Epsin potentiates *Notch* pathway activity in *Drosophila* and *C. elegans*

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Summary

Endocytosis and trafficking within the endocytosis pathway are known to modulate the activity of different signaling pathways. Epsins promote endocytosis and are postulated to target specific proteins for regulated endocytosis. Here, we present a functional link between the *Notch* pathway and epsins. We identify the *Drosophila* ortholog of epsin, *liquid facets* (*lqf*), as an inhibitor of cardioblast development in a genetic screen for mutants that affect heart development. We find that *lqf* inhibits cardioblast development and promotes the development of fusion-competent myoblasts, suggesting a model in which *lqf* acts on or in fusion-competent myoblasts to prevent their acquisition of the cardioblast fate. *lqf* and *Notch* exhibit essentially identical heart phenotypes, and *lqf* genetically interacts with the *Notch* pathway during multiple Notch-dependent events in *Drosophila.* We extended the link between the *Notch* pathway and *epsin* function to *C. elegans*, where the *C. elegans lqf* ortholog acts in the signaling cell to promote the *glp-1*/*Notch* pathway activity during germline development. Our results suggest that epsins play a specific, evolutionarily conserved role to promote *Notch* signaling during animal development and support the idea that they do so by targeting ligands of the *Notch* pathway for endocytosis.

Key words: *C. elegans Drosophila,* Epsins, Notch

Introduction

Endocytosis modulates the output of different signaling pathways and also maintains morphogen gradients during development (Gonzalez-Gaitan and Stenmark, 2003; Seto et al., 2002). For example, endocytosis positively regulates the *Decapentaplegic* (*Dpp*) and *Notch* pathways as trans-endocytosis of Dpp, a TGF-β family member, facilitates its spread (Entchev et al., 2000) and endocytosis of Delta appears essential for *Notch* signaling (Parks et al., 2000). Conversely, it is well established that endocytosis of cell surface receptors leads to signal downregulation, as for receptor tyrosine kinase and TGF-β signaling (Seto et al., 2002), and receptor-mediated endoctyosis followed by lysosomal clearing restricts the spread of Wingless (Piddini and Vincent, 2003).

Epsins are evolutionarily conserved proteins that appear to promote endocytosis. Binding of epsins to clathrin and AP2 protein complexes stimulates assembly of clathrin-coated vesicles and promotes vesicle internalization during endocytosis (Chen et al., 1998; Rosenthal et al., 1999; Wendland et al., 1999). Epsin family members harbor multiple motifs with putative endocytic functions and associate with the membrane via their epsin N-terminal homology domain and ubiquitin-binding motifs (UIMs) (Wendland, 2002; Itoh et al., 2001; Aguilar et al., 2003). Although epsins were first thought to play a general role in endocytosis, recent work suggests epsins target specific proteins for regulated endocytosis (Aguilar et al., 2003; De Camilli et al., 2002), consistent with the idea that epsins modulate the strength of specific signaling pathways. In this context, epsins have been proposed to use UIMs to target specific ubiquitinated-membrane proteins for regulated endocytosis (Shih et al., 2002; Wendland, 2002). However, epsins have not been shown to modulate the activity of any signaling pathway.

The *Notch* pathway is one of a handful of signaling pathways that act reiteratively to control the development of higher metazoans. *Notch* signaling is activated when a member of the DSL (Delta, Serrate LAG-2) family of Notch ligands on a signaling cell binds to the Notch receptor on the receiving cell (Mumm and Kopan, 2000). Ligand binding activates a series of proteolytic cleavage events to the Notch receptor that result in the release of its intracellular domain (Notch[intra]) from the membrane. Notch[intra] translocates into the nucleus where it complexes with transcription factors of the CSL (CBF, Su(H) and LAG-1) family and Mastermind or LAG-3 to activate target gene transcription (Mumm and Kopan, 2000).

Genetic and molecular studies in *Drosophila* indicate that *Notch* signaling requires Delta endocytosis in signaling cells. The link between Delta endocytosis and Notch activation arose from work that showed Delta internalization correlates with *Notch* signaling (Parks et al., 2000), presentation of secreted and intracellular truncated forms of ligand antagonize *Notch* signaling (Hukriede and Fleming, 1997; Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1996; Sun and Artavanis-Tsakonas, 1997) and genetic abrogation of endocytosis blocks *Notch* signaling (Seugnet et al., 1997).

Ubiquitination of Delta appears necessary for endocyosis of Delta. Two members of the *Notch* pathway, *neuralized* and *mind bomb*, encode E3-ubiquitin-ligases, ubiquitinate Delta and appear to be required for Delta internalization as loss of *neuralized* or *mind bomb* function causes excessively high levels of Delta at the cell membrane and blocks *Notch* signaling (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2001). Recently, the *Drosophila* homolog of epsin, *liquid facets* (*lqf*), has also been shown to promote Delta internalization (Overstreet et al., 2003), suggesting *lqf* may regulate *Notch* signaling even though the functional ramifications of *lqf* on *Notch* pathway activity have not been investigated.

Notch regulates sequential events during *Drosophila* heart development. The heart and dorsal somatic musculature arise from the dorsal-most region of the mesoderm. The cardiogenic mesoderm produces two types of heart cells: cardioblasts and pericardial cells. Cardioblasts are the contractile cells of the heart and coalesce to form the heart tube. Pericardial cells associate with cardioblasts and appear to filter the hemolymph (Bodmer and Frasch, 1999). The use of a *Notch* temperature-sensitive allele, N^{ts-1}, identified *Notch* as a critical regulator of heart development. During stage 11-12, *Notch* regulates the initial commitment of cells to the cardioblast and pericardial cell fate, as loss of *Notch* function leads to significant overproduction of both cell types (Hartenstein et al., 1992). *Notch* also appears to regulate the choice between the cardioblast and pericardial cell fate, as removal of *Notch* function later in development produces a heart with excess cardioblasts and few pericardial cells (Hartenstein et al., 1992).

