland with distinct functions: acting in the medullary zone (MZ) to regulate progenitor proliferation, acting in the posterior signaling center (PSC) to autonomously repress the size of the PSC, and acting in the MZ to non-autonomously regulate the PSC size. We also found that LqfR caused accumulation of the activated Notch (N) receptor, and its function requires N signaling. Our results provide a link from endocytic vesicle trafficking to N activation to hematopoiesis, and suggest that these functions are evolutionarily conserved.
http://zoolstud.sinica.edu.tw/Journals/51.7/1122.pdf

Key words: Epsin, Notch, Proliferation, Hemocyte, Leukemia.

Many regulatory and signaling mechanisms are conserved between humans and *Drosophila*. Of the 929 disease gene entries in the Online Mendelian Inheritance in Man (OMIM), 77% have homologs in *Drosophila* (Reiter et al. 2001). Studying the molecular mechanisms of these cellular processes in *Drosophila* can often yield important insights into similar processes in humans.

The basic approach is to express human genes in various tissues of the fly and examine the effect caused by their misexpression. This gain-of-function (GOF) approach is in contrast to the more classical loss-of-function (LOF) mutation approach. Loss-of-function mutations often cause no obvious phenotype, due to gene redundancy. In contrast, misexpression of a gene, although not physiological, often produces a phenotype suggestive of its physiological function. For example, the study of oncogenes initiated from the oncogenic phenotype due to their misexpression or activation eventually led to an understanding of their important roles in cell cycle control (reviewed by Varmus 1984). Cross-specific expression can also identify those mechanisms that are evolutionarily conserved.

We cloned full-length cDNA from 83 human genes into the pUAST-Flag vector, and generated transgenic fly lines. These human genes were based on previous studies demonstrating differential expressions in a hepatoma, compared to a normal liver, and also showed cell cycle-

*To whom correspondence and reprint requests should be addressed. E-mail:mbyhsun@gate.sinica.edu.tw

dependent expression (Yang et al. 2005). In total, 279 independent transgenic UAS lines for the 83 genes were tested. The construction of transgenic UAS lines and their screening were a consortium effort by multiple labs. We report here that in our overexpression screening, we identified human enthoprotin and its fly homolog, Liquid facet Related (LqfR), with conserved function as a regulator of hematopoiesis.

Drosophila and vertebrate hematopoietic systems employ conserved signaling pathways and transcription factors to regulate blood cell proliferation, differentiation, and lineage commitment. For example, Notch (N), Vascular endothelial growth factor receptor (VEGFR), Janus kinase (Jak)/STAT, and Toll signaling pathways all have conserved functional features in various aspects of hematopoiesis (see reviews by Evans et al. 2003, Lemaitre et al. 2007).

Both humoral and cellular innate immune responses against pathogens and parasites are important for *Drosophila* host defense (see review by Lemaitre et al. 2007). The humoral response is mainly induced in fat bodies, and involves production of antimicrobial peptides. The cellular response involves phagocytosis, melanotic mass formation, and encapsulation of pathogens mediated by different types of hemocytes.

Mature *Drosophila* hemocytes are constituted of 3 cell types: plasmatocytes, crystal cells, and lamellocytes. Plasmatocytes make up 90% - 95% of total embryonic and larval hemocytes, and function as phagocytes of invading bacteria and fungi, and apoptotic corpses. These cells persist through larval stages and metamorphosis, and can be detected in the adult fly (Holz et al. 2003). Crystal cells make up 5% of total hemocytes in embryos and larvae, but they disappear at the onset of metamorphosis. They have conspicuous crystalline structures composed of prophenoloxidase, which can be activated and causes the formation of melanin as an immune response (Nam et al. 2012). Lamellocytes are only found in larval stages and appear only after an infection (Rizki et al. 1992, Lanot et al. 2001, Sorrentino et al. 2002, Crozatier et al. 2004). They can engulf parasites and debris.

Drosophila circulating hemocytes have 2 temporally and spatially distinct origins (Tepass et al. 1994, Lanot et al. 2001, Holz et al. 2003). During the embryonic stage, hemocytes originate from the head mesoderm. During larval stages, hemocytes originate from the lymph gland. In late 3rd instar larvae, the primary lobe of the lymph gland has 3 distinct domains, the medullary zone (MZ), cortical zone (CZ), and posterior signaling center (PSC) (Jung et al. 2005). The MZ is mainly composed of hematopoietic progenitor cells, while the CZ is mainly composed of differentiated hemocytes (Jung et al. 2005). The PSC acts as the hematopoietic stem cell niche (Krzemien et al. 2007, Mandal et al. 2007, Minakhina and Steward 2010).

Epsins are membrane-associated proteins involved in vesicle trafficking that are characterized by an epsin-N-terminal homology (ENTH) domain (reviewed by Legendre-Guillemin et al. 2004, Horvath et al. 2007). They are conserved from yeast to humans and are also found in plants. They are classified, based on their location, into endocytic epsins and Golgi epsins (see Lee et al. 2009). The enthoprotin we identified in the expression screening of *Drosophila* belongs to the Golgi epsin class. Enthoprotin (Wasiak et al. 2002), also called Clint (Kalthoff et al. 2002), EpsinR (Hirst et al. 2003, Mills et al. 2003), and Epsin 4 (Pimm et al. 2005), contains an ENTH domain which can bind to phosphatidyl inositol (PtdIns) and is localized to Golgi membranes and endosomes. The ENTH domain can also bind to some soluble NSF attachment protein receptor (SNARE) proteins (Wang et al. 2011). The C-terminal portion of enthoprotin contains multiple motifs for binding to clathrin, clathrin adaptor protein AP-1, and Golgi-localized γ-ear containing ADP-ribosylation factor-binding protein 2 (GGA2). It promotes the formation of clathrin-coated vesicles and the trafficking of cargoes between early endosomes and the trans-Golgi network (Mills et al. 2003).

Drosophila has an enthoprotin homolog, encoded by *liquid facets-related* (*lqfR*; *CG31170*, *CG42250*, Lee et al. 2009) which was previously called *epsin-2* (Lloyd et al. 2000) and *epsin-like* (Tweedie et al. 2009). It is ubiquitously expressed and is localized to Golgi and early endosomes (Lee et al. 2009, Leventis et al. 2011), and is required for viability (Lee et al. 2009). When examined for its function in eye development, it was found to be required for cell proliferation, insulin-independent cell growth, morphogenetic movement, and ommatidia patterning (Lee et al. 2009). It is also required for oogenesis by acting in somatic follicle cells for egg chamber morphogenesis and non-autonomously affected oocyte maturation (Leventis et al. 2011). Two isoforms, due to alternative splicing, were reported (Lee et al. 2009, Leventis et al. 2011): a long (L) isoform (LqfRa in Lee et al.

2009, RD in FlyBase) of 1415 amino acid residues is encoded by a transcript containing exon 6; and a short (S) isoform (LqfRb in Lee et al. 2009, RC in FlyBase) of 649 residues is encoded by a transcript that skips exon 6 but contains exon 7 (Fig. 1A). Both isoforms have the conserved N-terminal ENTH domain and a shared C-terminal part containing 3 DLFs, 2 DLL clathrin-binding motifs, and 2 γ-adaptin ear-binding motifs (Fig. 1B, C).

Our results demonstrated a novel role for LqfR in promoting hemocyte proliferation, and showed that this is probably mediated by activating N signaling.

MATERIALS AND METHODS

Generation of UAS-transgenic lines for human cDNAs

Full-length cDNAs for the chosen set of 100 human genes were obtained from the Kazusa DNA Research Institute (http://zearth.kazusa.or.jp/huge/) in Japan. The open reading frames (ORFs) from these cDNAs were polymerase chain reaction (PCR)-amplified and cloned into the pUAST-FLAG vector (Yao et al. 2005). Only 83 of these were successfully cloned. These constructs were injected into fly embryos to generate transgenic flies following standard protocols (Jang et al. 2003). For each construct, 3-5 independent transgenic lines were generated and tested. In total, 279 transgenic lines were successfully generated and screened.

Generation of UAS-transgenic lines for LqfR

LqfR-S and *LqfR-L* cDNAs were separately amplified by a reverse-transcription (RT)-PCR with the primer sets of 5'-CCGCTCGAGCGGGATGGT GGATAAATTCATC-3' (with the *Xho I* site), 5'-GC TCTAGAGCTAGTTACGTTTGCCTTGG-3' (with the *Xba I* site), 5'-CCGCTCGAGCGGGATGGT GGATAAATTCATC-3' (with the *Xho I* site), and 5'-GCTCTAGAGCTCATTGAAACAAGTCGAA-3' (with the *Xba I* site). The PCR was performed by preheating at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30s, and extension at 72°C (1 min for the S form and 2.5 min for the L form), with a final extension at 72°C for 7 min. The products were cloned into a *pUAST-flag* vector within the *Xho I* and *Xba I* sites. The *LqfR-dsRNA* construct was made by respectively cloning the sense and antisense sequences of the *LqfR* Enth domain region into the *Bgl*II/*Xho*I and *Nhe*I/*Xba*I sites of the *pWIZ* vector (Lee and Carthew 2003). For the *LqfR* constructs, multiple independent transgenic lines (indicated by the number appended to the transgene) were established for each UAS construct. These gave qualitatively similar results, and only 1 on chromosome II and 1 on chromosome III were saved; *UAS- LqfR-S-1* (on II), *UAS- LqfR-S-25* (on III), *UAS- LqfR-L-11* (on III), *UAS- LqfR-L-17* (on II), *UAS- LqfR-dsRNA-8* (on III), and *UAS- LqfR-dsRNA-12* (on II). GenBank accession numbers for *LqfR-L* and *LqfR-S* are JQ860359 and JQ860360.

Drosophila stocks used

The following fly stocks were used in this study: *UAS-N^{DN}/TM6B*, *UAS-GFP*, *tub-GAL4/SM6-TM6B*, *UAS-dome$^{\Delta CYT}$/TM6B*, *vegfr^{C2195}/CyO* (Cho et al. 2002), *Dorothy-GAL4* on X (Kimbrell et al. 2002), *srpHemoGAL4* on X (Bruckner et al. 2004), *domeless-GAL4*/FM7 (Ghiglione et al. 2002), *Collagen-GAL4* on II (Jung et al. 2005), and *Antp-GAL4/TM6B* (Staehling-Hampton et al. 1994).

