**hephaestus** encodes a polypyrimidine tract binding protein that regulates Notch signalling during wing development in *Drosophila melanogaster*

David A. Dansereau¹,², Martine D. Lunke¹, Ariel Finkielsztein², Michael A. Russell² and William J. Brook¹,*

1 Genes and Development Research Group and Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta T2N 4N1, Canada
2 Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6E 4G2, Canada

*Author for correspondence (e-mail: brook@ucalgary.ca)

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**SUMMARY**

We describe the role of the *Drosophila melanogaster* hephaestus gene in wing development. We have identified several hephaestus mutations that map to a gene encoding a predicted RNA-binding protein highly related to human polypyrimidine tract binding protein and *Xenopus laevis* 60 kDa Vg1 mRNA-binding protein. Polypyrimidine tract binding proteins play diverse roles in RNA processing including the subcellular localization of mRNAs, translational control, internal ribosome entry site use, and the regulation of alternate exon selection. The analysis of gene expression in imaginal discs and adult cuticle of genetic mosaic animals supports a role for hephaestus in Notch signalling. Somatic clones lacking hephaestus express the Notch target genes wingless and cut, induce ectopic wing margin in adjacent wild-type tissue, inhibit wing-vein formation and have increased levels of Notch intracellular domain immunoreactivity. Clones mutant for both Delta and hephaestus have the characteristic loss-of-function thick vein phenotype of Delta. These results lead to the hypothesis that hephaestus is required to attenuate Notch activity following its activation by Delta. This is the first genetic analysis of polypyrimidine tract binding protein function in any organism and the first evidence that such proteins may be involved in the Notch signalling pathway.

Key words: Notch signalling, hnRNP I, Delta, Serrate, Polypyrimidine tract binding protein (PTB), RRM RNA-binding proteins, hephaestus

**INTRODUCTION**

Lateral inhibition describes the intercellular interactions that subdivide initially equivalent populations through competitive signalling (Greenwald and Rubin, 1992; Simpson, 1990). During lateral inhibition, one cell or group of cells within an equivalence group assumes a primary or dominant fate and inhibits surrounding cells from doing so. One mechanism commonly used for lateral inhibition is the *Notch* pathway (Artavanis-Tsakonas et al., 1999; Greenwald, 1998). In *Drosophila melanogaster*, the transmembrane Notch receptor is activated by interaction with its transmembrane ligands Serrate (SER) or Delta (DL) (Fleming, 1998). Subsequent Notch signalling involves a series of proteolytic steps that release both the Notch extracellular domain (NECD) and the Notch intracellular domain (NICD) in response to ligand binding (Mumm and Kopan, 2000). NECD is trans-endocytosed into ligand-expressing cells (Parks et al., 2000), while NICD is released into the cytoplasm of receiving cells (De Strooper et al., 1999; Mumm et al., 2000; Struhl and Greenwald, 1999; Ye et al., 1999). NICD is transported into the nucleus, where it interacts with the DNA-binding protein encoded by *Suppressor of Hairless [Su(H)]* to activate target gene expression (Fortini and Artavanis-Tsakonas, 1994; Fortini et al., 1993; Jarriault et al., 1995; Kao et al., 1998b; Klein et al., 2000; Lieber et al., 1993; Struhl et al., 1993) including *Enhancer of split Complex* genes [E(spl)-C] (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995).

Notch signalling plays several roles in wing pattern formation. Notch activation in cells along the DV compartment boundary of wing imaginal discs induces expression of the Wingless (WG) morphogen conferring organizer activity for the DV axis (de Celis et al., 1996b; Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1996; Neumann and Cohen, 1997; Rulifson and Blair, 1995). Later in pupal development, Notch signalling is required in vein competent regions to distinguish between vein and intervein boundary territories (de Celis et al., 1997; Huppert et al., 1997). In both cases, an initial broad domain of Notch activation is refined by the inhibition of Notch activity in adjacent ligand-expressing cells. Although the mechanisms regulating the decreases in Notch activation are not known, it is likely that the protein levels and activity of NICD and Notch effector genes such as the *E(spl)-C* transcription factors must be tightly regulated in order for cells to change Notch activation states.

This study shows that hephaestus (*heph*) is required to attenuate Notch activity after ligand-dependent activation during wing development. The original male sterile *heph* allele was identified in a genetic screen for loci required for
spermatogenesis (Castrillon et al., 1993). We have isolated new lethal alleles of heph that affect wing margin and wing vein pattern formation in genetic mosaics. We report that the Drosophila heph gene encodes the apparent homologue of mammalian poly(pyrimidine tract binding protein (PTB). PTB was first identified in vertebrates as a protein that binds to intronic poly(pyrimidine tracts preceding many 3’ pre-mRNA splice sites (Garcia-Blanco et al., 1989). Many different functions have been identified for vertebrate PTB, including the control of alternative exon selection (Carstens et al., 2000; Chan and Black, 1997; Chou et al., 2000; Cote et al., 2001; Lou et al., 1999; Mulligan et al., 1992; Perez et al., 1997; Southby et al., 1999; Zhang et al., 1999), translational control or internal ribosome entry site (IRES) use (Hunt and Jackson, 1999; Ito and Lai, 1999; Kim et al., 2000; Pilipenko et al., 2000), mRNA stability (Tillmar et al., 2002) and mRNA localization (Cote et al., 1999). PTB may also act as a transcriptional activator (Rustigian et al., 2002). This is the first report implicating such proteins in the regulation of the Notch pathway.