C. elegans contains two *Notch* homologs: *glp-1* and *lin-12* (Austin and Kimble, 1989; Yochem and Greenwald, 1989). Here, we focus on *glp-1*. *glp-1* promotes germ cell proliferation in the distal region of the gonad (Austin and Kimble, 1987; Seydoux and Schedl, 2001). GLP-1 protein is found on germ cells (Crittenden et al., 1994) while its ligand, LAG-2, is expressed on the somatic distal tip cell (DTC) (Fitzgerald and Greenwald, 1995; Henderson et al., 1994). The close proximity between the DTC and the germ cells is thought to bring ligand and receptor into contact and maintain distal germ cells in the proliferative state. As germ cells move proximally, away from the influence of the DTC, they leave the proliferative state and enter meiotic prophase. In wild-type animals, germ cells enter meiosis at ~19 germ cell diameters from the DTC (Crittenden et al., 1994; Hansen et al., 2004) – we refer to the region between the DTC and the meiotic cells as the proliferative zone. Demonstration that *glp-1* promotes the proliferative state of germ cells comes from loss- and gain-of-function *glp-1* alleles. Loss of *glp-1* causes premature entry of germ cells into meiosis in early larval development and thus no proliferative zone is present in adult animals (Austin and Kimble, 1987; Lambie and Kimble, 1991). Weak hypomorphic *glp-1* alleles reduce but do not eliminate the proliferative zone. Conversely, *glp-1* gain-of-function alleles increase proliferation and expand the proliferative zone (Berry et al., 1997; Pepper et al., 2003).

Our work on heart development led us to identify a functional link between *lqf* and *Notch* signaling. In a genetic screen we identified *lqf* as an inhibitor of cardioblast development. Our phenotypic studies support a model in which *lqf* acts on fusion-competent myoblasts to prevent their acquisition of the cardioblast fate. *lqf* and *Notch* exhibit similar heart phenotypes and our genetic studies reveal a broad role for *lqf* to promote specifically *Notch* signaling in *Drosophila*. Consistent with the model that epsins play an evolutionarily conserved role to potentiate *Notch* pathway activity, we found that the *C. elegans lqf* ortholog of *epsin* appears to mediate *Notch/glp-1* activity in the DTC during *C. elegans* germline development.

Materials and methods

Genetics

The following fly stocks were used: Oregon R or w^{1118} – as wild-type, lqf^{ARI}/*Tm3-ftzlacZ*, lqf^{AG}/*Tm3-ftzlacZ*, $Df(1)N^{81K}$, N^{ts-1}, N^{55e11}, Dl^{3}/*Tm6B*, Dl^{7}/*Tm2*, $neur^{1}$/*Tm3*, flb^{IF26}/*CyO*, wg^{cx4}/*CyO*, mam^{NN86}/*CyO* and htl^{42}/*CyO*.

The following double mutant flies were made by standard methods: lqf^{ARI} Dl^{3}/*Tm3-ftzlacZ*, lqf^{ARI} $neur^{1}$/*Tm3-ftzlacZ*, lqf^{ARI} htl^{42}/*Tm3-ftzlacZ*, flb^{IF26}/*CyO-ftzlacZ;*lqf^{ARI}/*Tm3-ftzlacZ*, wg^{cx4}/*CyO-ftzlacZ;*lqf^{ARI}/*Tm3-ftzlacZ*, N^{ts-1}/*Y;*lqf^{ARI}/+, lqf^{ARI} Dl^{3}/lqf^{ARI} +, lqf^{ARI} $neur^{1}$/lqf^{ARI} +, N^{ts-1}/+;lqf^{ARI}/+ and + Dl^{3}/lqf^{ARI} +.

C. elegans strains used were: N2 (wild type), *glp*-1(*bn18*) (Kodoyianni et al., 1992) and *rrf-1*(*pk1417*) (Sijen et al., 2001).

Immunohistochemistry and immunofluorescence analysis

Immunolabeling analyses were performed as described previously (Skeath, 1998). We used the following antibodies in our analysis of heart development: guinea-pig anti-Zfh1 (1:1000); anti-Mef2 (Lilly et al., 1995); anti-Eve (Frasch et al., 1987); anti-Lmd (Duan et al., 2001) and anti-FasIII and anti-MHC (Developmental Studies Hybridoma Bank, University of Iowa).

Antibody staining of dissected *C. elegans* gonads is described (Jones et al., 1996). Anti-REC-8 (Pasierbek et al., 2001) and anti-HIM-3 (Zetka et al., 1999) antibodies were used to detect proliferative and meiotic nuclei respectively (Hansen et al., 2004b).

Molecular cloning of *Ce-epn-1* RNAi feeding vector

We identified the *C. elegans* ortholog of *epsin*, *Ce-epn-1* (T04C10.2) through a BLAST search of the *C. elegans* genome using the Lqf protein sequence. *Ce-epn-1* was also identified computationally by other groups (De Camilli et al., 2002; Kay et al., 1999). Sequence corresponding to amino acids 1-238 was amplified from the first strand cDNA pool of all stage worm tissues. We cloned the amplified PCR fragment into pGEMT (Promega), and then excised and cloned it into the standard RNAi feeding vector *pPD129.36* (Timmons and Fire, 1998).

Double strand RNA interference (RNAi) in *C. elegans*

Wild-type (N2), *glp-1(bn18)* and *rrf-1(pk1417); glp-1(bn18)* eggs were placed on plates with bacteria expressing either *Ce-epn-1* or GFP double-stranded RNA at 20°C, the permissive temperature for *glp-1*(*bn18*). Initially, adult animals, rather than eggs, were placed on the RNAi plates with the intention of scoring the progeny, however the *glp-1(bn18)* animals grown on the *Ce-epn-1* plates produced dead embryos, preventing analysis of adult germline phenotypes. This probably results from reduction of *Ce-epn-1* activity enhancing the temperature-sensitive embryonic lethal phenotype of *glp-1(bn18)* (Austin and Kimble, 1987; Priess et al., 1987), however this phenotype was not analyzed in detail. By placing eggs on RNAi feeding plates, the dsRNA is not administered until the animal hatches and begins feeding (postembryonic RNAi), thereby bypassing the embryonic requirement for *Notch* signaling. The hatched animals were allowed to grow to one day past the fourth larval stage and then dissected, fixed and stained for analysis of the germline phenotypes. We noticed that both N2 and *glp-1(bn18)* animals grown on *Ce-epn-1*-expressing bacteria grew more slowly than when grown on GFP-expressing bacteria, requiring one additional day to reach the fourth larval stage.