RT-PCR of LqfR expression

To check *LqfR* overexpression and knock-down efficiency, 3 primers were used: a general 5'-TATTCAGGATGATGATCGTTTGCGC, 3'-ACGGTCATTGTATCGATAGTTACGT specific for *LqfR-S*, and 3'-CGGTCTTCGAACGAATGTTCA TGTT specific for *LqfR-L.* The RT-PCR of *LqfR-L* and *LqfR-S* was performed using total RNA from 3rd instar larvae, and *rp49* was used as an internal control. The PCR was performed by preheating at 95°C for 2 min, followed by 22 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 7 min.

Hemocyte counting

To count total circulating hemocytes from late 3rd instar larvae (Rus et al. 2006), hemolymph was collected from both *Dot>GFP* and *srpHemo>EGFP* coupled with *UAS-LqfRs*. Ten larvae from each experiment were washed with phosphate-buffered saline (PBS). They were torn open at the posterior end to let the hemolymph flow into 50 μl of fresh PBS at 4°C. The suspension of hemocytes was centrifuged at 93 xg for 10 min and washed

once with 50 μl PBS at 4°C. The process was repeated once. Fifteen microliters of resuspended hemocytes was counted in the hemocytometer for green fluorescent protein (GFP)-positive cells.

Immunohistochemistry

Immunostaining of lymph glands was as described by Krzemien et al. (2007). Late 3rd instar lymph glands were dissected out and fixed in 4% paraformaldehyde for 10 min, washed with PBST (PBS + 0.3% Triton) 3 times for 10 min each, and blocked with 10% normal goat serum for 30 min. The gland was then stained with primary antibodies: rabbit anti-phospho-H3 1:500, mouse anti-P1 1:500 (Kurucz et al. 2007; kindly provided by Istvan Ando), mouse anti-Lz 1:20, mouse anti-Antp 1:500, and rat anti-DE-cadherin 1:10 (DSHB) at 4°C overnight, washed with PBST 3 times for 10 min each, stained with the secondary antibody (rabbit-Cy3 1:500, mouse-Cy3 1:500, or rat-Cy5 1:500, Jackson ImmunoResearch Laboratory) for 2 h, and washed with PBST 3 times for 10 min each. Samples were mounted and observed under a confocal microscope.

For hemocyte immunostaining, hemocytes were collected from 5 larvae in 50 μl of 4°C PBS/PTU (containing 25 μM of phenolthiocarbamide, a phenoloxidase inhibitor that blocks the melanization reaction) (Ashburner 1989). Fifteen microliters of the mixture was loaded into 1 well of a 10-well printed slide for 30 min to allow hemocytes to attach to the slide, and then was fixed with 4% paraformaldehyde for 5 min. Hemocytes were stained in the same way as the lymph gland.

5-Bromo-2-deoxyuridine (BrdU) incorporation

Incorporation of BrdU into dividing cells was done as described by Tsai and Sun (2004).

RESULTS

Identification of *enthoprotin* in the screening

In our screening of the ubiquitous expressions of human transgenes driven by *tub-Gal4*, we found that *enthoprotin* (KIAA0171) expression caused the formation of melanotic masses in late larvae, pupae, and adults (Fig. 2A). Because the phenotype suggested an effect on hematopoiesis, we focused on this gene and its fly homolog.

LqfR is the fly homolog of enthoprotin. Two isoforms, *LqfRa* and *LqfRb*, were reported (Lee et al. 2009) (Fig. 1). By RT-PCR, we isolated cDNA for 2 other isoforms. The short isoform (*LqfR-S*, corresponding to the RA isoform previously annotated by FlyBase) contained exon 6. The longer isoform (*LqfR-L*, corresponding to the RB isoform previously annotated by FlyBase) skipped exon 6 but contained exon 7. These 2 isoforms are similar to the 2 reported previously, but with an important difference. Both *LqfRa* and *LqfRb* used a non-canonical GC… AG splice junction for the splice from exon 4 to exon 5, whereas our 2 isoforms both used the canonical GT…AG splice junction (Fig. 1A). These 2 splice donor sites are 4 bp apart (GCAAGT). The shift in the ORF in the *LqfR-S* isoform created a premature stop codon in exon 5, thereby creating a protein of 243 residues (Fig. 1B). The *LqfR-L* isoform also used an internal splice acceptor site in exon 5 (Fig. 1A), thereby creating a protein of 633 residues (Fig. 1B). *LqfR-L* and *LqfR-S* have identical 242 N-terminal residues, including the ENTH domain, which has 74.6% identity to the enthoprotin ENTH domain (Fig. 1). The C-terminal part of *LqfR-L* is identical to that of *LqfRb*, except for a 16-aa deletion (Fig. 1B). This part is highly conserved among 12 *Drosophila* species, and contains 3 DLFs, 2 DLL clathrin-binding motifs, and 2 γ-adaptin ear-binding motifs (Fig. 1C). The high sequence conservation suggests that this *LqfR-L* isoform is functional, and therefore evolutionarily conserved. Furthermore, the alternative splice acceptor and premature stop codon AG/ACG**TAA** (at the intron 4-exon 5 junction) is conserved among the *Drosophila* species we examined (*D. melanogaster*, *erecta*, *sechelia*, *yakuba*, *simulans*, and *ananassae*). The alternative splice donor, CAAG/GCAAGT (at the exon 4-intron 4 junction), is conserved in 5 of the 6 *Drosophila* species, except that *D. ananassae* has the sequence CAAG/GT. This suggests that the GT…AG splice site used in the *LqfR-L* and *LqfR-S* isoforms is more ancient. The conserved premature stop codon in exon 5 also suggests that *LqfR-S* is evolutionarily conserved.

Expressions of *LqfR-L* and *LqfR-S*, driven by *tub-GAL4*, can lead to melanotic mass formation (Fig. 2B). *LqfR-S* always caused a lower percentage of flies to exhibit the melanotic mass than *LqfR-L* (Fig. 2D). Two other FlyBase-reported isoforms, CP1 and CP7 (Fig. 1B), were cloned by a PCR from *LqfR-L* based on the sequence prediction. Both lacked the ENTH domain, and neither induced melanotic mass formation when

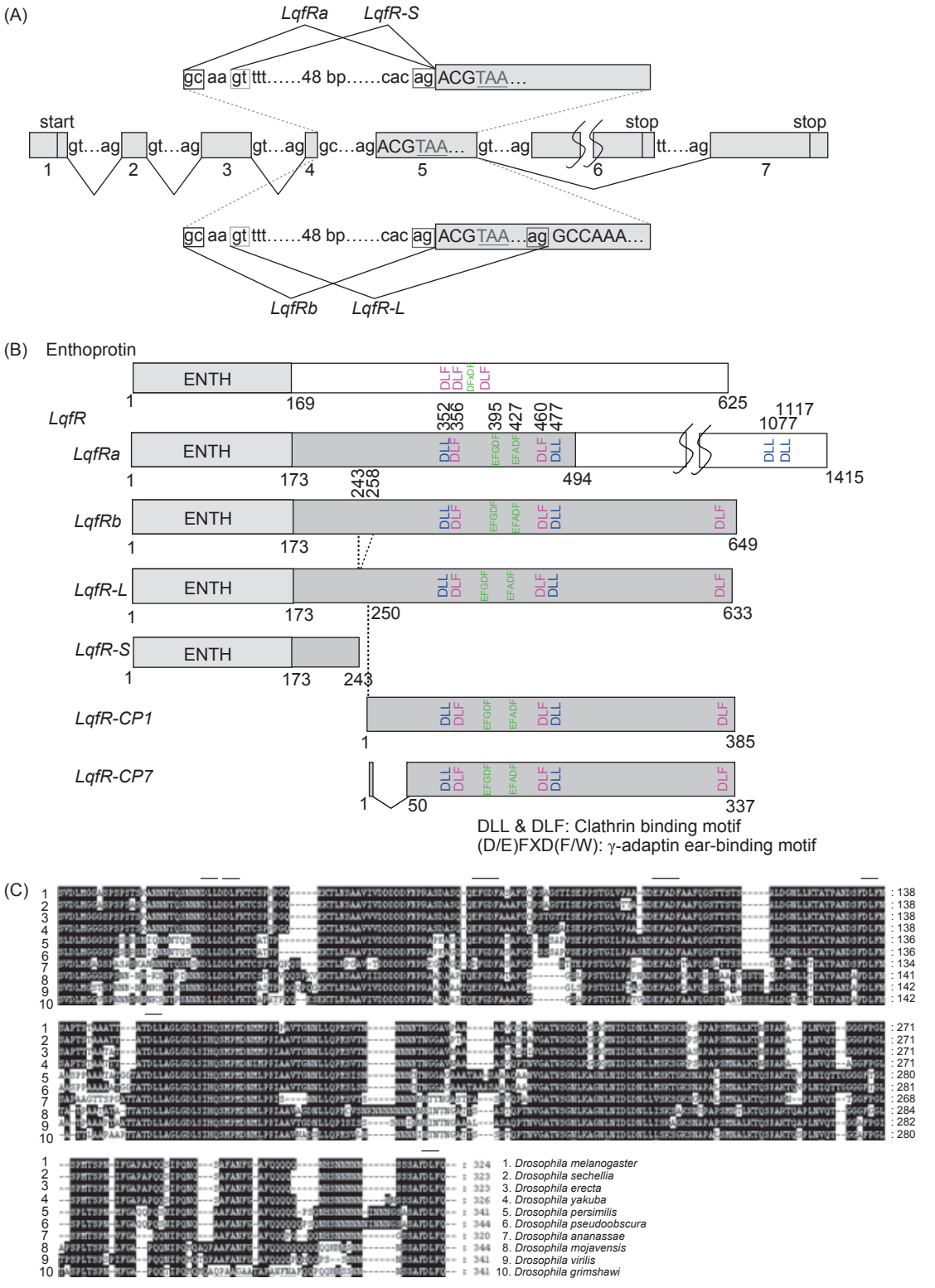
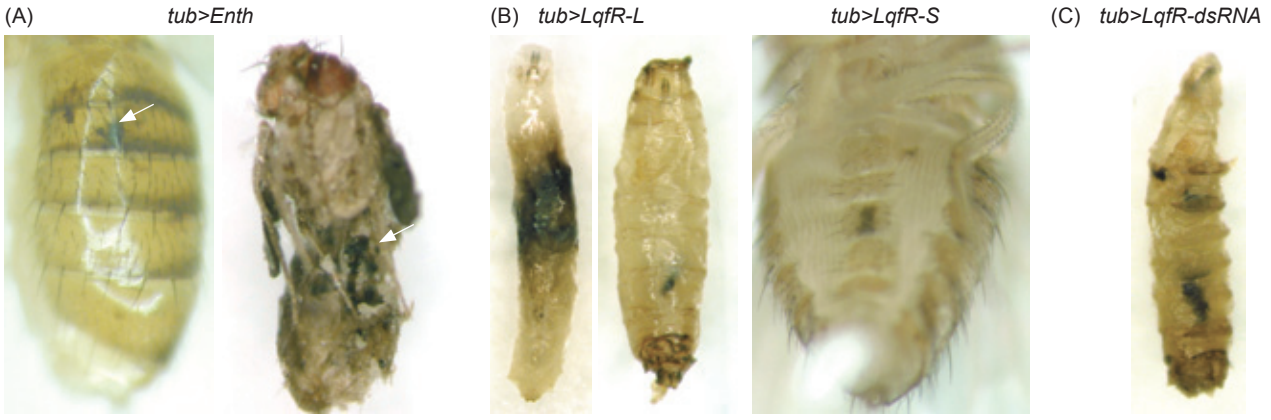
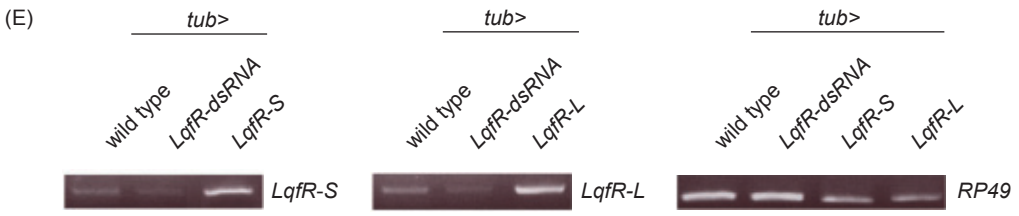


