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# Regulation of *Drosophila* wing vein patterning: *net* encodes a bHLH protein repressing *rhomboid* and is repressed by rhomboid-dependent Egfr signalling

**Doris Brentrup[1,2], Hans-Philipp Lerch[1], Herbert Jäckle[2] and Markus Noll[1,*]**

[1]Institut für Molekularbiologie, Universität Zürich, CH-8057 Zürich, Switzerland
[2]Max-Planck-Institut für biophysikalische Chemie, Abteilung Molekulare Entwicklungsbiologie, D-37070 Göttingen, Germany
*Author for correspondence (e-mail: noll@molbio.unizh.ch)

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## SUMMARY

**The stereotyped pattern of veins in the *Drosophila* wing is generated in response to local EGF signalling. Mutations in the *rhomboid* (*rho*) gene, which encodes a sevenpass membrane protein required to enhance signalling transmitted by the EGF receptor (Egfr), inhibit vein development and disrupt the vein pattern. By contrast, *net* mutations produce ectopic veins in intervein regions. We have cloned the *net* gene and show that it encodes a basic HLH protein that probably acts as a transcriptional repressor. *net* and *rho* are expressed in mutually exclusive patterns during the development of the wing imaginal disc. Lack of *net* activity causes *rho* expression to expand, and vice versa. Furthermore, ectopic expression of *net* or *rho* results in their mutual repression and thus suppresses vein formation or generates tube-like wings composed of vein-like tissue. Egfr signalling and *net* exert mutually antagonising activities during the specification of vein versus intervein fate. While Egfr signalling represses *net* transcription, *net* exhibits a two-tiered control by repressing *rho* transcription and interfering with Egfr signalling downstream of Rho. Our results further suggest that *net* is required to maintain intervein development by restricting Egfr signalling, which promotes vein development, to the Net-free vein regions of the wing disc.**

Key words: *net*, bHLH protein, *rho*, Egfr signalling, Wing vein patterning, *Drosophila*

## INTRODUCTION

Wing primordia originate in the *Drosophila* embryo from cells straddling the parasegmental compartment boundary of the second thoracic segment, and are subdivided into anterior and posterior compartments by the *engrailed* (*en*) gene, which is active in the posterior compartment (Lawrence and Morata, 1976; Kornberg et al., 1985; Hidalgo, 1994). During larval stages, pattern formation along the anteroposterior axis of the wing disc is mediated by posterior hedgehog (Hh) signalling, which activates in adjacent anterior cells the *decapentaplegic (dpp)* gene whose secreted TGFβ-like product acts as morphogen for both compartments (Tabata and Kornberg, 1994; Tabata et al., 1995; Guillen et al., 1995; Lecuit et al., 1996; Nellen et al., 1996; Hidalgo, 1998). While this aspect of wing pattern formation has attracted considerable interest as a model system for the analysis of cell communication and the signalling pathways underlying pattern formation (Díaz-Benjumea and Cohen, 1993), comparatively little is known about the genes and mechanisms that are turned on by the molecular prepatterns in the wing disc and establish the differentiated structures within the wing blade.

Adult wing blades consist of two planar epithelial cell layers that originate from two distinct cell populations after a further subdivision of the wing disc into a dorsal and ventral compartment. Wings show a stereotyped pattern of five longitudinal and two transverse veins, separating distinct sectors of intervein regions. Genetic and molecular analysis has revealed that vein versus intervein development is initiated in larval wing discs by the activity of prepattern genes and refined by multiple tiers of intercellular signalling in prepupal discs and pupal wings (García-Bellido, 1977; García-Bellido and de Celis, 1992; Sturtevant et al., 1993, 1994, 1997; de Celis et al., 1995, 1996, 1997; Sturtevant and Bier, 1995; Yu et al., 1996; Milán et al., 1997; Biehs et al., 1998; Roch et al., 1998; Guichard et al., 1999). Vein and intervein cells differ in several aspects. Vein cells are small, densely packed, and covered by a thick, darkly pigmented cuticle, whereas intervein cells are large, flat, and covered by a thin, transparent and lightly pigmented cuticle. In contrast to dorsal and ventral vein cells, which form tubular structures filled with haemolymph, intervein cells on opposite sides of the wing are tightly connected through a dense extracellular matrix. Finally, vein cells remain alive throughout adulthood, whereas intervein cells die upon eclosion of the fly.