MATERIALS AND METHODS

Drosophila stocks, cuticle preparation and mutagenesis

The heph alleles P[PZ]ms(3)heph2, P[PZ]l(3)03429 (heph03429) and P[LacW]l(3)119B (heph119B), and the stocks used for genetic mosaic analysis were obtained from the Bloomington Drosophila stock centre. Adult cuticle was stored and dissected in SH (30% glycerol, 70% ethanol), then mounted in Gur’s Aquamount (BDH Laboratory Supplies Inc.). To isolate heph point mutants, single ethyl methanesulphonate (EMS)-mutagenized (Lewis and Bacher, 1968) stock Drosophila Df(3R)G45. methanesulfonate (EMS)-mutagenized (Lewis and Bacher, 1968) analysis were obtained from the Bloomington Drosophila stocks, cuticle preparation and mutagenesis (Davis et al., 2000). Immunohistochemistry, clones were marked by loss of a green fluorescence (GFP) marker in clones and all but heph119B cause autonomous loss of wing vein differentiation in clones and all but heph119B, which is temperature-sensitive and likely to be hypomorphic, induce ectopic margin in genetic mosaics. All four lethal alleles fail to complement the male sterility of the previously identified P-element-induced male sterile mutation of hephaestus (ms(3)heph2) (Castrillon et al., 1993).

Immunoistochemistry

Mouse monoclonal antibodies for Achaete (working dilution: 1/25, Cut2B10 (1/100), NIDC C17.9C6 (1/10), NECD C458.2H (1/10) and WG 4D4 (1/10) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Monoclonal mouse anti-Distalless (1/100) was a gift from S. Cohen, monoclonal mouse anti-DL-202.9B (1/25) was a gift from M. Muskavitch and rat anti-SER polyclonal 2 (1/15) was kindly provided by K. Irvine. Secondary antibodies were goat anti-mouse Alexafluor546nm (1/500; Molecular Probes, Inc.) and goat anti-rat Cy3 (1/400; Jackson Immunoresearch). Immunostaining of imaginal discs was performed using protocols modified from those of Pattatucci and Kaufman (Pattatucci and Kaufman, 1991).

Isolation, sequencing and mapping of heph cDNAs

In reverse PCR was used to confirm the insertion point of heph03429 and to map the insertion point of heph119B. Sequence from the heph2 (AQ026438), heph03429 (G00761) and heph119B (AF373596) P-element insertions were mapped to Drosophila genomic DNA (AE003780), and ESTs were identified by sequence homology to this genomic DNA using BLAST. The heph P-elements mapped within a cluster of 3’ and 3’ ESTs that suggested a 145 kb transcription unit including CG2094 and CG2290. Full-length cDNA clones LD04329 (AY052367) and GH17441 (AF436844) were purchased from Research Genetics. A third cDNA sequence has been submitted to the GenBank Nucleotide Sequence Data Library under the name poly(pyrimidine tract binding protein (PTB); AF211191) (Davis et al., 2002).

PCR-based sequencing of EMS-derived heph alleles

Single homozygous heph+ or heph2 first instar larvae were identified by the absence of a P[Actin-GFP] balancer chromosome. Each heph exon and its splice sites were amplified by PCR and sequenced. Sequence from the mutants was compared with wild-type sequence using the same primers from the original non-mutagenized line. When several heph exons could not be amplified by PCR from homozygous heph+ DNA, Southern analysis confirmed that the heph2 allele is a deletion of several heph coding exons.

RESULTS

The hephaestus locus

As part of a genetic analysis of polytene region 100EF of chromosome 3 (D. A. D., M. A. R. and W. J. B., unpublished), we identified a lethal complementation group that causes wing defects in genetic mosaic animals. Four lethal alleles in the complementation group (heph2, heph2, heph119B and heph03429) cause autonomous loss of wing vein differentiation in clones and all but heph2, which is temperature-sensitive and likely to be hypomorphic, induce ectopic margin in genetic mosaics. All four lethal alleles fail to complement the male sterility of the previously identified P-element-induced male sterile mutation of hephaestus (ms(3)heph2) (Castrillon et al., 1993). The four lethal alleles and the male sterile allele of heph map to a transcription unit that is predicted to encode at least three isoforms of a protein with four RNA recognition motifs (RRMs) (Fig. 1A). The P-elements of ms(3)heph2, heph03429 and heph119B are inserted in large introns. heph+2 is an EMS-induced deletion of several coding exons including the coding region for RRM1, RRM2 and part of RRM3. The temperature-sensitive heph2 mutaion is a miss-sense mutation that changes a conserved glycine (G) residue to a glutamine (Q) residue in
the first predicted RRM domain of HEPH. The mapping of all five heph alleles to a single transcription unit indicates the lethality, ectopic margin, loss of vein differentiation and male sterile phenotypes are all due to loss of function in the same gene.

Because RRM domains are diagnostic of RNA-binding proteins and these domains are highly conserved among HEPH, human PTB and Xenopus Vg1RBP60 (Fig. 1B), it is likely that the RNA-binding function of these proteins has been conserved. A recent gene-expression report refers to this transcription unit as PTB because of its sequence similarity to vertebrate PTB (Davis et al., 2002). However, in accordance with Drosophila genetic nomenclature, we will use the name hephaestus, which has precedence (Castrillon et al., 1993).