Fig. 1. Human enthoprotin and fly *LqfR* isoforms. (A) The *Drosophila lqfR* gene has 7 exons. The 2 reported splice isoforms, *LqfRa* and *LqfRb* (Lee et al. 2009, Leventis et al. 2011), the 2 splice isoforms (*LqfR-L* and *LqfR-S*) reported in this study, and their corresponding splice sites are depicted. (B) Comparison of the 4 *LqfR* isoforms and enthoprotin. Regions shared among the *LqfR* isoforms are shaded. The ENTH domain is highly conserved between *LqfR* and enthoprotin. (C) Sequence comparison of the C-terminal part of *LqfR-L* among 10 *Drosophila* species. The residue that is identical to the 1 in *D. melanogaster* (top) is labeled as a white character on a black background.



(D)

	+	*LqfR-S-1(3)*	*LqfR-S-25*	*LqfR-L-7*	*LqfR-L-9*	*LqfR-L-11*	*LqfR-L-17*	*LqfR-dsRNA-8*	*LqfR-dsRNA-12*	*LqfR-dsRNA-35*
Dot-Gal4	0%	16%	14%	22%	26%	24%	28%	26%	26%	28%
srpHemo-Gal4	0%	18%	14%	26%	26%	30%	30%	30%	32%	34%



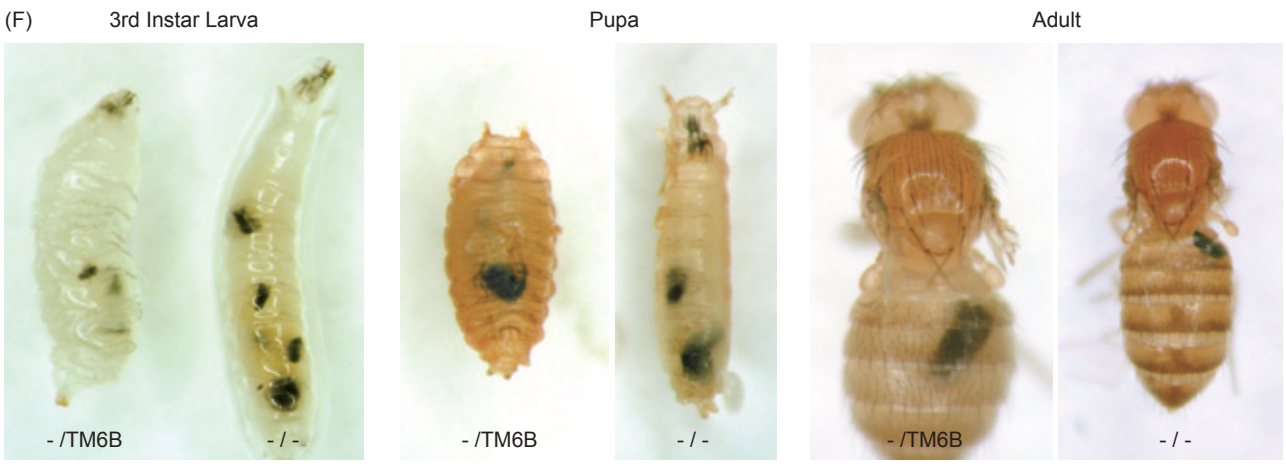


Fig. 2. Overexpression of enthoprotin and *LqfR* and knockdown of *LqfR* caused melanotic mass formation. (A) Overexpression of human *enthoprotin* by *tub-GAL4* (abbreviated as *tub>Enth*) led to melanotic mass formation visible in the adult abdomen (white arrows). Expression of *enthoprotin* driven by hemocyte-specific *Dot-GAL4* (abbreviated as *Dot>Enth*) caused melanotic masses in the larval abdomen (not shown). (B) Ubiquitous overexpression of *LqfR* isoforms (*tub>LqfR-L* and *tub>LqfR-S*) caused melanotic masses in the abdomens of larvae, pupae, and adults. (C) Knockdown of *LqfR* (*tub>LqfR-dsRNA*) also caused the formation of melanotic masses in larval and pupal abdomens. (D) The penetrance (% of flies with melanotic masses) for different independent transgenic lines driven by *Dot-Gal4* or *srpHemoGal4* is listed. The *LqfR-S* consistently caused weaker effects and lower penetrance than *LqfR-L* and *LqfR-dsRNA*. (E) RT-PCR was used to confirm the overexpression of *LqfR-S* and *LqfR-L* transcripts (by primers specific for each isoform) and the reduction of *LqfR* transcript due to *LqfR-dsRNA*, compared to levels in the wild-type. The level of *rp49* was used as a control for the amount of RNA used. (F) Heterozygotes (*-/TM6B*) and homozygotes (-/-) of the putative deletion mutation *PΔG18665* caused melanotic masses in late larvae, pupae, and adults.

overexpressed (data not shown). Although their endogenous presence has not been validated, they helped demonstrate that the ENTH domain is required for the effect on melanotic mass formation.

We next checked the LOS phenotype by knocking down endogenous *LqfR*. We designed a double-stranded (ds)RNA targeting the ENTH domain shared by *LqfR-L* and *LqfR-S* and overexpressed it using *tub-GAL4* (*tub>LqfR-dsRNA*). Surprisingly, the knockdown caused the same phenotype as the overexpression (Fig. 2C). The overexpression and knockdown experiments were checked by RT-PCR to confirm the increase and decrease in *LqfR* RNA (Fig. 2E). Another *lqfR* allele was generated by imprecise excision of a P element P{EP}G18665 inserted 419 bp upstream of *lqfR* (Bellen et al. 2004). This putative deletion mutant showed similar melanotic mass formation in homozygotes and heterozygotes (Fig. 2F), but was unfortunately lost before molecular mapping of the deletion extent. Melanotic mass was also reported for the strong hypomorphic mutation $lqfR^{D66}$ (Leventis et al. 2011). These results suggest that the level of LqfR needs to be tightly regulated, as too much and too little both caused defects.

Overexpression and knockdown of LqfR caused hemocyte overproliferation in larval lymph glands

Melanotic mass formation may occur in non-hematopoietic tissues when target tissues elicit an autoimmune attack from normal hemocytes, or by overproliferation of hemocytes, which then causes cell aggregation and melanization (see Minakhina et al. 2006). We tested whether LqfR acts in hematopoietic tissues by expressing it in the hematopoietic lymph gland, using *Dorothy-Gal4* (*Dot-Gal4*), which is expressed in the embryonic and larval lymph gland (Kimbrell et al. 2002, Jung et al. 2005), and hemocyte-specific *srpHemoGAL4* (Bruckner et al. 2004). Expression of *LqfR* or *enthoprotin*, driven by *Dot-Gal4* or *srpHemoGal4* (respectively abbreviated as *Dot>LqfR* and *srpHemo>LqfR*), also caused the formation of melanotic masses (summarized in Fig. 2D). This result suggests that the effect of LqfR acts on hemocytes. Larval hemolymph was collected to count the number of circulating hemocytes. When *LqfR* was overexpressed or knocked down, driven by the hemocyte-specific *Dot-GAL4* or *srpHemoGAL4* and co-expressed with *GFP* to trace hemocytes, the number of hemocytes significantly increased compared to the wild-type (WT) (Fig. 3A). Expression of human *enthoprotin* also caused a similar increase in circulating hemocytes (Fig. 3A). In the multiple transgenic lines tested, the frequency of melanotic mass formation in adult flies (Fig. 2D) was correlated with the increased number of circulating hemocytes in larvae (Fig. 3A).

Where do these excess hemocytes come from? The lymph gland is the major hematopoietic tissue during the larval stage (Lanot et al. 2001, Evans et al. 2003). Typically, there are few proliferating cells in the primary lobe in the late 3rd instar stage. When *LqfR* expression was driven by *Dot-GAL4* and *srpHemoGAL4*, significantly more proliferating cells (indicated by BrdU incorporation and phospho-histone 3) were detected in the primary lobe (Fig. 3B, C, E, F), but the primary lobe was not enlarged (Fig. 3D, G). Assuming that the cell density did not change, our results suggest that the increase in proliferating cells was due to a higher percentage of cells entering proliferation, rather than the total cell number increasing in the lymph gland. These results suggest that the origin of the excess hemocytes may be due to overproliferation in the lymph gland at the larval stage. Thus, an imbalance in the LqfR protein level may cause overproliferation of hemocytes, a hallmark of leukemia.

At late 3rd instar larvae, the primary lobe of the lymph gland can be characterized into 3 distinct domains, the MZ, CZ, and PSC (Jung et al. 2005). The MZ is mainly composed of hematopoietic progenitor cells, and the CZ is mainly composed of differentiated hemocytes (Jung et al. 2005). Both the MZ and CZ (marked by anti-P1) have higher numbers of proliferating cells of *Dot>LqfR-L* and *Dot>LqfR-dsRNA*, compared to the WT (Fig. 3H, I). It is possible that the primary effect of LqfR is on progenitor cells in the MZ, and the increase in proliferating cells in the CZ is an indirect consequence of more proliferating cells generated in the MZ which then enter the CZ. Alternatively, LqfR may directly affect both progenitor cells in the MZ and differentiated hemocytes in the CZ.