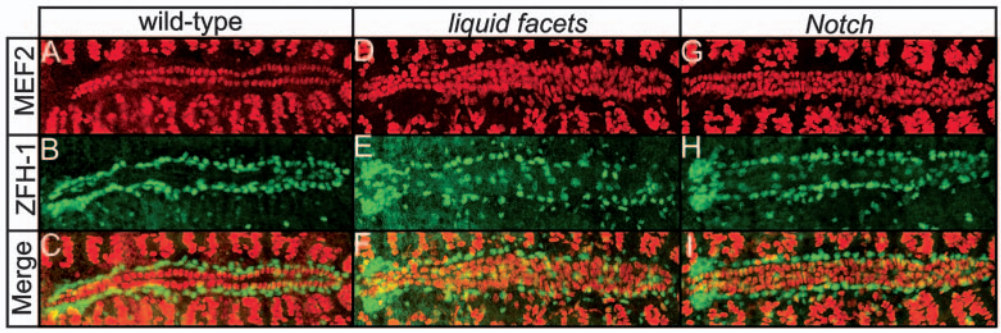


Fig. 1. *lqf* and *Notch* display similar heart phenotypes. Dorsal views of stage 16 wild-type (A-C), lqf^{ARI} (D-F) and $N^{ts\text{-}1}/Df(1)81k$ (G-I) embryos labeled with dMEF2 for cardioblasts (red) and Zfh-1 for pericardial cells (green). (A-C) In wild-type, cardioblasts align in two cell rows to form the heart (A,C); the pericardial cells flank the cardioblasts (B,C). (D-I) In lqf^{ARI} and $N^{ts\text{-}1}/Df(1)81k$ mutant embryos, excess cardioblasts form three to four poorly organized cell rows in the heart (D,F,G,I). In both *lqf* and *Notch* embryos relatively normal numbers of pericardial cells develop (E,F,H,I). Anterior – left.

Results

lqf represses cardioblast development during *Drosophila* heart development

We identified *lqf* as an inhibitor of cardioblast development in a screen of ~2000 third chromosome lethal P-element lines from the Hungarian P-element collection. Homozygous embryos from *l(3)0110/2* display an approximate doubling of cardioblasts (Fig. 1D). Molecular analysis localized the P element insertion site in *l(3)0110/2* to the first intron of *lqf*. We confirmed that loss of *lqf* function produces an excess cardioblast phenotype by showing that embryos homozygous mutant for each of four additional *lqf* alleles, lqf^{ARI}, lqf^{AG}, $lqf^{be25}$and lqf^{be428}(Cadavid et al., 2000; Overstreet et al., 2003) yield an excess cardioblast phenotype. The phenotypic severity of these alleles varied consistent with the following allelic series: $lqf^{ARI} \geqslant lqf^{AG} > lqf^{be25} > lqf^{be428} > lqf^{l(3)0110/27}$ (data not shown). Based on these data, we used the strong lqf^{ARI} and lqf^{AG} alleles in our studies and found that 200±41 cardioblasts (n=9) develop in lqf^{ARI} mutant embryos compared to 104±7 cardioblasts (n=18) in wild-type embryos (Fig. 1, Table 1). The presence of excess cardioblasts in *lqf* mutants demonstrates that *lqf* normally functions to oppose cardioblast development.

The *Drosophila* heart is composed of two distinct types of cardioblasts: Tinman-positive cardioblasts (Tin-CB) and Svp-lacZ-positive cardioblasts (Svp-CB) (Bodmer and Frasch, 1999). To determine if the *lqf* excess cardioblast phenotype arises due to a preferential expansion of one type of cardioblast, we compared the relative frequency of Tin-CBs and Svp-CBs in wild-type versus *lqf* embryos. In the wild-type heart, 104 cardioblasts develop of which 30±0 (n=8) are Svp-CBs, the remainder being Tin-CBs. Although on average 200 cardioblasts develop in *lqf* embryos, the number of Svp-CBs remains essentially unchanged (32± 3; n=10) demonstrating that loss of *lqf* function appears preferentially to enlarge the pool of Tin-CBs.

Table 1. *lqf* regulates cardioblast cell number

Cell type	Wild type (25°C)*	*lqf* (29-30°C)
Cardioblast (dMEF2$^+$ cell)/embryo	104±7 (n=18)†	200±41 (n=9)
Perocardial cell (Zfh-1$^+$ cell)/embryo	113±7 (n=12)	111±12 (n=16)
Svp-CB/embryo	30±0 (n=8)	32±3 (n=10)
Odd-PC/six posterior segments	27±3 (n=15)	25±3 (n=20)
Eve-PC/embryo	47±3 (n=10)	43±1 (n=8)
DA1 (Eve$^+$ nuclei)/muscle cell	11±2 (n=42)	8±2 (n=67)

*Numbers in parentheses after genotype indicate temperature at which embryos were raised.

†Numbers in parentheses indicate number of embryos or DA1 muscles scored.

lqf promotes the development of fusion-competent myoblasts

To investigate whether the excess cardioblasts in *lqf* embryos develop at the expense of a different mesodermal cell type we analyzed the development of the other derivatives of the dorsal mesoderm – pericardial cells, visceral mesoderm and dorsal somatic muscles. Markers specific for pericardial cells (Zfh-1) and visceral mesoderm (FasIII) revealed grossly normal development of these tissues in *lqf* embryos, although pericardial cell organization was slightly disrupted (Fig. 1B,E; Table 1; not shown for visceral mesoderm). In contrast, we observed a decrease in the size but not pattern of dorsal somatic muscles (Fig. 2A,C) while the pattern and size of ventral and lateral muscles appear normal (data not shown). Thus, in addition to restricting cardioblast development, *lqf* appears to promote dorsal somatic muscle development.