Because of the stereotyped wing pattern, subtle wing defects can be easily identified and therefore many mutants affecting the architecture or pattern of the wing have been isolated. One

of these mutants, *net*, discovered by Bridges in 1931 (Lindsley and Zimm, 1992), displays ectopic wing veins and often blisters on the wing surface, which indicates that the cell layers of opposing wing surfaces are not properly connected in the intervein regions (compare Fig. 4A with 4B). *net* was shown to interact genetically with *Notch* (*N*) (García-Bellido and de Celis, 1992). *N* belongs to the class of neurogenic genes and encodes a transmembrane receptor that, like all products of neurogenic genes, serves to single out a cell from a group of equivalent cells by a signalling process termed lateral inhibition (Artavanis-Tsakonas et al., 1983, 1995; Campos-Ortega, 1997). In the wing, N signalling is necessary to restrict vein cell development from seven- to eight-cell wide domains of vein-competent provein cells to rows of two to three cells in width (García-Bellido and de Celis, 1992; Sturtevant and Bier, 1995). This process depends also on the *rhomboid* (*rho*) gene (de Celis et al., 1997), which encodes a sevenpass membrane protein (Bier et al., 1990) necessary to promote the extracellular activation of Egfr, e.g. by the EGF-related ligand Vein (Vn) (Schnepp et al., 1996; Simcox et al., 1996; Simcox, 1997), in a process that further depends on the transmembrane protein Star (S) (Sturtevant et al., 1993; Guichard et al., 1999).

The *rho* gene is initially expressed in a series of discrete stripes corresponding to the longitudinal vein primordia of third instar wing discs and is subsequently maintained in differentiating vein cells throughout pupal development (Sturtevant et al., 1993; Sturtevant and Bier, 1995). Since all other known members of the Egfr signalling pathway are expressed uniformly, localized *rho* activity is thought to provide the decisive clue for vein formation and to maintain vein cell fate. This conclusion is based on several observations: (1) absence of *rho* activity causes loss of veins, whereas ectopic *rho* activity results in the formation of ectopic veins in intervein regions (Sturtevant et al., 1993; Noll et al., 1994; Sturtevant and Bier, 1995); (2) in pupal wing discs of *N* mutants, *rho* expression expands into the intervein regions and causes a broadening of the veins (Sturtevant and Bier, 1995; de Celis et al., 1997); and (3) in $N^{ts}$; $net^{1}$ double mutant wing discs, *rho* expression covers entire intervein regions (Sturtevant et al., 1997). These results further demonstrate that N signalling restricts *rho* expression and thus Egfr signalling to cells initiating vein development and suggest that *net* activity is required to inhibit vein-promoting *rho* activity in intervein regions (de Celis et al., 1997).

Here we show that *net* encodes a novel basic helix-loop-helix (bHLH) protein that may act as a transcriptional repressor. In wing discs, *net* is expressed in intervein regions, in a pattern complementary to that of *rho*. Ectopic expression of *rho* completely represses *net* transcription, whereas *net* expression expands into primordial vein regions in the absence of *rho* activity. Conversely, ectopic *net* expression represses *rho* transcription in vein primodia, whereas *rho* expression expands into intervein regions in *net* deficient wing discs. Thus, the transcription patterns of the two genes are complementary and their activities are mutually exclusive. Our results suggest that *net* is repressed by Rho-dependent Egfr signalling while its product in turn functions as negative regulator of *rho* and the Egfr signalling pathway. Our results imply that Net prevents vein development by the inhibition of Egfr signalling in intervein regions and thereby maintains intervein development.

## MATERIALS AND METHODS

### General procedures

Standard procedures, such as isolation and Southern blot analysis of genomic DNA, construction and screening of genomic libraries, chromosomal walking, isolation and northern blot analysis of poly(A)$^+$ RNA and PCR, were carried out essentially as described (Frei et al., 1985; Kilchherr et al., 1986; Sambrook et al., 1989; Fu and Noll, 1997).

### Isolation of genomic clones

To clone the *net* locus, a chromosomal walk was initiated from the genomic insert BPal-1 of a λ phage, which included the distal breakpoint of *Df(2L)al* (Schneitz et al., 1993), and extended for about 240 kb until it overlapped with genomic clones harbouring the *lethal(2)giant larvae* locus at the left telomere of the second chromosome (kindly provided by B. Mechler). The distal breakpoint of *Df(2L)al* is thus located about 300 kb from the telomere. To isolate the breakpoints of an inversion uncovering *net* and *u-shaped* (*ush*), a genomic library of the corresponding mutant stock *In(2L)TE99(Z)XR93/CyO* (generously provided by Pascal Heitzler) was prepared in λ DASH II and screened with the most proximal *Eco*RI fragment of Y38-4 (Fig. 1A). A breakpoint clone, XR93-11, which included the distal exon and 3′ downstream region of *net*, about 8 kb of TE99, and approximately 1 kb of the 5′ promoter region of the *ush* locus (Cubadda et al., 1997), and a wild-type clone of *net* from the *CyO* balancer chromosome, XR93-8 (Fig. 1A), were isolated.