The 3 types of hemocytes in *Drosophila*, plasmatocytes, crystal cells, and lamellocytes, have relatively stable ratios. Crystal cells are recognized by their larger size, specific expression of Lozenge (Lz), and crystalline structure inside the cytosol. Plasmatocytes can also be distinguished based on their smooth, round morphology and specific labeling by anti-P1. There were increases in both plasmatocytes (Fig. 4A, C) and crystal

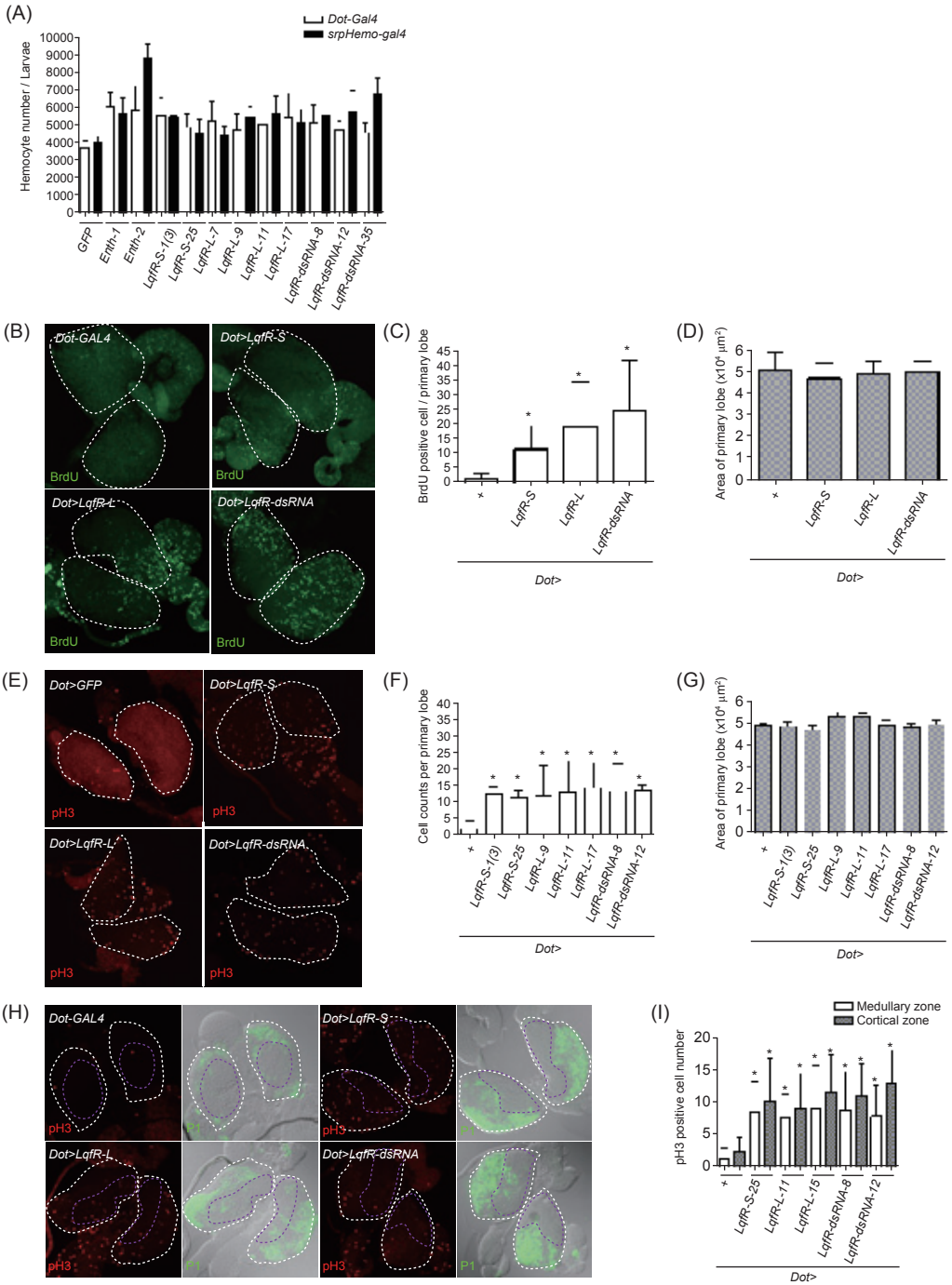


Fig. 3. Increased number of circulating hemocytes and proliferating cells in the medullary zone (MZ) and cortical zone (CZ) of the lymph gland. (A) Overexpression of *enthoprotin*, *LqfR-S*, and *LqfR-L* and knockdown of *LqfR* by *Dot-Gal4* or *srpHemoGal4* all caused an increase in the circulating hemocyte number. Multiple independent transgenic lines for each construct were tested and gave similar results ($n = 25$, * $p < 0.05$). (B-D) The effect of expression or knockdown, driven by *Dot-Gal4*, on cell proliferation as indicated by BrdU incorporation (green in B). The primary lobe of the lymph gland is marked by a white dotted line. (C) The average number of BrdU-positive cells in each pair of primary lobes for each genotype is summarized ($n = 20$, * $p < 0.05$). (D) The average area of the primary lobes did not significantly differ. (E-G) The effect of expression or knockdown, driven by *Dot-Gal4*, on cell proliferation, as indicated by phosphor-histone 3 (red in E). The primary lobe of the lymph gland is marked by a white dotted line. (F) The average number of pH3-positive cells in each pair of primary lobes for each genotype is summarized ($n = 20$, * $p < 0.05$). (G) The average area of the primary lobes did not significantly differ ($n = 20$, * $p < 0.05$). (H, I) More pH3-positive cells were found in both the MZ and CZ in lymph glands with *LqfR* overexpression and knockdown. Anti-P1 (green in H) marked the CZ. The average number of pH3-positive cells in the MZ and CZ are summarized in (I) ($n = 20$, * $p < 0.05$).

cells (Fig. 4B, D), in parallel with the increase in the total hemocyte number (Fig. 4D). The ratio of plasmatocytes to total hemocytes was 90% - 95% and was similar to that of the WT control (Fig. 4D). Lamellocytes are rare in adult hemolymph, and we did not observe any lamellocytes in *LqfR* LOF and GOF manipulations. However, whether their number or proportion changed cannot be determined. Effects on both plasmatocytes and crystal cell lineages suggest that *LqfR* may act on progenitor cells, and not on individual differentiated lineages.

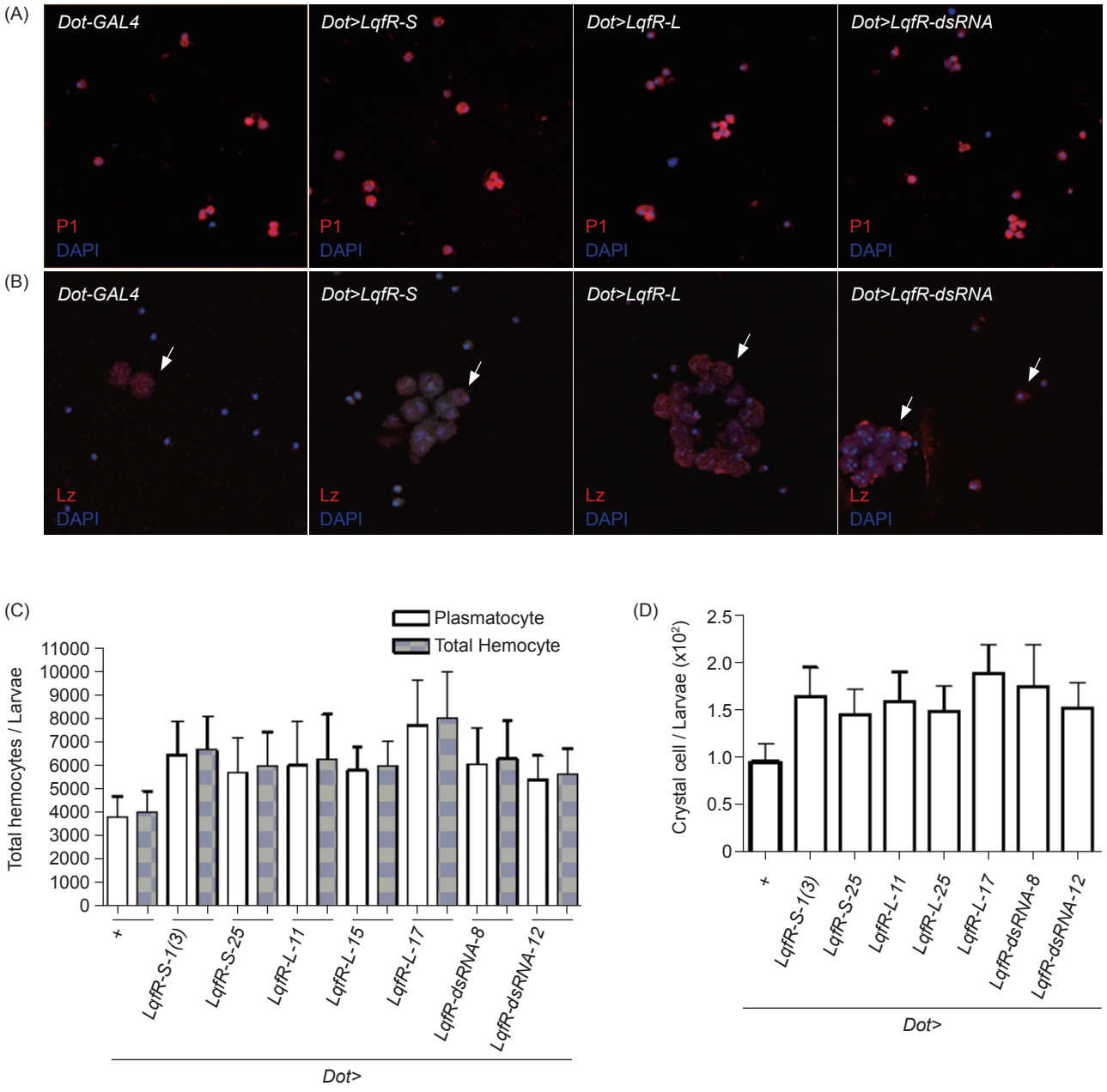


Fig. 4. Increases in both crystal cells and plasmatocytes. (A) Plasmatocytes in the hemolymph were identified by the specific marker, P1 (red), and the characteristic round and smooth morphology. Total circulating hemocytes were stained by DAPI (blue). (B) Crystal cells (arrow) in the hemolymph were identified by the specific marker, Lozenge (Lz, red), the crystalline structure inside the cell, and its larger size. (C) The average number of plasmatocytes and total hemocytes for the different genotypes are summarized. Numbers of plasmatocytes and total hemocytes increased in *LqfR* loss-of-function (LOF) and gain-of-function (GOF) conditions, but the proportion of plasmatocytes remained stable and ranged 90%-95%. (D) Numbers of crystal cells per larva for the different genotypes are summarized. Although the number increased, its proportion of total circulating hemocyte remained stable (n = 15, * p < 0.05).