Loss of heph in genetic mosaics induces ectopic wing margin

Based on our initial observations that heph mutant clones disrupted normal wing pattern formation, we performed several genetic mosaic analyses with heph mutations. We find similar results for genetic mosaics of all the strong heph alleles including heph point and P-element insertion mutants and with Df(3R)G45, a small deficiency that deletes heph along with a second lethal complementation group, modulo. Clones of Df(3R)G45 and of strong heph alleles are smaller than wild-type clones in twin spot experiments, indicating a growth disadvantage or increased cell death in the clone (Fig. 2A and clones marked with GFP in Figs 4-7). Using pixel dimensions as an estimate of clone size, heph clones induced during mid-second instar are about 65% of the corresponding twin size by late-third instar. When heph were given a growth advantage using the Minute technique, the clone sizes increased but even clones induced during the first instar never occupied more than a small fraction of the wing blade. In adult wings, cell polarity and cell size (trichome density) are not apparently affected by heph loss. Minute+ heph clones can differentiate all wing blade structures normally with the exception of veins.

Mutant clones induced in larval imaginal discs are associated with ectopic wing margin (Fig. 2A-G), loss of wing margin (Fig. 2H) and loss of wing veins (described in detail below). Clones induced throughout larval development are associated with ectopic wing margin when situated within a short distance of the endogenous wing margin (Fig. 2A-G). The ectopic margin of heph genetic mosaics always conforms to the original compartment identity and resembles the adjacent endogenous margin. The autonomy of the ectopic margin was tested in experiments marking heph mitotic clones with the bristle marker yellow (y). We also marked clones with pawn (pwn) and performed twin spot experiments marking the clones and twins with forked (f) and bald (bld), which affect both bristles and trichomes. In all of these experiments, the ectopic bristles are derived almost entirely from heph+ cells immediately adjacent to the clone with the occasional ectopic bristle induced from within the heph mutant tissue (Fig. 2C). The non-autonomy of bristle induction is especially clear when the heph growth disadvantage is partially rescued by generating marked Minute+ heph clones in a Minute background. The ectopic margin is induced along the border of the marked Minute+ heph mutant tissue when that border is close to the normal margin (Fig. 2B). Outside of this domain, mutant cells do not induce ectopic wing margin. The ectopic margin is associated with small outgrowths of wing blade tissue in clones located near the junction of the wing margin and the AP boundary (Fig. 2G). Dorsal (not shown) or ventral (Fig. 2H) heph mutant clones that apparently intersect the normal margin are associated with wing margin nicks.

Fig. 1. The hephaestus locus. (A) Five heph alleles map to a single, complex transcription unit predicted to encode an RNA-binding protein with four RNA-recognition motifs (RRM1-4). Three P-element alleles (hephj11B9, hephj18B9 and heph2) map to heph introns and two EMS-induced alleles (hephc and hephc2) alter conserved RNA-binding domains. Alternative splicing produces predicted protein variants: isoforms A and B include a bipartite nuclear localization signal (NLS). Only isoform A includes an N-terminal glutamine-rich domain (Q-rich). (B) The heph protein is highly related to the previously characterized RNA-binding proteins PTB/hnRNP1 (human, NM_002819) and Vg1RBP60 (Xenopus, AF091370). Percent amino acid identity is indicated and, in brackets, percent similarity.
heph clones induce the wing margin molecular markers wingless, cut and achaete

In vertebrates, PTB proteins regulate mRNAs involved in several cellular processes (Valcarcel and Gebauer, 1997; Wagner and Garcia-Blanco, 2001). The wing margin nicks and growth disadvantage caused by heph mutations could result from disruption of general processes required for cell survival. However, the ectopic margin phenotype indicates that heph plays a regulatory role in wing margin pattern formation. In order determine what processes heph is disrupting at the presumptive wing margin, we examined the expression of wing margin molecular markers in heph mosaic wing imaginal discs. In normal margin development, the WNT family member wingless (wg) is expressed in two or three rows of cells straddling the D/V compartment boundary (Fig. 3A) and diffuses to induce wing margin bristle fate in cells flanking the wg expression domain (Couso et al., 1994; Phillips and Whittle, 1993). Thus, the heph ectopic margin phenotype can be explained if heph mutant cells express wg ectopically. Using specific antibodies, we examined the expression of wg and cut, a second D/V boundary marker, in heph genetic mosaic wing discs. In agreement with the distribution of ectopic margin in adult wings, ectopic WG and CT were observed in those heph mutant cells located within a few cell diameters of the boundary stripe of WG and CT expression (Fig. 3A-F). Ectopic WG or CT expression was never seen in heph mutant tissue further away from the endogenous margin. As the expression of both wg and cut at the boundary depends on high levels of Notch activation, these results suggest that heph mutant cells near the endogenous boundary are Notch activated.

Induction of bristles of the anterior wing margin by WG depends on downstream target genes such as the proneural...
gene *achaete* (*ac*). Thus, there is a strong prediction that anterior *heph* mutant clones should induce anterior margin-promoting genes such as *ac*. As predicted from the adult phenotype, we observed ectopic AC expression surrounding *heph* mutant clones near the DV boundary with some ectopic AC expression in the clones (Fig. 3G-I). The association of ectopic *wg*, *cut* and *ac* expression with *heph* mutant tissue suggests that ectopic margin is induced around *heph* clones by the same mechanisms acting during normal development.