Muscle size correlates with the number of fusion-competent myoblasts (FCMs) that fuse with a given muscle founder cell, while the presence of individual muscles depends on the formation of appropriate muscle founder cells (Baylies et al., 1998). Thus, the reduced size of dorsal muscles suggests FCM development is impaired in *lqf* embryos. Even-skipped (Eve) labels all nuclei in the dorsal DA1 muscle and thus, can reveal the number of FCMs that contribute to this muscle. To assay whether fewer FCMs contribute to dorsal muscles in the *lqf* embryos, we used Eve to count the number of nuclei in DA1. In wild type, DA1 contains 11.1±1.8 (n=42) FCMs relative to 7.6±2.2 FCMs in *lqf* embryos (n=67; P<0.01; Fig. 2B,D; Table 1). Thus, *lqf* appears to promote either the development of FCMs or their ability to fuse with muscle founder cells.

To follow FCM development directly we assayed *lame duck* (*lmd*) expression in wild-type and *lqf* embryos. *lmd,* a Gli super family member, promotes FCM development and is expressed in all FCMs (Duan et al., 2001). Relative to wild-type embryos, we observe a moderate reduction of Lmd expression in the dorsal region of the somatic mesoderm in stage 12/13 *lqf*

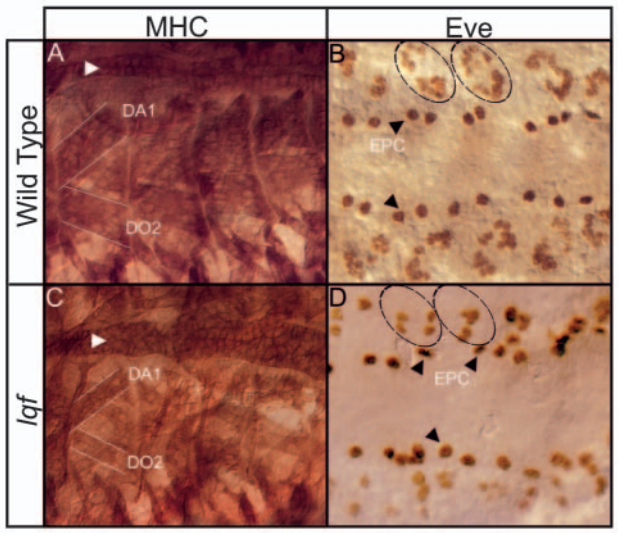


Fig. 2. *lqf* promotes dorsal muscle development. Dorsal views of stage 15 wild-type (A,B) and lqf^{AG} embryos (C,D) stained for myosin heavy chain (MHC; A,C) to label the heart and somatic muscles and Eve (B,D) to label nuclei within the DA1 muscle and Eve-pericardial cells (EPC). (A) MHC expression outlines the muscle pattern in the dorsal mesoderm. (C) In lqf^{AG} embryos, the muscle pattern appears grossly normal but the size of dorsal muscles appears reduced. To aid visualization of muscles, lines demarcate the size and orientation of the DA1 and DO2 dorsal muscles and arrowheads identify the heart (A,C). (B) In wild-type embryos, each DA1 muscle (dashed circles) contains roughly 11 Eve-positive nuclei while DA1 muscles in *lqf* embryos (D; dashed circles) contain on average eight Eve-positive nuclei. Arrowheads in B and D identify EPC. Anterior – left.

mutant embryos (data not shown) consistent with the idea that *lqf* promotes FCM development in the dorsal mesoderm.

The formation of excess cardioblasts, combined with the reduction of FCMs in *lqf* embryos, supports the model that ectopic cardioblasts arise at the expense of FCMs. However, it remains possible that over-proliferation of cardioblast progenitors produces the *lqf* cardioblast phenotype. To investigate this, we blocked cell division in *lqf* embryos by using a mutation in *Rca1*. *Rca1* is a cell cycle regulator and loss of *Rca1* function blocks the division of cardioblast progenitors (Han and Bodmer, 2003). Embryos mutant for *Rca1* contain 65±6 cardioblasts (n=14) while *Rca1;lqf* double mutant embryos contain 111±16 cardioblasts (n=13). The near doubling of cardioblasts in *Rca1; lqf* mutants relative to *Rca1* mutants indicates that over-proliferation of cardioblast progenitors does not play a primary role in the *lqf* heart phenotype. Taken together, our phenotypic data are consistent with a model in which *lqf* acts in or on presumptive dorsal FCMs to inhibit the cardioblast fate and promote the FCM fate.

lqf acts through the *Notch* pathway to repress cardioblast fate

Recent studies link endocytosis to the regulation of different signaling pathways. We therefore examined whether mutations in known signaling pathways yield heart phenotypes similar to *lqf* and whether any of these pathways genetically interact with *lqf* during heart development. Mutations in the *EGF*, *FGF*, *Wingless*, *Hedgehog* and *TGFβ* signaling pathways exhibit heart phenotypes distinct from that of *lqf* (reviewed by Bodmer and Frasch, 1999). However, *Notch* mutants display an excess cardioblast phenotype similar to that of *lqf* with the exception that *Notch* embryos also lack pericardial cells (Hartenstein et al., 1992). We re-examined the *Notch* heart phenotype using $N^{ts\text{-}1}/Df(1)81K$ embryos and found such embryos exhibit a ~twofold increase in cardioblasts, confirming the role of Notch in regulating cardioblast number (Fig. 1G); however, these embryos also exhibit grossly normal pericardial development (Fig. 1H). Thus, the *lqf* and *Notch* heart phenotypes appear essentially identical, supporting the idea that *lqf* and *Notch* act together to regulate heart development.