LqfR regulated N signaling

There are 4 major signaling pathways affecting hematopoietic progenitor cell proliferation and hemocyte cell fate determination (Evans et al. 2003, Wood et al. 2007). The N signaling pathway induces crystal cell differentiation. The VEGFR signaling pathway affects plasmatocyte cell fate determination (Munier et al. 2002, Bruckner et al. 2004). Lamellocytes, which are induced by immune challenge, are regulated by the Jak/STAT and Toll/Cactus signaling pathways (Harrison et al. 1995, Luo et al. 1995, Qiu et al. 1998, Hou et al. 2002). In addition to regulating hemocyte specification, these 4 pathways are all related to hematopoietic progenitor cell proliferation in the larval lymph gland.

Because overexpression of *LqfR* led to hemocyte overproliferation, we checked whether blocking any of the 4 signaling pathways could block *LqfR*'s effect. Neither reducing the VEGFR dosage (in *vegfr/+* heterozygotes) nor co-expressing a dominant negative form of the Upd receptor (*UAS-dome*$^{\Delta CYT}$) significantly affected the excess circulating hemocyte phenotype (Fig. 5A, B, respectively). These results do not formally rule out involvement of the VEGF and Upd/Jak/STAT signaling pathways, because the function might not be sensitive to the receptor dosage, or the interaction may be a step downstream of the receptor, as the ENTH domain may interact with transcription factors (Hyman et al. 2000).

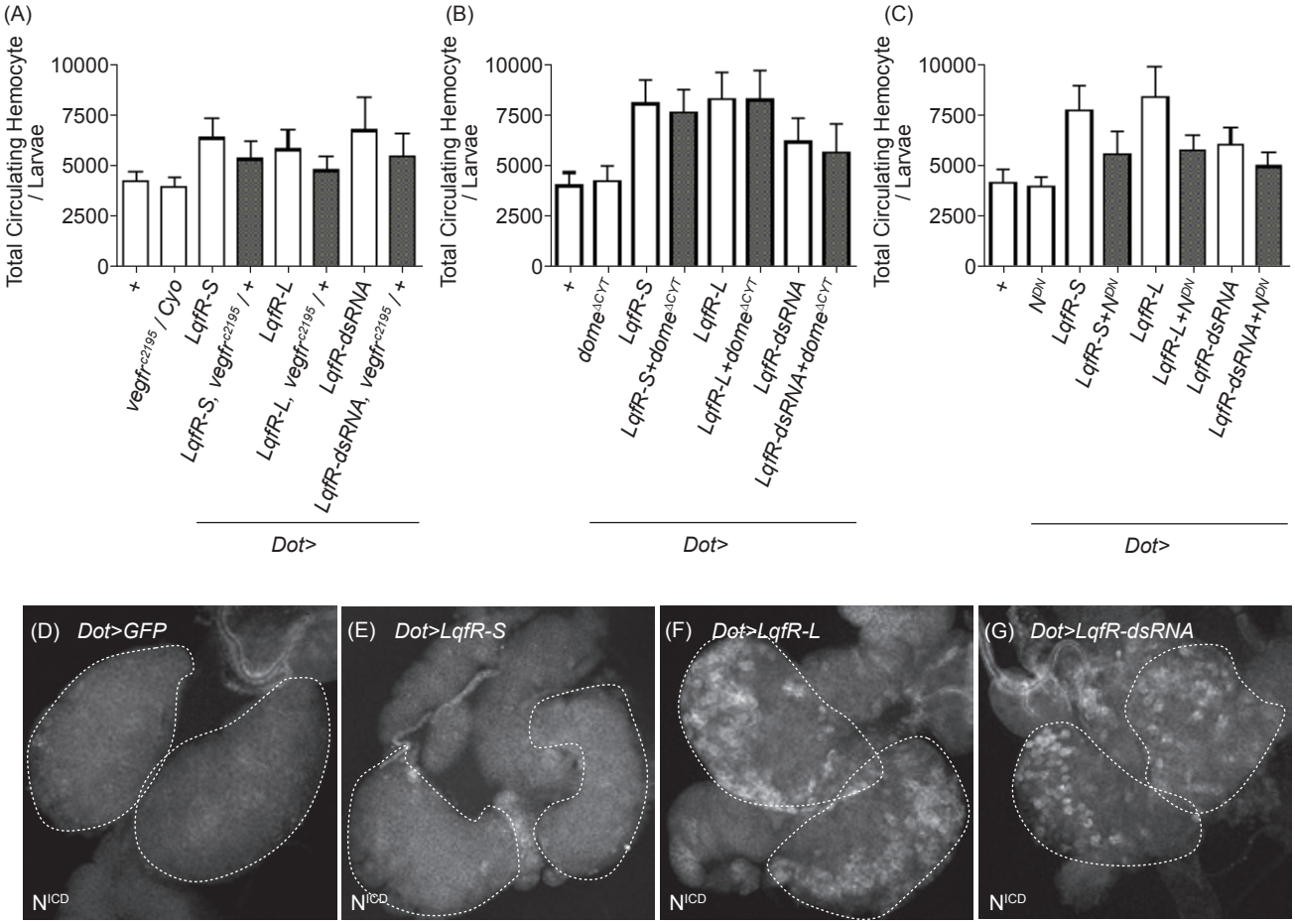


Fig. 5. *LqfR* activates Notch (N) and requires N signaling for its effect. (A) Reducing the *vegfr* gene dosage (in *vegfr*^{C2195}/+ heterozygotes) did not significantly affect the increase in total circulating hemocytes caused by *LqfR* gain-of-function (GOF) and loss-of-function (LOF) conditions. (B) Blocking the Jak/STAT signal by co-expressing the dominant negative *Dome*$^{\Delta CYT}$ did not significantly affect the increase in total circulating hemocytes caused by *LqfR* GOF and LOF conditions. (C) Blocking the N signal by co-expressing the dominant-negative *N*^{DN} significantly reduced the increase in total circulating hemocytes caused by *LqfR* GOF and LOF conditions (n = 20, * $p < 0.05$). (D-G) The intracellular domain of N (N^{ICD}) represents activated N. It was significantly enhanced in *Dot>LqfR-L* (F) and *Dot>LqfR-dsRNA* (G) compared to *Dot>GFP* (D) (n = 20).

Co-expressing a dominant negative form of N, (*UAS-N^{DN}*, Rebay et al. 1993) can partially block the excess hemocyte phenotype due to LqfR (Fig. 5C). This result suggests that N signaling may act downstream of LqfR and is required for LqfR's action. Activation of N results in its cleavage at the membrane and the release and nuclear localization of its intracellular domain (N^{ICD}). In *Dot>LqfR-L* and *Dot>LqfR-dsRNA*, there was a significant increase in N^{ICD} accumulation in primary lobe cells of the lymph gland (Fig. 5F, G, compared to 5D). The increase in N^{ICD} suggests that LqfR can activate N signaling at or upstream of the level of the receptor. N signaling is critical for specifying the crystal cell lineage, but also has an early role in the proliferation of hematopoietic cells in the lymph gland, similar to the proliferative role of N in imaginal discs (Go et al. 1998, Artavanis-Tsakonas et al. 1999, Lebestky et al. 2003).

When *UAS-N^{DN}* and *UAS-dome$^{\Delta CYT}$* were driven by *Dot-GAL4* to respectively repress the N and JAK/STAT pathways, there was no significant change in the number of circulating hemocytes in 3rd instar larvae (Fig. 5A-C, respectively), consistent with previous reports showing that the *N^{ts}* mutation bypassed the embryo stage and the zygotic *hop* amorphic mutation did not affect hemocyte numbers or cell type distributions (Remillieux-Leschelle et al. 2002, Lebestky et al. 2003). It is possible that in normal hematopoiesis, the multiple signaling pathways have redundant roles; therefore, blockage of any one would not result in a phenotype.

PSC formation is affected by LqfR

The PSC in the lymph gland functions as a niche to maintain hematopoietic progenitors and block their differentiation (Krzemien et al. 2007, Mandal et al. 2007). We tested whether LqfR affected PSC cells to relieve the quiescence of hematopoietic progenitor cells and cause them to enter proliferation. In *Dot>LqfR-S*, the Antp-expressing PSC can be expanded (Fig. 6B) or lost (Fig. 6C). In contrast, *LqfR* knockdown (in *Dot>LqfR-dsRNA*) only caused PSC expansion (Fig. 6D). Another PSC marker, *Ser-lacZ* (Lebetsky et al. 2003), showed similar results (data not shown).

Dot-GAL4 is expressed in the entire lymph gland from the embryonic stage, but expression becomes restricted to the PSC during the late 3rd instar larval stage (Kimbrell et al. 2002, Jung et al. 2005). We further tested *Gal4* drivers specific for different regions of the lymph gland. *Antp-Gal4* was specific to the PSC (Mandal et al. 2007). PSC-specific overexpression (in *Antp>LqfR-S*) resulted in the absence of Antp-expressing cells (Fig. 6G), while knockdown (in *Antp>LqfR-dsRNA*) caused PSC expansion (Fig. 6H). *domeless-GAL4* (*dome-GAL4*) is specific for the MZ (Mandal et al. 2007, Sinenko et al. 2009). MZ-specific overexpression (in *dome>LqfR-S*) and knockdown (in *dome>LqfR-dsRNA*) both led to PSC expansion (Fig. 6K, L, respectively). Both *Antp-Gal4-* and *dome-Gal4*-driven overexpression and knockdown also caused increases in the number of circulating hemocytes (Fig. 6Q). *Collagen-GAL4* (*Cg-GAL*4) is expressed by embryonic hemocytes, larval circulating hemocytes, and in the CZ of the larval gland (Asha et al. 2003, Jung et al. 2005). *Cg-Gal4*-driven overexpression (in *Cg>LqfR-S*) or knockdown (in *Cg>LqfR-dsRNA*) had no effect on the PSC size or the number of circulating hemocytes, compared to the WT (Fig. 6M-Q). These results suggest that LqfR does not act in the CZ or on circulating lymphocytes.