**Ser and DI expression are reduced in *heph* mutant tissue**

The precise expression of WG in DV boundary cells that is present by the late third instar evolves through interaction between the Notch and WG signalling pathways. In mid-second instar wing discs, the Notch pathway is activated to high levels along the boundary between dorsal and ventral cells by *Ser*, which is expressed dorsally, and *DI*, which is expressed predominantly ventrally (de Celis et al., 1996b). These ligands induce a broad domain of Notch-activated cells at the DV interface that express *wg* (de Celis and Bray, 1997; Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995). Expression of *DI* and *Ser* is dynamic, and the initial DV asymmetry disappears as the two ligands become expressed under the control of WG signalling (Milan and Cohen, 2000). By mid third instar, a broad stripe of cells along the DV boundary express *Ser*, *DI* and *wg* (de Celis and Bray, 1997; Micchelli et al., 1997; Rulifson et al., 1996). During late third larval instar, this broad domain evolves into a narrow stripe of cells expressing *wg* but not *Ser* or *DI*, flanked on either side by cells expressing *Ser* and *DI* but not *wg* (Fig. 4A). WG secreted by the boundary cells is required to maintain high levels of *DI* and *Ser* expression in the flanking cells (de Celis and Bray, 1997; Micchelli et al., 1997). The high levels of DL and SER

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**Fig. 3.** *heph* clones autonomously misexpress WG and CT, and induce ectopic AC expression in surrounding tissue. Normal WG (A), CT (D), AC (G) and DLL (J) expression from *heph* heterozygous wing discs. (B,C) A *heph* mosaic wing disc with ectopic WG (arrows) within *heph* mitotic clones that are close to the normal WG-expressing cells. (E,F) A *heph* mosaic wing disc with ectopic CT (arrows) within *heph* mitotic clones that are close to the normal CT-expressing boundary cells. Notice that CT is preferentially expressed along the *heph* clone border (F,F'). (H,I) A *heph* mosaic wing disc with ectopic AC (arrow) in cells adjacent to *heph* clones that are close to the normal DV boundary (marked by the normal gap between domains of AC expression). Notice in I that some *heph* mutant cells express AC. (K,L) Loss of *heph* in clones does not affect expression of the WG target gene *Dll*. 

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in the flanking cells serve two roles. First, they signal back to adjacent boundary cells to maintain the high levels of Notch activation required for wg and cut expression. Secondly, in the flanking cells, DL and SER autonomously inactivate Notch signalling, which restricts Notch-dependent expression of wg and cut to the boundary. High levels of Notch signalling in late third instar boundary cells activates the expression of cut, which encodes a homeodomain protein required to repress Ser and Dl expression in the boundary cell domain.

The ectopic wing margin phenotype and association of ectopic wg, cut and ac expression with heph mutant tissue suggests that heph mutant cells situated near the endogenous DV boundary are highly Notch-activated and thus behave like boundary cells and induce wing margin fate in adjacent flanking cells. The complex interdependent signalling network at the DV boundary (Fig. 4A) offers several possible mechanisms that could lead to Notch activation and the ectopic margin phenotype. Ectopic boundary cell fate and ectopic margin are induced by clones of cells mutant for dishevelled (dsh) (Rulifson et al., 1996), which are deficient for WG signal transduction, or by clones mutant for both Dl and Ser (Micchelli et al., 1997). In both cases, clones of cells in the flanking domains lose Dl and Ser expression and Notch becomes activated through signalling from the adjacent wild-type Dl- and Ser-expressing cells (de Celis and Bray, 1997; Micchelli et al., 1997). In heph mutant cells, the levels of both DL and SER are autonomously decreased independent of clone position within the wing disc (Fig. 5). The decrease in DL and SER protein levels in heph clones is sufficient to account for the ectopic induction of wg and cut expression in cells flanking the margin where DL and SER normally repress Notch. This reduction of DL and SER could be the result of loss of WG signal transduction or to loss of Dl and Ser expression. Finally, autonomous activation of Notch signalling could result in heph mutant cells in the flanking domains assuming a boundary fate.

**heph clones are not defective for wg signalling**

Disruption of WG signal transduction is not a likely explanation for the loss of Ser and Dl expression in heph mutant clones. Clones mutant for heph in the antenna and leg have no pattern phenotypes (data not shown) and heph mutations do not enhance the phenotype of ‘dishevelled-weak’ (data not shown), a genetic background that is highly sensitive to dose changes in WG pathway signalling components (Haerry et al., 1997). Furthermore, the WG target gene acheate can be activated in heph mutant cells (Fig. 3G-I), and expression of the WG target gene Distal-less is not affected in heph clones (Fig. 3J-L). These results suggest that clones lacking heph are able to transduce the wg signal and that the primary effect of heph is not on wg signalling.

Further support that Notch signalling and not wg signalling is disrupted in heph mutants comes from a genetic interaction observed between heph mutants and fringe$^{D4}$ (fringe$^{D4}$) (Fig. 6). fringe$^{D4}$ is a gain-of-function allele of fringe, a gene encoding a Notch-modifying glycosyltransferase (Bruckner et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000). A DV fringe expression boundary is required for maximal activation of Notch signalling and proper induction of the DV organizer (Fleming et al., 1997; Irvine and Wieschaus, 1994; Kim et al., 1995; Klein and Arias, 1998; Panin et al., 1997). Ectopic transcription of fringe$^{+}$ on fringe$^{D4}$ results in decreased Notch activation and wg expression at the DV boundary, causing loss of the wing margin and much of the wing blade (de Celis and Bray, 2000; Irvine and Wieschaus, 1994). A decrease in the dose of a wg pathway component would be predicted to enhance the fringe$^{D4}$ phenotype. However, flies heterozygous for
both heph and fngD4 have considerably more wing margin and wing blade than do flies heterozygous for fngD4 alone (Fig. 6). A similar suppression has been reported for flies heterozygous for fngD4 and activating ‘Abruptex’ alleles of Notch (N Ax) (de Celis and Bray, 2000). The Abruptex phenotype probably results from an inability to repress Notch activation (de Celis and Bray, 2000; de Celis et al., 1996b), and like heph, N Ax mitotic clones are associated with ectopic margin within a few cell diameters of the endogenous margin (de Celis and Garcia-Bellido, 1994a; de Celis et al., 1996b), and cause a cell-autonomous loss of vein differentiation (de Celis and Garcia-Bellido, 1994a; de Celis and Garcia-Bellido, 1994b).