### Mapping of *net* rearrangements

The breakpoint of the inversion *In(2L)TE99(Z)XR93* was mapped to the *net* locus by Southern blot analysis, and a clone that included the proximal breakpoint was isolated form a genomic library (s. above). In addition, the position of the breakpoint was determined precisely by DNA sequencing. The breakpoints of the terminal deficiencies *Df(2L)PM4*, *Df(2L)net*$^{18}$, and *Df(2L)U314* were mapped by whole-genome Southern analysis. The deviations from wild type in $net^{K1}$ and $net^{1}$ were mapped to the 0.58kb *Sal*I-*Hin*dIII fragment and that of $net^{K7}$ to the 2.0 kb *Hin*dIII fragment (Figs 1B and 2A) by Southern blot analysis. The precise insertion sites of $net^{K1}$, $net^{1}$, and $net^{PM50}$ were determined by nested PCR with the genomic DNA of these mutants and subsequent DNA sequencing.

### Isolation of cDNA clones and 5′ RACE

Nine *net*-cDNA clones were isolated from $7.5\times10^5$ phages of a 4-8 hour embryonic cDNA library in λ UNI-ZAP XR (Schneitz et al., 1993) by screening it with a 10.3 kb *Eco*RI fragment from XR93-8. To clone a cDNA that includes the transcriptional start site of the *net* transcript, the 5′ RACE technique was applied to poly(A)$^+$ RNA from 4-8 hour old embryos and from third instar larvae, using the 5′ Amplifinder RACE kit and following the instructions of Clontech. The RACE products of RNAs from both stages extended the longest *net*-cDNA, netce2, by the same length of 19 base pairs (Fig. 2A). DNA sequencing and comparison with the wild-type genomic DNA sequence revealed that the netce1 cDNA had a frame shift mutation that was due to the insertion of an additional A into the string of seven As at positions 941-947 (Fig. 2A). Hence the 0.58 kb *Sal*I-*Hin*dIII fragment was exchanged in netce1 by the corresponding genomic fragment from XR93-8 to generate the corrected *net*-cDNA clone, netce1-ΔA.

### DNA sequencing and homology searches

DNA sequencing was carried out on both strands of the *net*-cDNA netce1 (Fig. 2A) and the corresponding genomic DNA, including both intron-exon boundaries and about 2.2 kb of the intron, with a DNA sequencer model 373A using dye terminators (Applied Biosystems). Searches of the data bases for similarities were performed with the

WWW-based BLAST program provided by the National Center for Biotechnology Information (NCBI) of the NIH (Bethesda, MD; Altschul et al., 1990) and enhanced with BEAUTY (Baylor College of Medicine, Houston, TX; Worley et al., 1995). Sequence alignments were made using the Gelassemble and Pileup modules of the Genetics Computer Group Wisconsin Package (GCG).

### In situ hybridization to wing discs and pupal wings

In situ hybridization to imaginal discs and pupal wings with DIG-labeled probes was carried out essentially as described (Tautz and Pfeifle, 1989), using a DIG DNA-labelling and detection kit (Boehringer) and following the manufacturer's application manual. Prepupae and pupae were staged. The outer case of prepupae was cut open, and prepupae were fixed in 4% formaldehyde for at least 60 minutes at room temperature or at 4°C overnight. For the preparation of pupal wings, the brown case and the transparent pupal cuticle surrounding the wings were removed, and the pupal wings were pulled out and fixed in 4% formaldehyde (or 4% paraformaldehyde for RNA probes) for 1 hour at room temperature or at 4°C overnight. DIG-labeled probes for *net* RNA consisted of the entire 2.2 kb insert of netce1-ΔA and for *rho* RNA of a 2.5 kb *Eco*RI fragment of a *rho*-cDNA (a gift from E. Bier). DIG-labeled antisense RNA probes were produced as run-off-transcripts for *net* from the netce1-ΔA template cleaved by *Eco*RI and for *rho* from the 2.5 kb *rho*-cDNA clone digested with *Hin*dIII. No signal was detectable in wing discs or pupal wings with a sense RNA probe for *net*. A *knirps* (*kni*) RNA-labeled probe was kindly supplied by C. Wolf.