While we failed to detect genetic interactions between *lqf* and the *EGF* or *FGF* (*heartless*) pathways during heart development (not shown), we observed dominant genetic interactions between *lqf* and three components of the *Notch* pathway. When raised at 18°C, 10% of lqf^{ARI} mutant embryos exhibit an excess cardioblast phenotype. However, removal of one copy of *Delta* or *neur* from lqf^{ARI} embryos raised at 18°C causes the majority of such embryos to develop excess cardioblasts (62/65 for *Delta* and 32/36 for *neur*) (Fig.

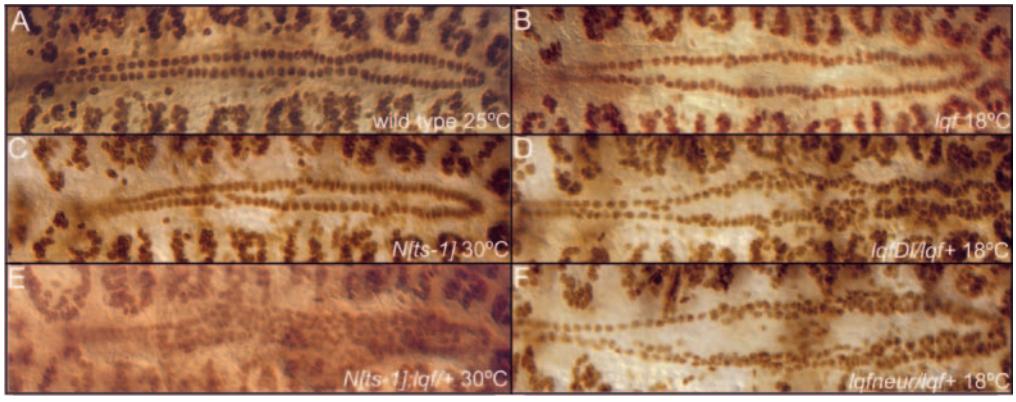


Fig. 3. *lqf* collaborates with the *Notch* pathway to regulate cardioblast development. Dorsal views of stage 16 wild-type (A), lqf^{ARI} (B), $N^{ts\text{-}1}$ (C), $lqf^{ARI}Dl^{3}/lqf^{ARI}$+ (D), $N^{ts\text{-}1};lqf^{ARI}/$+ (E) and lqf^{ARI} $neur^{1}/lqf^{ARI}$+ (F) embryos labeled for cardioblasts. (A) Wild-type cardioblast pattern. (B,C) $N^{ts\text{-}1}$ embryos grown at 30°C (C), and lqf^{ARI} embryos grown at 18°C (B), exhibit near wild-type cardioblast patterns with very mild increases in cardioblast number. (D) $N^{ts\text{-}1};lqf^{ARI}/$+ embryos grown at 30°C exhibit a clear increase in cardioblast number as do *lqf* Dl^{3}/lqf^{ARI}+ (E) and lqf^{ARI} $neur^{1}/lqf^{ARI}$+ (F) embryos grown at 18°C. Anterior – left.

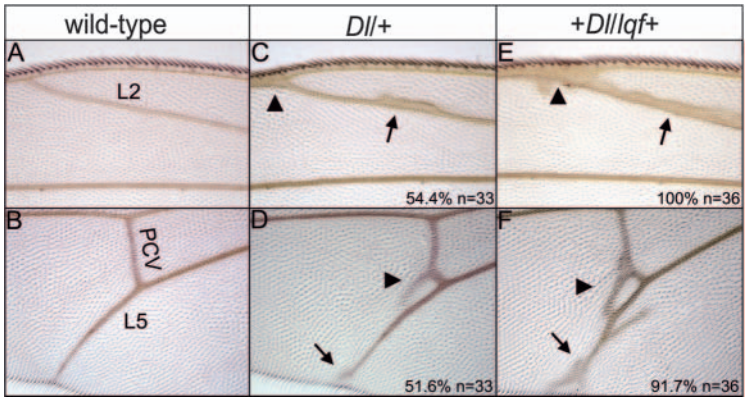


Fig. 4. *lqf* genetically interacts with Delta during wing vein formation. Dorsal views of wild-type (A,B), Dl^3/+ (C,D) and Dl^3+/+lqf^{ARI} (E,F) wings. (A,B) Wild-type patterns of the L2 vein (A) as well as of the L5 vein and posterior cross vein (PCV) (B). (C) Dl^3/+ wings exhibit vein thickening at the tip (arrowhead) and along the length (arrow) of L2. (D) Dl^3/+ wings also exhibit thickening of L5 (arrow) and vein bifurcation at the junction of the posterior cross vein and L5 (arrowhead). (E,F) Dl^3+/+lqf^{ARI} wings exhibit severe vein thickening at the tip and along the length of L2 (E; arrow, arrowhead) as well along L5 (F, arrow). The bifurcation of the L5 and posterior cross vein is also more severe in Dl^3+/+lqf^{ARI} wings (F, arrowhead). The percentage of flies of indicated genotype that exhibit noted phenotypes is given in the bottom right of each panel. Proximal – right.

3B,D,F). Similarly, a 50% reduction of *lqf* function in a $N^{ts\text{-}1}$ background dominantly enhances the weak $N^{ts\text{-}1}$ excess cardioblast phenotype (compare Fig. 3E to 3C). The near identity of the *lqf* and *Notch* heart phenotypes, together with the dominant genetic interactions between *lqf* and *Notch* pathway members, suggest that *lqf* promotes *Notch* pathway function during heart development.

lqf genetically interacts with the *Notch* pathway during wing vein and bristle development