**Notch levels are increased in heph mutant cells**

The ectopic margin phenotype is probably caused either by loss of Ser and Dl expression or autonomous activation of the Notch pathway in heph mutant cells. One consequence of Notch activation is the cleavage of the full-length receptor, which releases the Notch intra-cellular domain (NICD), allowing it to translocate from the membrane to the nucleus (Gho et al., 1996; Kidd et al., 1998). To determine if Notch is activated by loss of heph activity, we examined the distribution of Notch immunoreactivity in heph genetic mosaic wing discs. An increase in Notch immunoreactivity was found in heph mutant cells, regardless of their position within the imaginal disc (Fig. 7). This increase is specific to an antibody that recognizes the intracellular domain of Notch (NICD) and is found in the cell body away from the apical surface of the cell (Fig. 8). As this effect was not observed with an antibody to the extracellular domain of Notch, and the apical levels of Notch are very similar in heph mutant and wild-type tissue, accumulation of the full-length Notch is not apparently increased in heph mutant cells. Consistent with this observation, comparable changes in NICD immunostaining are found along the DV boundary, where the Notch pathway is active and Notch target genes are expressed at high levels (Fig. 8). As further evidence that the changes in NICD immunoreactivity represent Notch activation, we find that heph clones generated in Su(H) mutant discs do not alter the levels or localization of NICD (Fig. 9). While it is not possible to conclude that the increased levels of NICD are localized to the nucleus, these results are consistent with an increase in Notch activation in heph mutant cells, and

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Fig. 5. Delta and Serrate expression are reduced in heph clones. Normal DL (A) and SER (D) expression in heph03429 heterozygous wing discs. heph03429 mosaic wing discs stained for DL (B,C) or SER (E,F) protein expression. The relative levels of DL and SER are reduced within heph03429 clones (arrows). Although not dependent on proximity to the normal margin, this effect on DL and SER levels can be observed most clearly in cells flanking the boundary cells, where DL and SER expression is normally highest.

Fig. 6. heph03429 partially rescues the fngD4 margin loss phenotype. (A) An example of a wing from fngD4/+ male. Most or all of the wing margin is missing. This phenotype is partially rescued in fngD4/heph03429 male (B) or in N Ax–1/+; fngD4/+ transheterozygous females (C). All flies were cultured at 25°C. The fngD4 phenotype is variable and these images are representatives of the most common phenotype observed in flies of each genotype.
with the proposed role for SU(H) in transporting NICD to the nucleus (Gho et al., 1996; Kidd et al., 1998). These data suggest hepH acts in all wing disc cells to repress Notch pathway function. This is consistent with a report that hepH mRNA is present uniformly in imaginal discs (Davis et al., 2002).

**Delta levels are decreased in hepH mutant cells in a Su(H)-dependent manner**

We find that the level of Notch is elevated (Figs 7, 8) and the level of DL is decreased (Fig. 5A-C) in hepH mutant cells regardless of their position in the imaginal disc. This reciprocal relationship is typical of most tissues where Notch signalling is acting and is generally the result of interdependent signalling causing autonomous inhibition of DI expression in Notch-activated cells (Artavanis-Tsakonas et al., 1999). DL itself is sometimes required to repress Notch activation autonomously (Artavanis-Tsakonas et al., 1999; de Celis and Bray, 1997; Doherty et al., 1996; Micchelli et al., 1997) so the observed decrease in DL levels could be either a cause or an effect of increased Notch activation. In order to distinguish between these possibilities, we examined DI expression in cells mutant for both hepH and Su(H). In mature third instar discs, DI is expressed ubiquitously at a low level, and in elevated levels at the DV margin, in the presumptive wing veins and in the proneural clusters of the thorax. Discs from third instar larvae mutant for strong alleles of Su(H) lack most of the wing pouch, because of the absence of Notch signalling along the DV boundary, but they retain the low ubiquitous expression of DI. We reasoned that if DI expression were still reduced in hepH cells in the absence of Su(H), then hepH might act directly on DI expression. However, we observed no change in the low levels of DI expression in hepH clones generated in Su(H) imaginal discs (Fig. 8A-C), indicating that the decrease in DI expression in hepH cells depends on Su(H). The implication of this result is that hepH directly affects Notch activity and indirectly reduces ligand expression.