### Rescue of *net* mutants

To rescue the net phenotype, the 10.3 kb *Eco*RI fragment of XR93-8 was cloned into the *Eco*RI site of the P-element vector pW6, which carries the miniwhite gene as marker (Klemenz et al., 1987), to produce the construct RI (Fig. 1B). In addition, RIIa and RIIb were constructed by cloning the 3.3 kb *Nhe*I-*Xho*I and 3.9 kb *Nhe*I-*Sal*I fragments of phage Y38-4, respectively, with the 8.8 kb *Eco*RI-*Nhe*I fragment of XR93-8 (Fig. 1B) into the *Eco*RI/*Xho*I cleaved polylinker of pW6. Each construct was injected together with pUChspΔ2-3 P-element helper plasmid (D. Rio, unpublished), carrying the transposase gene, into 30-60 min old $w^{1118}$ or *y w* embryos and $w^+$ transformants were selected (Rubin and Spradling, 1982). Transformed lines whose *net* transgene was not located on the second chromosome were crossed to $w^{1118}$; $net^1$ flies, their progeny crossed inter se, and the $w^+$ F2 progeny screened for the absence of the net phenotype.

### Construction and activation of *UAS-net*

The *UAS-net* transgene was constructed by cloning the *Eco*RI-*Xho*I insert of netce1-ΔA into the P-element vector pUAST (Brand and Perrimon, 1993), and transformed lines carrying the *UAS-net* transgene were obtained as described above. The lines 4U16-3.5, 8U14-7.1, and 9U5-1 were crossed to the GAL4-enhancer trap lines MS1096, MS209, C765, 10 (gifts from K. Basler), hGAL4, enGAL4, ptcGAL4, dppGAL4, 69BGAL4 and 71BGAL4 (all obtained from the *Drosophila* stock center in Bloomington). The progeny was tested for ectopic *net* expression with DIG-labeled *net*-cDNA probes. In addition, the GAL4-lines were crossed to *UAS-lacZ* (a gift from K. Basler) and histochemically stained for β-galactosidase activity (Boehringer) or with an anti-β-galactosidase antibody (Cappel). All these GAL4 lines strongly drive expression of *net* in the wing disc as well as in other tissues. Since MS1096 produced the strongest vein