DISCUSSION

Implications for human leukemia

In *Drosophila*, both hemocyte overproliferation and autoimmune responses can result in melanotic mass formation (Minakhina and Steward 2006). Our results showed that the overexpression of human enthoprotin and its fly homolog, LqfR, can lead to melanotic mass formation in *Drosophila*, and the phenotype is caused by overproliferation of hemocytes. Thus, the function of enthoprotin/LqfR is evolutionarily conserved. Whether enthoprotin plays a role in human hematopoiesis has not been studied. We noted that another ENTH domain protein, clathrin assembly lymphoid myeloid leukemia protein (CALM, also known as phosphatidylinositol-binding clathrin assembly protein or PICALM), is required for erythrocyte maturation (Suzuki et al. 2012). Fusion of CALM and AF10 genes is associated with a variety of leukemias in humans (Dreyling et al. 1998) and was used to establish a mouse model of acute myeloid leukemia (AML) (Deshpande et al. 2006). Therefore, our study identified enthoprotin as a candidate that participates in human hematopoiesis and leukemia.

Our results also suggest that N activation is required for LqfR-induced hemocyte overpro-

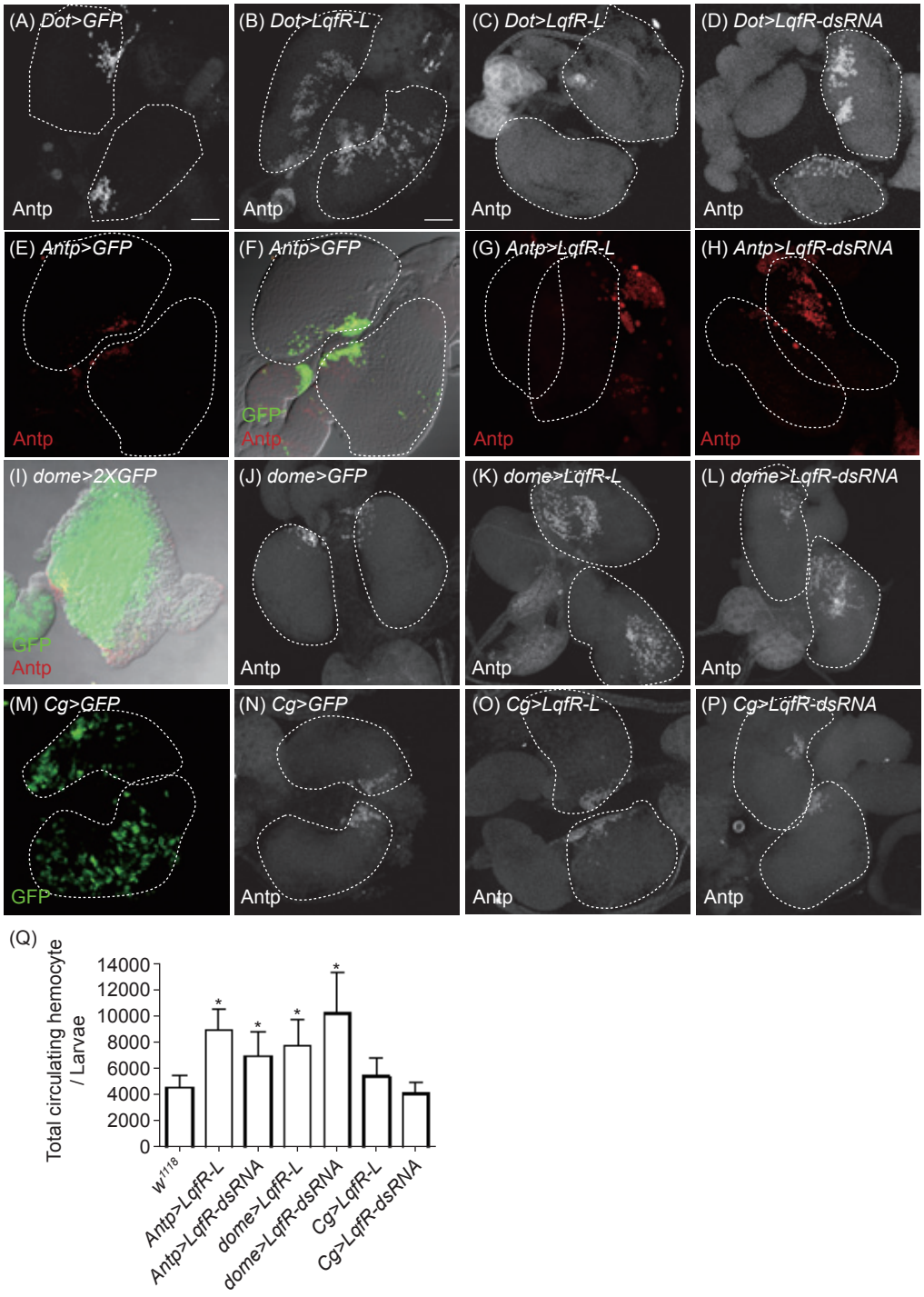


Fig. 6. *LqfR* regulates the posterior signaling center (PSC) size. (A) The PSC (marked by Antp, white) is located at the posterior part of the primary lobe of the lymph gland. *Dot>LqfR-L* caused PSC expansion (B; 60%, *n* = 35) or PSC loss (C; 17%, *n* = 35). The scale bar is 50 μm. (D) *Dot>LqfR-dsRNA* caused only PSC expansion (67%, *n* = 45). (E-H) Specific expression in the PSC driven by *Antp-Gal4*. (F) *Antp>GFP* showed expression in the PSC. (G) *Antp>LqfR-L* caused PSC loss (100%, *n* = 10). (H) *Antp>LqfR-dsRNA* caused PSC expansion (100%, *n* = 10). (I-L) Specific expression in the medullary zone (MZ) driven by *dome-Gal4*. (I) *dome>2XGFP* showed the area of the MZ. (K) *dome>LqfR-L* and (L) *dome>LqfR-dsRNA* both caused PSC expansion (*n* = 25). *dome>LqfR-S* caused no effect (not shown). (M-P) Specific expression in the cortical zone (CZ) driven by *Cg-Gal4*. (M) *Cg>GFP* showed the region of the CZ. (O) Neither *Cg>LqfR-L* nor (P) *Cg>LqfR-dsRNA* caused an effect on the PSC size (*n* = 20). (Q) *LqfR* loss of function (LOF) and gain of function (GOF) driven by *Antp-Gal4* or *dome-Gal4* caused significant increases in the total circulating hemocyte number, but *Cg-Gal4* caused no significant effect (* $p < 0.05$, *n* = 20).

liferation. N signaling is known to be important for specifying crystal cell lineages in normal development and for lamellocyte proliferation upon parasitization (Duvic et al. 2002). The link of the LqfR endosomal protein to N activation is consistent with previous findings that endocytosis can enhance N signaling (reviewed by Baron 2012). The endocytic epsin Liquid facets (*Lqf*) can potentiate N activity by internalizing the ubiquitinated N ligands, Ser and Delta (Overstreet et al. 2004, Tian et al. 2004, Wang and Struhl 2004). Accumulation of activated N (N^{ICD}) may lead to prolonged signaling activity, thereby causing greater cell proliferation in the lymph gland. Mutation in another endocytic protein, Asrij, resulted in accumulation of N^{ICD} in endosomes and caused premature hemocyte differentiation but did not affect hemocyte proliferation (Kulkarni et al. 2011). Thus different steps of endosomal trafficking of the N receptor may have different functional effects on N signaling.

The N signaling mechanism is evolutionarily conserved (Artavanis-Tsakonis et al. 1999). It is involved in the proliferation and differentiation of lymphocytes in mammals (reviewed by MacDonald et al. 2001, Izon et al. 2002). A recent study also pointed to a conserved interaction between N and hypoxia-inducible factor (HIF)-α in hemocyte survival that was conserved between flies and mammals (Mukherjee et al. 2011). Misregulation of N signaling can lead to T and B lymphocyte malignancies (reviewed by Mirandola et al. 2011). Therefore, our results in *Drosophila* suggest the endocytic pathway as a potential target for therapeutic development of human lymphoid malignancies.

The C-terminal part of *LqfR* is not essential for its functions

The human enthoprotin and *Drosophila LqfR* belong to the epsin family of adaptor proteins required for clathrin-coated vesicle (CCV) formation. Epsins have an N-terminal ENTH domain and multiple protein interaction motifs in their C-terminal domain which function in the endocytic process (Mills et al. 2003). The ENTH domain can bind to inositol phospholipids, inserts into membranes to induce membrane curvature, as well as binding to SNARE proteins (Wang et al. 2011). The C-terminal part of enthoprotin contains multiple motifs for binding to clathrin, clathrin adaptor protein (AP)-1, and Golgi-localized γ-ear-containing ADP ribosylation factor-binding protein 2 (GGA2).

The *Drosophila LqfR* homolog has 4 isoforms, 2 reported earlier (*LqfRa* and *LaqfRb*, Lee et al. 2009, Leventis et al. 2011) and 2 reported in this study (*LqfR-L* and *LqfR-S*). All 4 contain the ENTH domain. However, a construct in which the ENTH domain is deleted can rescue *lqfR* larval lethality and eye defects (Lee et al. 2009), similar to Lqf which does not contain an ENTH domain (Overstreet et al. 2004). Those results suggest that the ENTH domain is not essential. However, we found that the 2 ENTH-less isoforms, CP1 and CP7, failed to induce melanotic masses, thus suggesting that the ENTH domain may be required for LqfR's function in hemocyte proliferation. LqfRa, LqfRb, and LqfR-L have multiple clathrin-binding motifs in their C-terminal part. LqfR-S is truncated shortly after the ENTH domain, and therefore does not contain these motifs. We found that overexpression of *LqfR-S* produced essentially the same, although consistently weaker, effects as *LqfR-L*. Thus, clathrin-binding motifs are not essential but contribute to the strength of LqfR activity.

GOF and LOF effects of *LqfR*

Overexpression of *LqfR* caused melanotic mass formation and overproliferation of hemocytes. LOF conditions, including knock-down by dsRNA and deletion mutations, also caused melanotic mass formation and overproliferation of hemocytes. It seems that both GOF and LOG can cause the same phenotype by perturbing a delicate balance of the *LqfR* dosage. However, examination of the effect on the PSC size demonstrated that the GOF and LOF conditions can cause different phenotypes. When expression was driven by *Dot-Gal4*, GOF caused either a reduction or expansion of the PSC, while LOF caused only expansion. When expressed in the PSC by *Antp-Gal4*, GOF caused a reduction, while LOF caused an expansion of the PSC. These results suggest that although both GOF and LOF caused similar excesses of circulating hemocytes, the underlying mechanism may differ.