**hepH causes a cell autonomous and Delta-dependent loss of vein differentiation**

On balance, the effects of hepH on wing margin formation suggest that hepH represses Notch pathway activity. The hepH loss-of-function phenotype in the wing veins also suggests that hepH directly affects Notch signalling. Lateral inhibition involving Notch and Epidermal Growth Factor Receptor (EGFR) signalling is required to refine pro-vein territories in the wing blade (Fig. 4B) (de Celis and Garcia-Bellido, 1994b; Huppert et al., 1997). The position of veins is set by the expression of rhomboid (rho) in stripes of cells oriented perpendicular to the DV compartment boundary in the wing pouch (Sturtevant et al., 1993). RHO facilitates signalling through EGFR and EGFR activation is required for the vein fate (Diaz-Benjumea and Garcia-Bellido, 1990). Loss of EGFR activity is epistatic to the wide vein phenotype of

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**Fig. 7.** The amount of NICD is higher in hephaestus clones relative to normal surrounding tissue. (A,C) Normal NICD staining in a heterozygous hepH03429 wing disc. The position of the DV boundary is noted in A (arrowhead). (B-B″,D-D″) A typical example of a hepH03429 mosaic wing disc with elevated levels of NICD staining corresponding to the positions of hepH mutant clones that are marked by loss of a GFP reporter transgene. (C,D-D″) Higher magnification view of the discs in A,B-B″, respectively. The positions of the XZ sections through C,D-D″ are marked by a broken line. Each image is a projection of 20 laser scanning fluorescent images (spanning a 30 μm total thickness) of GFP from a P(Ubi-GFP) transgene (green; B′,D′) and mouse anti-NICD primary antibody labelled with an anti-mouse Alexa-fluor594nm secondary antibody (red; A-D) in control flies of genotype y w P( hsFLP); P(neoFRT) P(πM) Sb hepH03429/TM6B (A,C) or target class flies of genotype y w P( hsFLP); P(neoFRT) P(πM) Sb hepH03429/P(neoFRT) P[hsGFP] (B-B″,D-D″).
Notch mutants, indicating that EGFR activation induces pro-vein regions, then Notch functions to restrict vein fate by refining the domain of rho expression (de Celis et al., 1997). Dl mutant clones that span a vein territory produce thicker veins than normal because DL is required in the vein to activate Notch in adjacent lateral provein cells. By contrast, heph clones covering a vein territory cell autonomously fail to differentiate as vein (Fig. 10A,B). Only when dorsal and ventral clones coincide does a vein appear to be completely missing. This phenotype is consistent with ectopic Notch activation in heph clones as it resembles the effects of activating Notch by a variety of different genetic manipulations (de Celis et al., 1997; de Celis et al., 1996a; de Celis and Garcia-Bellido, 1994a; de Celis and Garcia-Bellido, 1994b; Huppert et al., 1997; Schweisguth and Lecourtois, 1998; Struhl et al., 1993). Furthermore, despite the reduction of Dl expression in heph clones, the heph mutant clones have a wing vein phenotype opposite to that of Dl mutant clones (Fig. 10A-D). This is strong support for the interpretation that the reduction of Dl expression in heph clones is a consequence rather than a cause of the Notch activated heph phenotype.

To determine the epistatic relationship between heph and Dl, we compared the wing vein phenotypes of double mutant clones with clones lacking only heph or Dl. Clones of cells mutant for both heph and Dl cause a thick vein phenotype (Fig. 10E,F) that is indistinguishable from the effects of Dl mutant clones (Fig. 10C,D). These phenotypes indicate that heph is not required for specification of vein fate, i.e. heph is not directly required for rho expression or EGFR activity. The parsimonious interpretation that heph acts to repress Delta contradicts the loss of DL staining in heph mutant tissue, and the lack of requirement for Dl in specifying vein fate. Another interpretation is that Notch must be activated by DL before heph is required. That is, heph may attenuate the Notch signalling pathway in cells where Notch has already been activated by DL.

DISCUSSION

Loss of heph function resembles Notch activation

Ectopic Notch activation is sufficient to induce margin formation in adjacent tissue in any wing compartment without affecting the compartment identity of those cells (de Celis and Bray, 1997; Diaz-Benjumea and Cohen, 1995). This similarity in ectopic margin phenotypes and the expression of the Notch target genes wg and cut in heph clones suggests that heph acts as a repressor of Notch activation. Inhibition of vein differentiation is also a characteristic phenotype of ectopic activation of the Notch signalling pathway. Autonomus loss of vein differentiation in heph clones is the same phenotype observed in clones of Hairless (de Celis et al., 1997; Schweisguth and Lecourtois, 1998), which encodes a transcriptional co-repressor of Notch target gene expression (Bang et al., 1995; Brou et al., 1994; Kao et al., 1998a; Schweisguth and Lecourtois, 1998; Schweisguth and Posakony, 1994). Vein loss is also characteristic of gain-of-function Abruptex alleles of Notch, ectopic expression of the genes of the E(Spl)-C, or expression of low levels of the activated form of Notch (de Celis et al., 1996a; de Celis and Garcia-Bellido, 1994b; Huppert et al., 1997; Struhl et al., 1993).