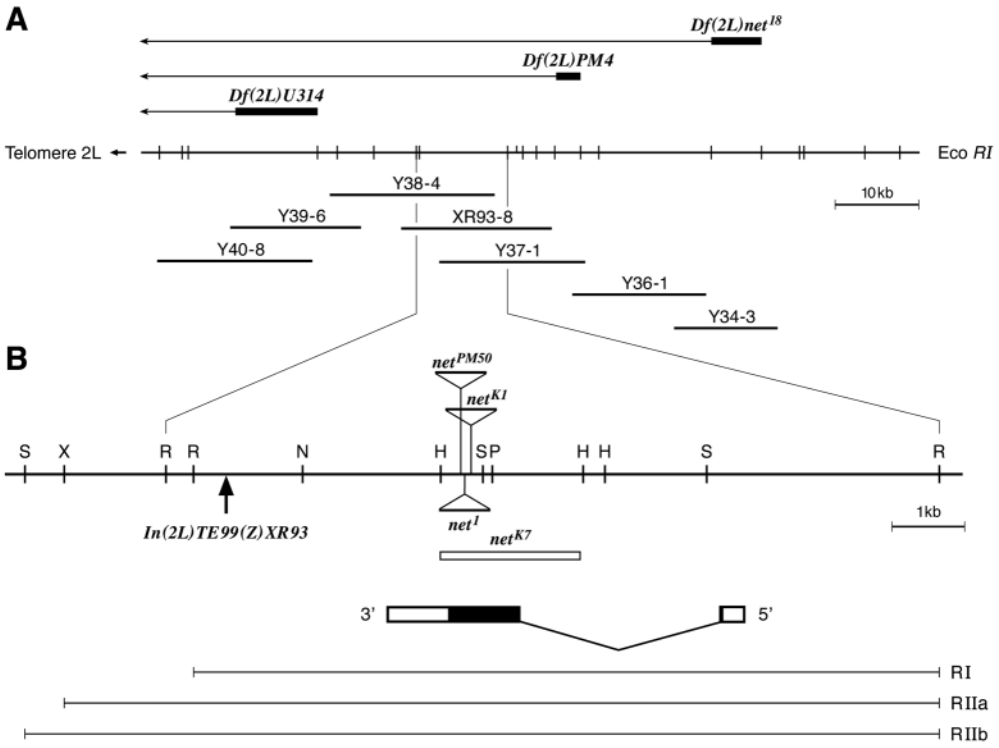


**Fig. 1.** Isolation and molecular characterization of the *net* locus. (A) Chromosomal walk covering the *net* locus. Three telomeric deficiencies *Df(2L)U314*, *Df(2L)PM4*, and *Df(2L)net*$^{18}$ are mapped (filled boxes delimit the regions including the proximal breakpoints) with respect to a genomic *Eco*RI map (two *Eco*RI fragments whose order has not been determined are separated by a short vertical line) of the chromosomal region between 21A2 and 21B1. Below the *Eco*RI map, the distal portion of a 240 kb chromosomal walk (Y34-3 to Y40-8 are inserts isolated from a *Kr*$^{SB1}$*/CyO* library in EMBL4; XR93-8 is a wild-type DNA insert isolated from a *In(2L)TE99(Z)XR93/CyO* library in λ DASH II) is shown, which overlaps at its proximal end with a chromosomal walk including the region uncovered by *Df(2L)al* (Schneitz et al., 1993) and at its distal end with a 40 kb chromosomal walk including *l(2)gl* and the left telomere (Mechler et al., 1985). (B) Transcriptional organization of the *net* gene and map of mutations disrupting *net*. The locations of the B104 insertion *net*$^{K1}$, the 8 kb insertion *net*$^{K7}$ (mapped to the 2.0 kb *Hin*dIII fragment), *net*$^{PM50}$, as well as of the small *net*$^1$ duplication and of the distal breakpoint of the inversion *In(2L)TE99(Z)XR93* uncovering *net* and *ush* (personal communication and kind gift from P. Heitzler), are shown with respect to an enlarged restriction map of the *net* gene (as defined by the three fragments indicated at the bottom, RI, RIIa and RIIb, that were tested as transgenes for rescue of the *net* mutant phenotype) and its intron/exon structure below (open reading frame in black). The *net*$^{PM50}$ allele is a lost P-element insertion that was kindly provided by C. Caggese. H (*Hin*dIII), N (*Nhe*I), P(*Pst*I), R (*Eco*RI), S (*Sal*I), X (*Xho*I).