The phenomenon of GOF and LOF causing similar effects was observed for other proteins required for endocytic vesicle trafficking and sorting. The hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) is associated with early endosomes as part of the endosomal sorting complex required for transport (ESCRT) complex in sorting ubiquitinated proteins to lysosomes

(see review by Raiborg et al. 2002, Hurley 2010). Both overexpression and knockdown of Hrs led to inhibition of trafficking of many receptors for lysosomal degradation (Hasdemir et al. 2007).

The primary action on hemocyte proliferation may be on progenitor cells in the MZ

Changes in *LqfR* levels caused the formation of melanotic masses in adults, pupae, and late larvae. The penetrance of melanotic mass formation correlated well with the number of circulating hemocytes, i.e., more hemocytes correlated with a higher frequency of melanotic masses (Fig. 2B), suggesting that melanotic masses were caused by excessive hemocytes. We traced the origin of the increased hemocytes to an increase in cell proliferation in the larval lymph gland.

The lymph gland has an MZ with a hematopoietic progenitor and a CZ with differentiated hemocytes. Overproliferation was found in both zones (Fig. 3E). Overproliferation in the MZ can result in more hematopoietic progenitor cells. The excess proliferating cells in the CZ may have originated from the MZ and migrated into the CZ. Alternatively, cell proliferation induced by *LqfR* may occur directly within the CZ. Both plasmatocytes and crystal cells increased, in parallel with total circulating hemocytes, suggesting that there was no lineage bias. These results suggest that *LqfR* affects the proliferation of progenitor cells but not hemocyte differentiation. Therefore, we propose that the primary site of action may be in MZ to regulate the proliferation of the progenitor cells.

***LqfR* acts in the PSC and MZ to affect the PSC size**

We found that the size of the PSC was affected by *LqfR* overexpression. It is not clear whether the change in the PSC size was due to changes in cell proliferation, cell size, or simply expression of the marker genes, Antp and Ser. Wg expressed by the PSC is required for the maintenance and proliferation of PSC cells in an autocrine or paracrine manner (Sinenko et al. 2009). Dpp from the PSC antagonizes Wg signaling in the PSC to restrict the PSC size by restricting PSC cell proliferation (Pennetier et al. 2012). The effect of *LqfR* was similar to the effect of Dpp signaling, i.e., loss of *LqfR* caused expansion of the PSC, while *LqfR* overexpression caused PSC loss. Whether *LqfR* directly promotes Dpp signaling or represses Wg signaling awaits further study.

We attempted to define in which of the 3 regions (the PSC, MZ, or CZ) of the lymph gland *LqfR* exerts its effect on the PSC size. PSC-specific overexpression caused loss of the PSC, while knockdown caused expansion of the PSC. Thus, *LqfR* has autonomous activity in repressing the size of the PSC.

Interestingly, MZ-specific overexpression and knockdown both caused expansion of the PSC. Thus, *LqfR* can function in MZ to non-autonomously affect PSC. Previous studies showed that the PSC acts as a signaling center to regulate cell proliferation and differentiation in the MZ (Krzemien et al. 2007, Mandal et al. 2007). Hh expressed in the PSC acts on the MZ to prevent progenitor cells from differentiation, thus maintaining their stem cell properties (Crozatier et al. 2007). Our result of changes in the MZ being able to affect the PSC is a novel finding, and suggests that signaling between the PSC and MZ is bi-directional. This may be similar to the interaction of niche and germline stem cells (GSCs) in *Drosophila* ovaries. The niche sends transforming growth factor (TGF)β to GSCs to maintain their population (Song et al. 2004). GSCs can signal back to niche cells through the Delta-Notch pathway to maintain the niche (Ward et al. 2006, Song et al. 2007). Our results also imply that N signaling acts downstream of *LqfR* in hemocyte proliferation. *LqfR* was also found to affect oogenesis in somatic follicle cells (Leventis et al. 2011).

Our results suggest that *LqfR* has 3 functions. First, it acts in the MZ to regulate progenitor proliferation. Second, it acts in the PSC to autonomously repress the size of the PSC. Third, it acts in the MZ to non-autonomously regulate the PSC size. While it can function in the PSC and MZ, it does not function in the CZ. A previous study showed that the circulating hemocyte number can increase in either PSC loss or expansion conditions (Crozatier et al. 2004). Therefore, the number of circulating hemocytes and the size of the PSC, as determined by the number of Antp-expressing cells, may be independent events.

Opposite effects of *LqfR* overexpression in the PSC and MZ were found. Overexpression in the PSC caused PSC reduction, while overexpression in the MZ caused PSC expansion. Both reduction and expansion can occur when *LqfR* is expressed in the entire lymph gland, possible reflecting the combined effect of expression in the PSC (causing

reduction) and MZ (causing expansion). The final outcome may depend on fluctuations in the relative expression levels of LqfR in the PSC versus the MZ.

Acknowledgments: We are grateful to I. Ando, U. Banerjee, S. Cohen, D. Kimbrell, S. Noselli, N. Perrimon, the Bloomington Stock Center, and the VDRC Stock Center for providing fly stocks and reagents. We are grateful to C.L. Hsu and Y.C. Yang for preparing fly food and maintaining fly stocks, to C.Y. Tang for making transgenic flies, and S.P. Lee and the IMB Imaging Core for help with confocal microscopy. This study was supported by a grant to Y.H.S. (AS91IMB4PP) from Academia Sinica, Taiwan.

REFERENCES

Artavanis-Tsakonas S, MD Rand, RJ Lake. 1999. Notch signaling: cell fate control and signal integration in development. Science **284:** 770-776.

Asha H, I Nagy, G Kovacs, D Stetson, I Ando, CR Dearolf. 2003. Analysis of ras-induced overproliferation in *Drosophila* hemocytes. Genetics **163:** 203-215.

Ashburner M. 1989. *Drosophila*: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Baron M. 2012. Endocytic routes to Notch activation. Semin. Cell Develop. Biol. (in press)

Bellen HJ, RW Levis, G Liao, Y He, JW Carlson, G Tsang et al. 2004. The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. Genetics **167:** 761-781.

Bruckner K, L Kockel, P Duchek, CM Luque, P Rorth, N Perrimon. 2004. The Pdgf/Vegf receptor controls blood cell survival in *Drosophila*. Develop. Cell **7:** 73-84.

Cho NK, L Keyes, E Johnson, J Heller, L Ryner, F Karim et al. 2002. Developmental control of blood cell migration by the *Drosophila* Vegf pathway. Cell **108:** 865-876.

Crozatier M, M Meister. 2007. *Drosophila* haematopoiesis. Cell Microbiol. **9:** 1117-1126.

Crozatier M, JM Ubeda, A Vincent, M Meister. 2004. Cellular immune response to parasitization in *Drosophila* requires the Ebf orthologue Collier. PLoS Biol. **2:** E196.

Deshpande AJ, M Cusan, VP Rawat, H Reuter, A Krause, C Pott et al. 2006. Acute myeloid leukemia is propagated by a leukemic stem cell with lymphoid characteristics in a mouse model of CALM/AF10-positive leukemia. Cancer Cell **10:** 363-374.

Dreyling MH, K Schrader, C Fonatsch, B Schlegelberger, D Haase, C Schoch et al. 1998. MLL and CALM are fused to AF10 in morphologically distinct subsets of acute leukemia with translocation t(10;11): Both rearrangements are associated with a poor prognosis. Blood **91:** 4662-4667.

Duvic B, JA Hoffmann, M Meister, J Royet. 2002. Notch signaling controls lineage specification during *Drosophila* larval hematopoiesis. Curr. Biol. **12:** 1923-1927.

Evans CJ, V Hartenstein, U Banerjee. 2003. Thicker than blood: conserved mechanisms in *Drosophila* and vertebrate hematopoiesis. Develop. Cell **5:** 673-690.

Ghiglione C, O Devergne, E Georgenthum, F Carballes, C Medioni, D Cerezo et al. 2002. The *Drosophila* cytokine receptor Domeless controls border cell migration and epithelial polarization during oogenesis. Development **129:** 5437-5447.

Go MJ, DS Eastman, S Artavanis-Tsakonas. 1998. Cell proliferation control by Notch signaling in *Drosophila* development. Development **125:** 2031-2040.

Harrison DA, R Binari, TS Nahreini, M Gilman, N Perrimon. 1995. Activation of a *Drosophila* Janus kinase (Jak) causes hematopoietic neoplasia and developmental defects. EMBO J. **14:** 2857-2865.

Hasdemir B, NW Bunnett, GS Cottrell. 2007. Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) mediates post-endocytic trafficking of protease-activated receptor 2 and calcitonin receptor-like receptor. J. Biol. Chem. **282:** 29646-29657.

Hirst J, A Motley, K Harasaki, SY Peak Chew, MS Robinson. 2003. Epsinr: an ENTH domain-containing protein that interacts with AP-1. Mol. Biol. Cell **14:** 625-641.

Holz A, B Bossinger, T Strasser, W Janning, R Klapper. 2003. The two origins of hemocytes in *Drosophila*. Development **130:** 4955-4962.

Horvath CA, D Vanden Broeck, GA Boulet, J Bogers, MJ De Wolf. 2007. Epsin: inducing membrane curvature. Int. J. Biochem. Cell Biol. **39:** 1765-1770.

Hou SX, Z Zheng, X Chen, N Perrimon. 2002. The Jak/STAT pathway in model organisms: emerging roles in cell movement. Develop. Cell **3:** 765-778.

Hurley JH. 2010. The ESCRT complexes. Crit. Rev. Biochem. Mol. Biol. **45:** 463-487.

Hyman J, H Chen, PP Di Fiore, CP De, AT Brunger. 2000. Epsin 1 undergoes nucleocytosolic shuttling and its eps15 interactor NH(2)-terminal homology (ENTH) domain, structurally similar to Armadillo and HEAT repeats, interacts with the transcription factor promyelocytic leukemia $Zn(2)^{+}$ finger protein (PLZF). J. Cell Biol. **149:** 537-546.

Izon DJ, JA Punt, WS Pear. 2002. Deciphering the role of Notch signaling in lymphopoiesis. Curr. Opin. Immunol. **14:** 192-199.

Jang CC, JL Chao, N Jones, LC Yao, DA Bessarab, YM Kuo et al. 2003. Two Pax genes, *eye gone* and *eyeless*, act cooperatively in promoting *Drosophila* eye development. Development **130:** 2939-2951.

Jung SH, CJ Evans, C Uemura, U Banerjee. 2005. The *Drosophila* lymph gland as a developmental model of hematopoiesis. Development **132:** 2521-2533.

Kalthoff C, S Groos, R Kohl, S Mahrhold, EJ Ungewickell. 2002. Clint: a novel clathrin-binding enth-domain protein at the Golgi. Mol. Biol. Cell **13:** 4060-4073.