Direct evidence of Notch activation was also observed in heph mutant clones situated throughout the wing imaginal disc. Increased levels of the Notch intracellular domain (NICD) were found in the cell body of normal boundary cells and in heph mutant cells. Although we do not know that these changes represent genuine nuclear accumulation, the altered

![Fig. 8. The intracellular localization of NICD is altered in hephaestus clones. A heph mosaic disc stained for NICD (A'-A'') or NECD (B'-B''). Loss of heph is marked by loss of GFP from a P{Ubi-GFP} transgene (A,B). Each image is an XY projection representing one of three 10 μm apical (A',B') to basal (A'',B'') sections through each disc. The increased relative amount of NICD staining in heph(3429) clones appears to be inside of the mutant cells, not bound to the apical membrane. No corresponding increase is apparent using the NECD antibody. The position of the DV boundary is noted with an arrowhead, the position of heph clones are marked with arrows.](image-url)
distribution of NICD, which is absent in Su(H) mutant tissue, is consistent with models of Notch activation involving SU(H)-mediated translocation of NICD to the nucleus (Gho et al., 1996; Kidd et al., 1998). The position independence of this effect indicates that heph acts in all wing disc cells to modulate Notch pathway function and is consistent with reports that low levels of Notch pathway activation are required throughout the developing disc for cell proliferation (Baonza and Garcia-Bellido, 2000; de Celis and Garcia-Bellido, 1994b) and for the weak general expression of Notch target genes such as E(spl)mβ (Cooper et al., 2000; de Celis et al., 1996a).

Although the effects of heph on DL, SER and NICD levels are position independent, the margin-inducing effects of heph are restricted to a competent region within a few cell diameters of the endogenous wing margin. By contrast, clones of cells expressing NICD under the control of a strong promoter are able to induce wg and cut expression, as well as ectopic margin and outgrowths throughout the wing blade, independent of the position of the clone (de Celis and Bray, 1997). The effects of heph clones, including vein loss and the restricted location of ectopic margin, more closely resemble the effects reported for clones expressing NICD under a weak promoter (Diaz-Benjumea and Cohen, 1995; Struhl and Basler, 1993). The competent region corresponds roughly to the domain of Dl and Ser expression in the regions flanking the boundary. As defined by the refinement of wg expression, cells in this region are initially Notch activated and express wg...
during late second and early third instar. The flanking cells subsequently repress Notch activity and lose wg expression in response to increased levels of DL and SER. It is likely that the band of cells that maintains wg expression begin with higher levels of Notch activation and that this bias is what allows them to maintain Notch activation and wg expression (Blair, 1997). Even small increases in Notch activity caused by the loss of heph activity in the cells of the flanking domains may bias the signalling required to refine the wg and DI/Ser domains in favour of maintaining Notch activity in heph clones. Thus, the competent region for heph-induced margin formation may reflect the high levels of Notch activation required for margin induction.

The maintenance of Notch activation in heph cells in the flanking regions may cause them to become more like boundary cells. Thus, they maintain wg expression and decrease DI and Ser expression. The reduction in DI/Ser in heph clones would reduce Notch inhibition to allow further increases in Notch activation by adjacent DI/Ser-expressing cells. This is consistent with the induction of ectopic cut expression, which may require the highest levels of Notch activation (Micchelli et al., 1997), only in heph mutant cells abutting the DI/Ser domain. A bias toward Notch activation in heph mutant cells may also explain the unusual shape of the boundary cell domain that is often observed in heph mosaic discs (Fig. 3B). Boundary Notch activation is required to maintain the lineage restriction property of the DV boundary and perturbing the spatial pattern of Notch activation can alter the shape of the boundary (Micchelli and Blair, 1999; Rauskolb et al., 1999).

Notch-dependent repression of ligand expression

It has been reported that Notch activation increases DI and Ser expression in the wing pouch (de Celis et al., 1996b; Doherty et al., 1996; Kim et al., 1995; Panin et al., 1997). However, these effects are due to a positive feedback loop mediated indirectly through wg (de Celis and Bray, 1997; Micchelli et al., 1997). Cells mutant for heph located away from the wing margin do not activate wg expression and do not activate DI and Ser expression, perhaps because of insufficient levels of Notch activation. Instead, we observe an autonomous decrease in DI and Ser expression throughout heph clones. We have shown that the decrease in DI expression depends on Su(H), and by implication is downstream of Notch activity in heph mutant cells. These observations are consistent with genetic evidence that Notch activity represses Delta activity (de Celis and Bray, 2000; de Celis and Garcia-Bellido, 1994b; Vässin et al., 1985), and with instances of Notch signalling that require the progressive restriction of ligand and receptor to reciprocal cells (e.g. during wing margin and wing vein patterning; Fig. 4).

Is heph specific to the Notch signalling pathway?

It is unlikely that the Drosophila PTB homologue (heph) is a dedicated Notch pathway component considering that several target RNAs not involved in Notch signalling have been identified for vertebrate PTBs (Valcarcel and Gebauer, 1997; Wagner and Garcia-Blanco, 2001). This is especially clear when considering heph-induced wing margin nicks and the under-proliferation or lack of survival of heph mutant cells, neither of which are Notch gain-of-function phenotypes. Both of these defects may result from the disruption of heph targets required for cell survival. Strong decreases in cell survival in clones would mask the enhancement of proliferation provided by Notch activation. Cell death in clones in the wing margin could result in wing margin nicks through nonspecific disruption of wg expression at the DV boundary. This may explain the greater recovery of nicked wings resulting from clones induced prior to the formation of the DV boundary, which are able to occupy both the dorsal and ventral surfaces of the wing blade (Garcia-Bellido et al., 1973).