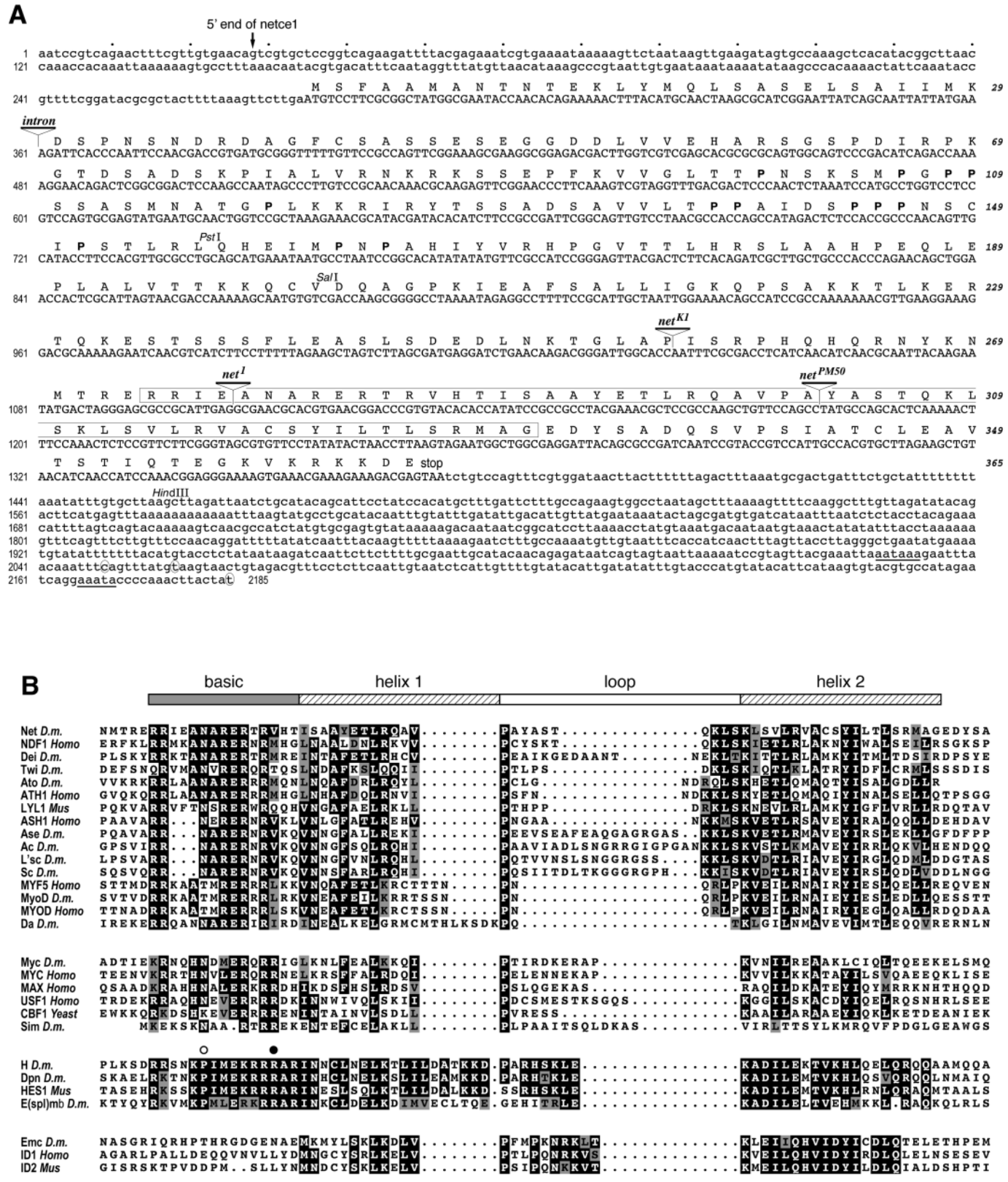
A
5' end of netce1
intron
PstI
SalI
netKI
net1
netPM50
HindIII
stop
B
basic
helix 1
loop
helix 2
Net D.m.
NDF1 Homo
Dei D.m.
Twi D.m.
Ato D.m.
ATH1 Homo
LYL1 Mus
ASH1 Homo
Ase D.m.
Ac D.m.
L'sc D.m.
Sc D.m.
MYF5 Homo
MyoD D.m.
MYOD Homo
Da D.m.
Myc D.m.
MYC Homo
MAX Homo
USF1 Homo
CBF1 Yeast
Sim D.m.
H D.m.
Dpn D.m.
HES1 Mus
E(spl)mb D.m.
Emc D.m.
ID1 Homo
ID2 Mus

**Fig. 2**

**Fig. 2.** cDNA and protein sequence of the *net* gene and sequence comparison of the Net bHLH domain with those of other (b)HLH transcription factors. (A) Sequence of *net* exons and Net protein. The nucleotide and putative amino acid sequences derived from netce1, 5′ RACE, and genomic DNA sequences are shown. The bHLH domain of the Net protein is boxed and prolines of the Pro-rich region (amino acids 100 to 164) are shown in bold letters. The positions of the 2.75 kb intron, the B104 $net^{K1}$ insertion (the preceding 5 bp are the duplicated target site), the 244 bp $net^{1}$ duplication (the preceding 244 bp are duplicated), and the P-element insertion $net^{PM50}$ (the preceding 10 bp were duplicated in this lost allele) are indicated by vertical lines below triangles. The 5′ end of the sequenced *net*-cDNA, netce1, is marked by a vertical arrow. The last nucleotides of two cDNAs whose transcript was cleaved after the underlined canonical poly(A) addition signal AATAAA are encircled, while the third encircled poly(A) addition site, found in all other cDNAs, occurs after a putative noncanonical AAATA signal (also underlined). Restriction sites shown in Fig. 1B are indicated. The GenBank Accession number for the nucleotide and amino acid sequence of the *net* gene and its mutant alleles is AF303350. (B) Net is a bHLH protein of class A. The Net bHLH domain is aligned with those of class A (NDF1 to Da), class B (Myc to Sim), and class C bHLH proteins (H to E(spl)mβ), and with those of non-basic HLH proteins (Emc to ID2). These four classes have been defined by Ohsako et al. (1994). Class A proteins, which include the *ASC* products (Campuzano et al., 1985), bind to CA(C/G)CTG target sites, act as transcriptional activators, and lack an arginine in position 13 of the basic region, which is present in class B and C proteins (marked by a black circle). Class B proteins bind to CA(C/T)GTG target sites, but do not bind to class A sites, a property that is based on the specific interaction of an arginine residue at position 13 in the basic region of class B bHLH domains with the G4 nucleotide of class B DNA binding sites (Ferre-D'Amare et al., 1993). Class C (or proline-bHLH) proteins, which have a proline residue at position 6 of their basic region (indicated by an open circle), act as transcriptional repressors and include proteins encoded by *hairy* (*h*) (Ohsako et al., 1994; Barolo and Levine, 1997) and the *Enhancer of split* complex (Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992) as well as their mammalian homologue HES (Ishibashi et al., 1994). Class C proteins also have an arginine at position 13 of their bHLH domain and bind to both class B and class C (CACG(C/A)G) sites, whereas class B proteins bind only weakly to class C sites. Class C proteins further include a C-terminal WRPW motif known to interact with the co-repressor groucho. Members of the last class lack a basic region and hence cannot bind to DNA by themselves, but are able to form heterodimers with class A or class B bHLH proteins and hence act as dominant-negative repressors by dimerization with bHLH activator proteins. Proteins have been aligned to maximize number of amino acid identities (black background) and homologies (grey background) that have been conserved within a given class.