Next, we asked if *lqf* genetically interacts with the *Notch* pathway during other *Notch*-mediated developmental events. *Notch* signaling regulates wing vein formation and bristle development. For example, flies heterozygous for *Delta* exhibit mild vein thickening along the length and at the distal tip of L2, bifurcation of the posterior cross vein and the distal tip of L5 (Fig. 4C,D) as well as thickening of L4 at its junction with the anterior cross vein (data not shown). Flies heterozygous for *lqf* exhibit wild-type veination. However, a 50% reduction of *lqf* dominantly enhances the expressivity and penetrance of all haploinsufficient *Delta* vein phenotypes (Fig. 4E,F). For example, 54.4% of Dl^3/+ wings (*n*=33) exhibit vein thickening along L2 and 51.6% (*n*=33) exhibit bifurcation of the posterior cross vein and the distal tip of L5. In contrast, 100% of Dl^3+/+lqf^{ARI} wings (*n*=36) exhibit more severe vein thickening along L2 and 91.7% (*n*=36) exhibit a more severe bifurcation of the posterior cross vein and the distal tip of L5. In addition, while only 12% of Dl^3/+ wings (*n*=33) exhibit thickening of L4 at its junction with the anterior cross vein, 83.3% of Dl^3+/+lqf^{ARI} wings (*n*=36) exhibit more severe thickening of L4 in this region (data not shown). The dominant enhancement of *Delta* by *lqf* is not allele specific as we observe similar genetic interactions between lqf^{AG}, lqf^{be428} and Dl^7 (data not shown).

lqf and *Notch* also exhibit dominant genetic interactions during bristle development. Flies heterozygous for N^{55e11}, a hypomorphic allele of *Notch*, exhibit a wild-type bristle pattern (*n*=27) while 2.5% of flies heterozygous for *lqf* exhibit one extra notal bristle (*n*=40). However, 40% of flies doubly heterozygous for *Notch* and *lqf* contain extra notal bristles (*n*=45; Fig. 5B). The dominant genetic interactions of *lqf* with *Notch* and *Delta* indicate that *lqf* acts with the *Notch* pathway to promote bristle and wing development. Together with our results in the heart, these data suggest that *lqf* promotes *Notch* signaling during multiple developmental events in *Drosophila*, consistent with *lqf* playing a general role to promote *Notch* pathway activity.

Ce-epn-1 promotes *glp-1* activity during germline development in *C. elegans*

Our experiments in *Drosophila* suggest *lqf* promotes *Notch* pathway function in most *Notch*-dependent developmental events. To test whether *lqf* has an evolutionarily conserved role in contributing to *Notch* signaling we investigated the functional relationship between the *C. elegans lqf* ortholog and *Notch* signaling during *C. elegans* germline development. We identified the *C. elegans lqf* ortholog – T04C10.2 (referred to as *Ce-epn-1*) – and cloned a region of *Ce-epn-1* (amino acids 1-238) into the standard *C. elegans* RNAi feeding vector, *pPD129.36* (Timmons and Fire, 1998). This region of *Ce-epn1* bears no significant similarity to any other *C. elegans* gene and should specifically target *Ce-epn-1* for RNAi-mediated gene interference.

We focused our analysis on the role of *Ce-epn-1* in germline development and used RNAi-mediated gene interference (Fire et al., 1998) to assay the effect of postembryonic loss of *Ce-epn-1* function on germline development and to determine

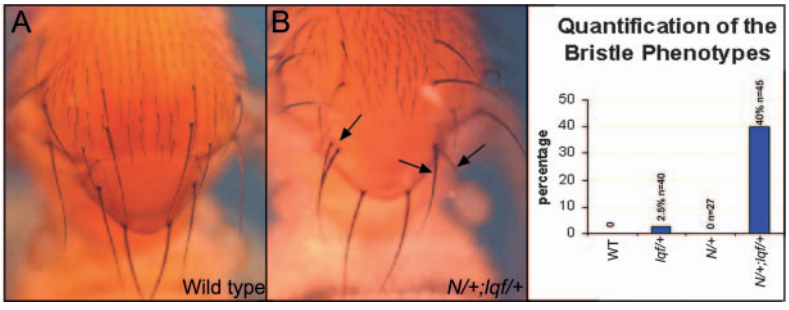


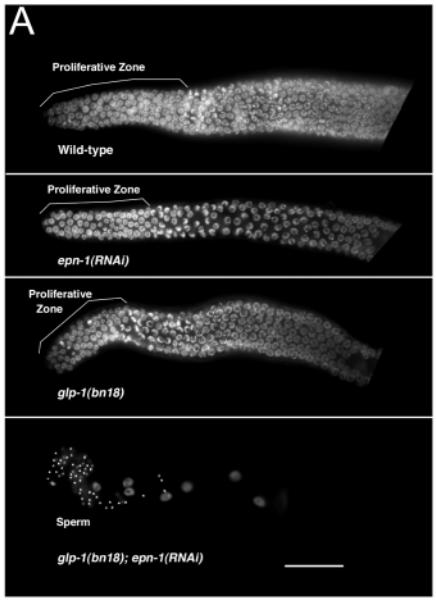
Fig. 5. *Notch* and *lqf* genetically interact to inhibit bristle development. Dorsal views of wild-type (A) and N^{55e11}/+; lqf^{ARI}/+ (B) nota and a graphical representation of bristle phenotypes in N^{55e11}/+, lqf^{ARI}/+ and N^{55e11}/+; lqf^{ARI}/+ nota. (A) Wild-type notal and scutellar bristle pattern. (B) Ectopic bristles develop on the notum and scutellum of N^{55e11}/+; lqf^{ARI}/+ flies (arrows). (C) Quantification of the percentage of flies that exhibit ectopic notal and scutellar bristles for the genotypes – lqf^{ARI}/+, N^{55e11}/+, and N^{55e11}/+;lqf^{ARI}/+.