Kimbrell DA, C Hice, C Bolduc, K Kleinhesselink, K Beckingham. 2002. The Dorothy enhancer has Tinman binding sites and drives hopscotch-induced tumor formation. Genesis **34:** 23-28.

Krzemien J, L Dubois, R Makki, M Meister, A Vincent, M Crozatier. 2007. Control of blood cell homeostasis in *Drosophila* larvae by the posterior signalling centre. Nature **446:** 325-328.

Kulkarni V, RJ Khadilkar, MS Srivathsa, MS Inamdar. 2011. Asrij maintains the stem cell niche and controls differentiation during *Drosophila* lymph gland

hematopoiesis. PLoS One **6:** e27667.

Kurucz E, B Vaczi, R Markus, B Laurinyecz, P Vilmos, J Zsamboki et al. 2007. Definition of *Drosophila* hemocyte subsets by cell-type specific antigens. Acta Biol. Hung. **58(Supplement):** 95-111.

Lanot R, D Zachary, F Holder, M Meister. 2001. Postembryonic hematopoiesis in *Drosophila*. Develop. Biol. **230:** 243-257.

Lebestky T, SH Jung, U Banerjee. 2003. A Srrate-expressing signaling center controls *Drosophila* hematopoiesis. Genes Develop. **17:** 348-353.

Lee JH, E Overstreet, E Fitch, S Fleenor, JA Fischer. 2009. *Drosophila liquid facets-Related* encodes Golgi epsin and is an essential gene required for cell proliferation, growth, and patterning. Dev. Biol. **331:** 1-13.

Lee YS, RW Carthew. 2003. Making a better RNAi vector for *Drosophila*: use of intron spacers. Methods **30:** 322-329.

Legendre-Guillemin V, S Wasiak, NK Hussain, A Angers, PS McPherson. 2004. ENTH/ANTH proteins and clathrin-mediated membrane budding. J. Cell Sci. **117:** 9-18.

Lemaitre B, J Hoffmann. 2007. The host defense of *Drosophila* melanogaster. Annu. Rev. Immunol. **25:** 697-743.

Leventis PA, TR Da Sylva, N Rajwans, S Wasiak, PS McPherson, GL Boulianne. 2011. Liquid facets-related (*Lqfr*) is required for egg chamber morphogenesis during *Drosophila* oogenesis. PLoS One **6:** e25466.

Lloyd TE, P Verstreken, EJ Ostrin, A Phillippi, O Lichtarge, HJ Bellen. 2000. A genome-wide search for synaptic vesicle cycle proteins in *Drosophila*. Neuron **26:** 45-50.

Luo H, WP Hanratty, CR Dearolf. 1995. An amino acid substitution in the *Drosophila* Hop^{tum-l} Jak kinase causes leukemia-like hematopoietic defects. EMBO J. **14:** 1412-1420.

MacDonald HR, A Wilson, F Radtke. 2001. Notch 1 and T-cell development: insights from conditional knockout mice. Trends Immunol. **22:** 155-160.

Mandal L, JA Martinez-Agosto, CJ Evans, V Hartenstein, U Banerjee. 2007. A Hedgehog- and Antennapedia-dependent niche maintains *Drosophila* haematopoietic precursors. Nature **446:** 320-324.

Mills IG, GJ Praefcke, Y Vallis, BJ Peter, LE Olesen, JL Gallop et al. 2003. EpsinR: an AP1/clathrin interacting protein involved in vesicle trafficking. J. Cell Biol. **160:** 213-222.

Minakhina S, R Steward. 2006. Melanotic mutants in *Drosophila*: pathways and phenotypes. Genetics **174:** 253-263.

Minakhina S, R Steward. 2010. Hematopoietic stem cells in *Drosophila*. Development **137:** 27-31.

Mirandola L, P Comi, E Cobos, WM Kast, M Chiriva-Internati, R Chiaramonte. 2011. Notch-ing from T-cell to B-cell lymphoid malignancies. Cancer Lett. **308:** 1-13.

Mukherjee T, WS Kim, L Mandal, U Banerjee. 2011. Interaction between Notch and Hif-alpha in development and survival of *Drosophila* blood cells. Science **332:** 1210-1213.

Munier AI, D Doucet, E Perrodou, D Zachary, M Meister, JA Hoffmann et al. 2002. PVF2, a PDGF/VEGF-like growth factor, induces hemocyte proliferation in *Drosophila* larvae. EMBO Rep. **3:** 1195-1200.

Nam HJ, IH Jang, H You, KA Lee, WJ Lee. 2012. Genetic evidence of a redox-dependent systemic wound response via hayan protease-phenoloxidase system in *Drosophila*. EMBO J. **31:** 1253-1265.

Overstreet E, E Fitch, JA Fischer. 2004. Fat facets and liquid facets promote delta endocytosis and delta signaling in the signaling cells. Development **131:** 5355-5366.

Pennetier D, J Oyallon, I Morin-Poulard, S Dejean, A Vincent, M Crozatier. 2012. Size control of the *Drosophila* hematopoietic niche by bone morphogenetic protein signaling reveals parallels with mammals. Proc. Natl. Acad. Sci. USA **109:** 3389-3394.

Pimm J, A McQuillin, S Thirumalai, J Lawrence, D Quested, N Bass et al. 2005. The Epsin 4 gene on chromosome 5q, which encodes the clathrin-associated protein enthoprotin, is involved in the genetic susceptibility to schizophrenia. Am. J. Hum. Genet. **76:** 902-907.

Qiu P, PC Pan, S Govind. 1998. A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. Development **125:** 1909-1920.

Raiborg C, KG Bache, DJ Gillooly, IH Madshus, E Stang, H Stenmark. 2002. Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat. Cell Biol. **4:** 394-398.

Rebay I, RG Fehon, S Artavanis-Tsakonas. 1993. Specific truncations of *Drosophila* Notch define dominant activated and dominant negative forms of the receptor. Cell **74:** 319-329.

Reiter LT, L Potocki, S Chien, M Gribskov, E Bier. 2001. A systematic analysis of human disease-associated gene sequences in *Drosophila melanogaster*. Genome Res. **11:** 1114-1125.

Remillieux-Leschelle N, P Santamaria, NB Randsholt. 2002. Regulation of larval hematopoiesis in *Drosophila melanogaster*: a role for the *multi sex combs* gene. Genetics **162:** 1259-1274.

Rizki TM, RM Rizki. 1992. Lamellocyte differentiation in *Drosophila* larvae parasitized by leptopilina. Dev. Comp. Immunol. **16:** 103-110.

Rus F, E Kurucz, R Markus, SA Sinenko, B Laurinyecz, C Pataki et al. 2006. Expression pattern of Filamin-240 in *Drosophila* blood cells. Gene Express. Patterns **6:** 928-934.

Sinenko SA, L Mandal, JA Martinez-Agosto, U Banerjee. 2009. Dual role of Wingless signaling in stem-like hematopoietic precursor maintenance in *Drosophila*. Develop. Cell **16:** 756-763.

Song X, GB Call, D Kirilly, T Xie. 2007. Notch signaling controls germline stem cell niche formation in the *Drosophila* ovary. Development **134:** 1071-1080.

Song X, MD Wong, E Kawase, R Xi, BC Ding, JJ McCarthy, T Xie. 2004. Bmp signals from niche cells directly repress transcription of a differentiation-promoting gene, *bag of marbles*, in germline stem cells in the *Drosophila* ovary. Development **131:** 1353-1364.

Sorrentino RP, Y Carton, S Govind. 2002. Cellular immune response to parasite infection in the *Drosophila* lymph gland is developmentally regulated. Develop. Biol. **243:** 65-80.

Staehling-Hampton K, PD Jackson, MJ Clark, AH Brand, FM Hoffmann. 1994. Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in *Drosophila* embryos induced by *decapentaplegic* but not *60A*. Cell Growth Diff. **5:** 585-593.

Suzuki M, H Tanaka, A Tanimura, K Tanabe, N Oe, S Rai et al. 2012. The clathrin assembly protein picalm is required for erythroid maturation and transferrin internalization in mice. PloS One **7:** e31854.

Tepass U, LI Fessler, A Aziz, V Hartenstein. 1994. Embryonic

origin of hemocytes and their relationship to cell death in *Drosophila*. Development **120:** 1829-1837.

Tian X, D Hansen, T Schedl, JB Skeath. 2004. Epsin potentiates Notch pathway activity in *Drosophila* and *C. elegans*. Development **131:** 5807-5815.

Tsai L, YH Sun. 2004. Long-range effect by Upd, a ligand for Jak/STAT pathway, on cell cycle in *Drosophila* eye development. Genesis **39:** 141-153.

Tweedie S, M Ashburner, K Falls, P Leyland, P McQuilton, S Marygold et al. 2009. Flybase: enhancing *Drosophila* gene ontology annotations. Nucleic Acids Res. **37:** D555-D559.

Varmus HE. 1984. The molecular genetics of cellular oncogenes. Annu. Rev. Genet. **18:** 553-612.

Wang J, M Gossing, P Fang, J Zimmermann, X Li, GF von Mollard et al. 2011. Epsin N-terminal homology domains bind on opposite sides of two SNAREs. Proc. Natl. Acad. Sci. USA **108:** 12277-12282.

Wang W, G Struhl. 2004. *Drosophila* Epsin mediates a select endocytic pathway that DSL ligands must enter to activate notch. Development **131:** 5367-5380.

Ward EJ, HR Shcherbata, SH Reynolds, KA Fischer, SD Hatfield, H Ruohola-Baker. 2006. Stem cells signal to the niche through the Notch pathway in the *Drosophila* ovary. Curr. Biol. **16:** 2352-2358.

Wasiak S, V Legendre-Guillemin, R Puertollano, F Blondeau, M Girard, HE de Heuvel et al. 2002. Enthoprotin: a novel clathrin-associated protein identified through subcellular proteomics. J. Cell Biol. **158:** 855-862.

Wood W, A Jacinto. 2007. *Drosophila melanogaster* embryonic haemocytes: masters of multitasking. Nat. Rev. Mol. Cell Biol. **8:** 542-551.

Yang CW, JY Su, AP Tsou, GY Chau, HL Liu, CH Chen et al. 2005. Integrative genomics based identification of potential human hepatocarcinogenesis-associated cell cycle regulators: RHAMM as an example. Biochem. Biophys. Res. Commun. **330:** 489-497.

Yao JG, YH Sun. 2005. Eyg and Ey Pax proteins act by distinct transcriptional mechanisms in *Drosophila* development. EMBO J. **24:** 2602-2612.