suppression in wings when combined with a *UAS-net*, only results obtained with this driver line are reported. Combination of a rhoGAL4 line (gift from C. Klämbt) with *UAS-net* caused embryonic lethality and hence could not be tested for vein suppression.

### Mounting of fly wings

Wings were removed from flies stored in 70% ethanol, transferred directly into a drop of Advantage Permanent Mounting Media (Innovex), covered with a cover slip and photographed under bright-field optics through a compound microscope.

### Drosophila strains

*Df(2L)*$net^{18}$*/CyO*, *Df(2L)U314/CyO*, $net^{K7}$*/CyO*, and $net^{K1}$ stocks were kindly provided by B. Mechler. *Df(2L)PM4/CyO* and $net^{PM50}$ stocks, and the *In(2L)TE99(Z)XR93/CyO* stock were generous gifts from C. Caggese and P. Heitzler. The *UAS-rho* line was kindly provided by C. Klämbt; GAL4-enhancer trap lines MS1096, MS209, C765, 10 and the *UAS-lacZ* line were supplied by K. Basler; and *UAS-kni* and *UAS-knrl* by R. Schuh; $px^{72}$ by S. Hayashi. hGAL4, enGAL4, ptcGal4, dppGAL4, 69BGAL4, 71BGAL4 and *UAS-Dras1*$^{V12}$; $net^{1}$, $px^{1}$, *ri*, $w^{1118}$ and *y w* stocks, as well as the enhancer trap lines $bs^{P1292}$ (synonym: $bs^{l(2)03267}$) and $bs^{l(2)k07909}$ were obtained from the Bloomington and Umea stock centres.

## RESULTS

### Molecular identification and characterization of the *net* locus

Cloning of the *net* gene was initiated by positional cloning. We started a chromosomal walk from the distal breakpoint of the deficiency *Df(2L)al* (Schneitz et al., 1993), which complements *net*. The walk was extended distally by screening genomic λ phage libraries for overlapping inserts until the *lethal(2) giant larvae* locus at the left telomere of the second chromosome was reached (Mechler et al., 1985). To delimit the *net* gene within the cloned genomic DNA, we took advantage of chromosomal aberrations including terminal deficiencies that either complement or uncover *net*. As illustrated in Fig. 1A, the proximal breakpoint of the deficiency *Df(2L)U314*, which complements *net*, is located distal to the proximal breakpoints of the larger deficiencies *Df(2L)PM4* and *Df(2L)*$net^{18}$, both of which uncover *net*. Hence, at least part of the *net* gene is included in the 30-40 kb region between the proximal breakpoints of *Df(2L)U314* and *Df(2L)PM4*. To identify *net* gene sequences, we analysed this region in the spontaneous mutants $net^{1}$, $net^{K1}$, $net^{K7}$ by Southern blot analysis and cloned the distal breakpoint of the X-ray induced inversion *In(2L)TE99(Z)XR93*, previously shown to uncover *ush* and *net* (P. Heitzler, personal communication).