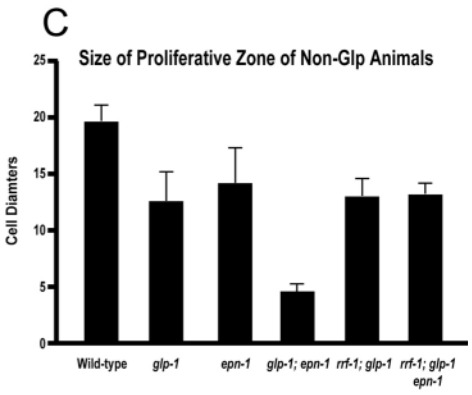
whether *Ce-epn-1* genetically interacts with *glp-1*. We tested the effect of reducing *Ce-epn-1* function on germline development by placing wild-type *C. elegans* embryos, as well as embryos carrying the weak temperature-sensitive loss-of-function mutant *glp-1(bn18)* on plates with bacteria containing the *Ce-epn-1*-encoding RNAi feeding vector at 20°C. As controls, we performed parallel experiments using bacteria containing the RNAi feeding vector with a GFP insert. At 20°C, *glp-1(bn18)* yields a weak premature entry into meiosis phenotype with a slightly smaller proliferative zone, and provides a sensitized background within which to assay *Ce-epn-1* function. We find that postembryonic RNAi-mediated reduction of *Ce-epn-1* function in wild-type individuals reduces the size of the adult proliferative zone relative to control worms (Fig. 6A,C). The size of the proliferative zone is similar to that observed in weak *glp-1* alleles indicating that *Ce-epn-1* acts in the same direction as *glp-1* to promote germline proliferation. In addition, we find that postembryonic RNAi-mediated reduction of *Ce-epn-1* function in *glp-1(bn18)* worms significantly enhances the *glp-1(bn18)* phenotype such that 95% of such animals (*n*=43) lack a proliferative zone, phenocopying a strong loss of *glp-1* phenoytpe (Fig. 6A,B). These results suggest that *Ce-epn-1* promotes GLP-1 signaling during *C. elegans* germline development, and support the idea that *epsins* play an evolutionarily conserved role to potentiate *Notch* signaling.

To distinguish whether *Ce-epn-1* acts non-autonomously in the signaling cell, the somatic DTC, or autonomously in the germ line receiving cell we fed *C. elegans* embryos of the genotype *rrf-1;glp-1(bn18)* on either *Ce-epn-1* RNAi bacteria or control *GFP* RNAi bacteria. The *rrf-1* mutation renders somatic cells deficient for RNAi while the germline remains sensitive to RNAi (Sijen et al., 2001). Thus, a failure to observe enhancement of the *glp-1(bn18)* phenotype in an *rrf-1* background would suggest that *Ce-epn-1* acts in the somatic DTC, where RNAi does not work, as part of the signaling mechanism to promote germ cell proliferation. In agreement with this, postembryonic *Ce-epn-1* RNAi treatment does not enhance the *glp-1(bn18)* proliferative zone phenotype in an *rrf-1* background (Fig. 6B,C). Thus, Ce-EPN-1 appears to act in the soma to help transmit the LAG-2 signal to GLP-1-expressing germ cells. These data agree with the observation that *lqf* acts cell non-autonomously in the *Drosophila* eye (Cadavid et al., 2000).

Discussion

Our work links epsins with *Notch* pathway activity. However, these results raise many questions with respect to epsin/Notch function during heart development and the molecular understanding of *Notch* pathway activation. For example, what is the precise developmental role of *lqf/Notch* during heart





B

Genotype	Percentage Glp[a]	*N*[b]
Wild-type *GFP(RNAi)*	0	100
glp-1(bn18); GFP(RNAi)	10[c]	106
epn-1(RNAi)	0	34
glp-1(bn18); epn-1(RNAi)	95	43
rrf-1(pk1417); glp-1(bn18); GFP(RNAi)	8	76
rrf-1(pk1417); glp-1(bn18); epn-1(RNAi)	9	85

Fig. 6. *Ce-epn-1(RNAi)* enhances the premature meiotic entry defect of *glp-1(bn18)* mutants. (A) Dissected gonad arms stained with DAPI to visualize DNA morphology. Distal is left and the proximal portions of the gonad arms are not shown. Brackets mark the regions of gonad arms containing proliferative nuclei. Wild-type (top), *Ce-epn-1 (RNAi)* (second from top) and *glp-1(bn18)* (third from top) animals all contain a proliferative zone one day past the fourth larval stage, however the proliferative zones are smaller in the *epn-1(RNAi)* and *glp-1(bn18)* animals. *glp-1(bn18); epn-1(RNAi)* animals (bottom) of the same age lack a proliferative zone, but rather have sperm extending to the distal end. All animals were grown at 20°C. Scale bar: 20 μm. (B) Quantification of percentages of worms that exhibit strong *glp-1* phenotype, lack of proliferative zone phenotype. [a]Gonad arms were determined to be Glp if they had sperm or oocytes at the very distal end of the gonad or if no proliferative cells were present as evidenced by HIM-3(plus)/REC-8(minus) cells at the very distal end of the gonad. [b]*N* refers to the number of gonad arms examined. [c]This percentage of Glp is somewhat higher than what has previously been described for *glp-1(bn18)* at 20°C, however this is probably due to the plates being slightly above 20°C prior to placing the eggs on them. (C) Quantification of proliferative zone size in worms that contain reduced proliferative zone based on HIM-3 and REC-8 staining. Error bars represent s.d.

development? How specific is the role of epsins to *Notch* signaling? And, how do epsins regulate *Notch* signaling at the molecular level?

The roles of *liquid facets* and *Notch* in cell-type specification in the heart

Our phenotypic studies of *lqf* embryos are consistent with the model that *lqf* acts in a subpopulation of FCMs to inhibit their acquisition of the cardioblast fate. How might *lqf* and *Notch* inhibit cardioblast development? In lateral and ventral regions of the mesoderm, Notch-mediated lateral inhibition helps select individual somatic muscle progenitor cells from clusters of equipotential cells. Cells in these clusters express *lethal of scute* (*l'sc*) and can adopt either the muscle progenitor or FCM fate. Cells that retain *l'sc* become progenitor cells while cells that lose *l'sc* expression become FCMs (Carmena et al., 1995). In these clusters, *Notch* inhibits *l'sc* expression and the progenitor fate, thereby promoting the FCM fate (Corbin et al., 1991; Hartenstein et al., 1992; Bate, 1993; Giebel, 1999). We speculate that *lqf* and *Notch* may act similarly to regulate the cardioblast progenitor/FCM decision in the dorsal mesoderm with *Notch* functioning to inhibit *tin* expression and the cardioblast progenitor fate and, in so doing, promoting the FCM fate. In this model, loss of *lqf/Notch* activity would lead to excess cardioblast progenitors at the expense of FCMs. Consistent with this, clusters of Tin-expressing cells in the dorsal mesoderm resolve to individual heart cells during the stages when *lqf* and *Notch* inhibit cardioblast development.