In addition to the heph wing phenotypes discussed above, we have observed the effects of heph mutations on other imaginal tissues, and clearly heph affects some but not all Notch dependent development. For example, heph clones cause ommatidial pattern defects, whereas we see little or no effect in the formation of leg joints or in the development of thoracic microcheatae (data not shown). This suggests that the Notch pathway requires modification to accommodate the diversity of processes it regulates and that, as in the case of modifiers such as numb and Suppressor of deltex, heph may be essential for only a limited subset of Notch signalling events (Busseau et al., 1994; Cornell et al., 1999; Fostier et al., 1998; Santolini et al., 2000). The specificity of heph phenotypes also argues against Notch activation in heph clones being due to the amplification of generic defects in signal transduction or transcription as a result of the requisite dosage sensitivity of Notch signalling. Indeed, the lateral inhibition signalling that regulates the spacing of adult thoracic microcheatae, perhaps the most dosage-sensitive Notch process (Heitzler and Simpson, 1991), is relatively unaffected by loss of heph activity (data not shown). Thus, although heph is unlikely to be dedicated to the Notch signalling pathway, it is most likely to play a specific role in the regulation of some Notch signalling events.

How does heph regulate Notch activity?

This study has linked together for the first time the PTB/hnRNPI RNA-binding proteins and the Notch signalling pathway. Given the strong sequence similarity shared between heph and vertebrate PTBs, it is probable that heph regulates the processing, stability or translation of a Notch pathway mRNA. However, the heph mosaic wing phenotypes most closely resemble the effects of low level ectopic Notch activation and cannot be easily correlated with an effect on any particular known element in the Notch pathway. The phenotypes of clones mutant for Delta and heph are most informative in explaining where heph acts in the Notch pathway. The epistasis of DI over heph in double mutant clones indicates that the Notch activation in heph clones depends on DI. This ligand dependency excludes the possibilities that Notch target genes are generally de-repressed, or that the Notch receptor is constitutively activated, in heph mutant cells. Rather, it suggests that in the absence of heph, existing Notch activity is amplified and/or maintained. Therefore, our favoured explanation is that heph is required to attenuate Notch activity after ligand-dependent activation.

The phenotypic consequences of heph are most prominent in the wing margin cells and wing vein cells. Both of these cell types require decreases in the levels of Notch activity during development and the heph phenotype results from persistent Notch activity in these cells. As described above, the wing margin cells lose Notch activation and wg expression during the refinement of wg and DI/Ser expression during the late
second and early third instar. During larval development, the cells that will ultimately give rise to the vein express low levels of Notch and Notch target genes such as E(spl)mβ (Cooper et al., 2000; de Celis et al., 1996a; Fehon et al., 1991; Koo et al., 1993), indicating that these cells have low levels of Notch activation prior to the repression of Notch transcription in pupal vein cells. Although it is not certain how these cells normally lose Notch activation, one possibility is that NICD stability is tightly regulated in order for cells to change Notch activation states and that heph+ may be required for cells to degrade NICD following ligand activation of the Notch receptor.

Several lines of evidence suggest that regulated degradation of NICD may be crucial. NICD includes a PEST domain, a characteristic of proteins with very short half-lives (Rogers et al., 1986), and mutations of Notch that delete the PEST domain are associated with N(gf) phenotypes (Ramain et al., 2001), albeit in different Notch signalling events than those affected by heph. The ubiquitin-proteasome pathway has been implicated in regulating the degradation of a NICD related protein in C. elegans, where the Notch family receptor LIN-12 is negatively regulated by sel-10 (Hubbard et al., 1997; Sundaram and Greenwald, 1993). The C. elegans and mammalian SEL-10 proteins both contain F-box motifs, and are thought to form part of a ubiquitin ligase complex that regulates NICD protein stability (Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001). In Drosophila, the gene most similar to sel-10, archipelago (ago), is required to destabilize Cyc1 E proteins (Moberg et al., 2001). However, available ago alleles do not affect the accumulation of NICD (Moberg et al., 2001). The expression of a dominant-negative proteasome subunit (Schweisguth, 1999) stabilizes NICD in the Drosophila wing disc, and wild-type levels of proteasome activity are required for alternative cell fate decisions during sense organ development. In addition, treatment of cells with chemical proteasome inhibitors increases the accumulation of nuclear NICD (Schweisguth, 1999). In this model, heph would regulate the mRNA for some element of the proteasome-dependent degradation of NICD.

The most intriguing possibility is that heph may negatively regulate the translation of E(spl)-C mRNAs. The E(spl) complex bHLH genes are transcribed in response to Notch signalling (Bailey and Posakony, 1995; Jennings et al., 1994; Lecourtois and Schweiguth, 1995) and this is counteracted by inhibition of translation by the 3'-UTR’s of E(spl)-C mRNAs (Lai et al., 1998; Lai and Posakony, 1997). This inhibition is presumably mediated through the binding of factors to conserved sequences found in most E(spl)-C mRNAs as well as in genes of the Bearded family, another group of Notch mediators (Lai et al., 2000; Lai et al., 1998; Lai and Posakony, 1997; Leviten et al., 1997). In this model, loss of heph function would increase the stability of E(spl)-C mRNAs, resulting in amplification of the effects of transcriptional activation by Notch signalling. Increased expression of E(spl)-C members has been demonstrated to inhibit wing vein differentiation (de Celis et al., 1996a), although the ectopic expression of individual E(spl)-C members has not been demonstrated to induce ectopic wing margin. However, it is possible that the stabilization of multiple E(spl)-C mRNAs could result in more dramatic effects on the wing margin. Furthermore, E(spl)-C members have different transcription patterns and may have divergent roles downstream of Notch (Cooper et al., 2000; de Celis et al., 1996a; Ligoxygakis et al., 1999; Nellesen et al., 1999). If heph were to regulate a subset of the E(spl)-C mRNAs, it would explain the limited requirement of heph in various Notch-mediated signalling events.

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