If *lqf* plays a general role to promote *Notch* activity, why do we observe defects only during heart development in *lqf* embryos? One explanation is that maternal *lqf* product masks earlier requirements for *lqf* during *Notch*-dependent events. Consistent with this, temperature shift experiments indicate *lqf* acts during late stage 12 to restrict cardioblast development (data not shown). Nearly all well-characterized *Notch*-dependent events in the embryo occur before stage 12. Thus, the apparent specificity of the *lqf* phenotype for the heart may simply arise due to the late stage at which this *Notch*-dependent event occurs combined with the masking effect of *lqf* maternal product. Unfortunately, we could not assay embryos devoid of maternal and zygotic *lqf* function as *lqf* germline clones failed to produce eggs.

In the case of postembryonic *Ce-epn-1* RNAi treatment, we observed a weak *glp-1* loss-of- function germline phenotype, which could be significantly enhanced in a *glp-1* temperature-sensitive background at the permissive temperature. Strong *lin-12* loss-of-function phenotypes were not observed in these experiments (unpublished observation). The weak *glp-1* loss-of-function and absence of *lin-12* phenotypes in a wild-type background is very likely because the postembryonic feeding RNAi treatment only partially depletes *Ce-epn-1* mRNA. The isolation and characterization of a null mutation will greatly facilitate uncovering all roles of *Ce-epn-1* in *C. elegans*.

Is *liquid facets/epsin* function specific to *Notch* pathway regulation?

As epsins appear to regulate endocytosis, and endocytosis regulates the activity of most signaling pathways (reviewed by Wendland, 2002), *lqf* might act broadly to regulate the output of many signaling pathways rather than acting specifically on the *Notch* pathway. However, existing data support a specific interaction between *lqf* and *Notch* activity. For example, we failed to detect genetic interactions between *lqf* and the *EGF* or *FGF* pathways during heart development, and *lqf* does not appear to interact with the dominant *Egfr[ellipse]* allele during eye development (data not shown). Furthermore, *lqf* mutant clones in the *Drosophila* eye exhibit phenotypes consistent with the specific loss of Notch activity (Cadavid et al., 2000; Overstreet et al., 2003; Overstreet et al., 2004). Thus, *lqf* appears to display specific interaction with the *Notch* pathway. Although it is important to assay whether *lqf* alters the activity of other signaling pathways regulated by endocytosis, such as the *TGFβ*, *Wingless*, and *Hedgehog* pathways, these data support a model in which Lqf plays a relatively specific role to target a component of the *Notch* pathway for endocytosis and in so doing promotes *Notch* signaling.

Are epsins core components of the *Notch* pathway?

Our work shows that *lqf*/epsins promote *Notch* pathway activity in *Drosophila* and *C. elegans*. Notably, epsins participate in Notch-mediated lateral inhibition signaling during bristle and perhaps heart development, as well as Notch-mediated inductive signaling in the *C. elegans* germline. These data argue that epsins are essential evolutionarily conserved components of the *Notch* pathway, potentially required for most if not all Notch-mediated processes.

Genetic studies have the potential to identify all components of a signaling process, however, they do not necessarily differentiate between the roles different genes play in a signaling process. Here, we distinguish between core components of a signaling pathway – those factors that actively take part in the signal transduction event – and factors that set the stage for signal transduction but do not actively transmit the signal. For example, Notch, DSL ligands, presenillins and CSL effectors are core components of the *Notch* pathway as they actively transmit the signal – DSL ligands bind Notch, induce the metalloprotease-mediated S2 cleavage followed by the presenilin dependent intramembrane (S3) cleavage of Notch that releases Notch[intra], which translocates to the nucleus and complexes with CSL-class proteins to activate *Notch* target genes (Mumm and Kopan, 2000). However, many other proteins set the stage for signaling by ensuring each core member of a pathway is present in the right location and correct form such that signal transduction will occur given the proper stimulus. For example, presentation of a functional Notch receptor on the cell membrane appears to require S1-mediated cleavage of Notch by furin-type proteins (see Mumm and Kopan, 2000). Although furins do not actively take part in the signaling event, furin activity and its requirement for presentation of Notch is a prerequisite for *Notch* signaling. Similarly, *ras* signaling requires Ras localization to the cell membrane and prenylation of Ras constitutively targets it to the cell membrane (Zhang and Casey, 1996).

In support of *lqf/epsins* as core components of the *Notch* pathway, Delta endocytosis appears essential for *Notch* signaling (Parks et al., 2000) and *lqf* appears essential for Delta endocytosis (Overstreet et al., 2003). Furthermore, epsins are thought to target ubiquitinated membrane proteins for regulated endocytosis via their ubiquitin-interacting motif (UIM) (Aguilar et al., 2003; Shih et al., 2002) and ubiquitination of Delta appears necessary for Delta endocytosis and active *Notch* signaling (Deblandre et al., 2001; Lai et al.,

2001; Pavlopoulos et al., 2001; Yeh et al., 2001). Thus, Lqf/epsins may act as part of a complex that specifically targets Delta for internalization after ubiquitination and as such be core members of the *Notch* pathway.

It remains possible, however, that *lqf/*epsin function is a prerequisite for *Notch* signaling. For example, some endocytic proteins function in protein transport in the secretory pathway and epsin1 family members could in principle enable transport of Delta to the membrane. In such a capacity, epsins would not be considered core components of the *Notch* pathway. Clearly, future experiments that test the requirement of specific domains of epsins, such as the UIM, for *Notch* signalling, as well as those that identify the protein complexes within which epsins act, should help elucidate the molecular basis through which *lqf/*epsins potentiate *Notch* signaling.

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