Molecular lesions associated with the mutant alleles $net^{K1}$ and $net^{1}$ affected the same 0.58 kb *Sal*I-*Hin*dIII DNA fragment (Fig. 1B) and suggest that it includes functional sequences of the *net* gene. Using a DNA probe overlapping with this fragment, we isolated nine cDNAs derived from a single 2.2 kb mRNA expressed in embryos, larvae and pupae. To confirm that this transcript is derived from the *net* gene, we attempted to rescue *net* mutants to a wild-type phenotype by P-element-mediated germline transformation. The first rescue construct, RI, which extended about 0.4 kb beyond the *net* inversion breakpoint of *In(2L)TE99(Z)XR93* in the downstream region of the 2.2 kb transcript (Fig. 1B), showed no rescue of the *net* wing phenotype. A rescue construct including additional downstream sequences, RIIa, rescued about 45% or 80% of the flies to a wild-type phenotype when present as one or two copies. Finally, the *net* wing phenotype was rescued in all flies harbouring a single transgene, RIIb, which included an additional 0.54 kb of downstream sequences (Fig. 1B). These results suggest that the 2.2 kb mRNA encodes the Net protein and that downstream sequences of the gene are necessary to provide proper *net* activity in wing discs.

### The *net* gene encodes a novel bHLH protein

Sequence analysis of the *net*-cDNAs and mapping the transcriptional start site of *net* by 5′ RACE showed that the longest cDNA is nearly full-length, missing only 19 bp at its

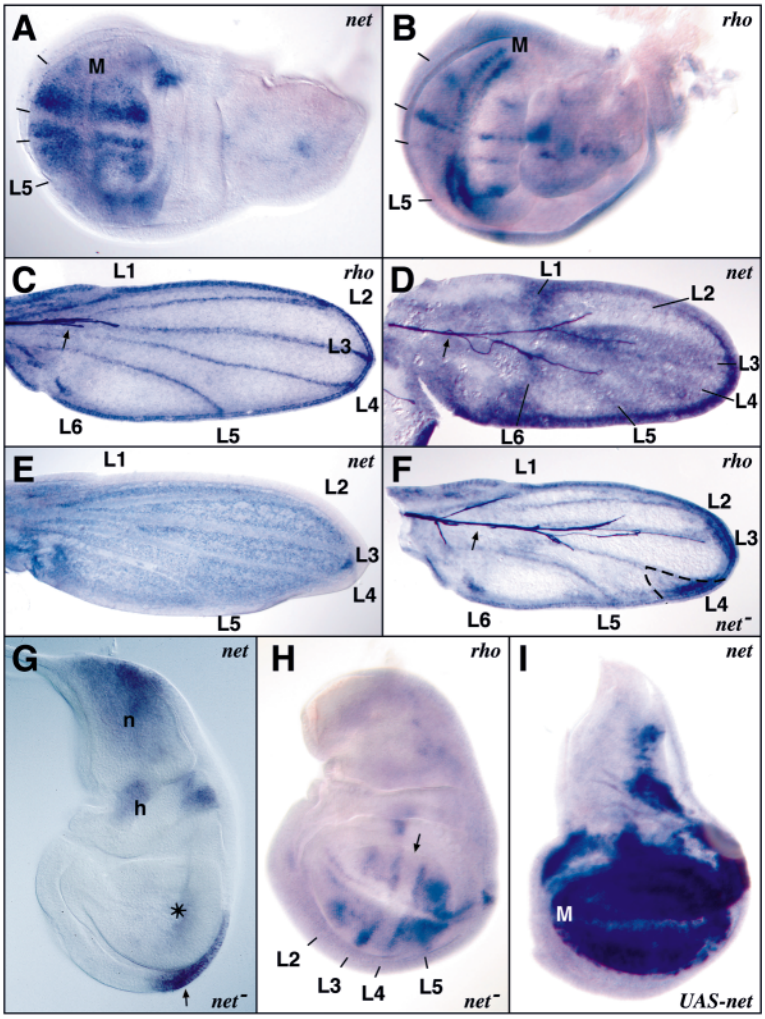


**Fig. 3.** Expression of *net* and *rho* mRNA in late third instar wing discs and pupal wings of wild-type and *net*$^{-}$ mutants. (A-E) Expression patterns of *net* and *rho* in developing wild-type wings. Expression patterns of *net* (A,D,E) and *rho* (B,C) transcripts were analysed by in situ hybridization of DIG-labeled probes to late third instar wing discs (A,B) and staged pupal wings (C-E). *net* transcripts appear in intervein regions of the wing pouch (A) and are excluded from vein primordia (B) where *rho* transcripts accumulate in wing discs (the wing margin (M) and the positions of L2 to L5 are indicated). Expression of *net* along the wing margin (A) is flanked on both sides by *rho* expression (B). Note that absence of *net* RNA from L2 is unclear only because L2 is out of focus in (A). Pupal wings at 28-30 hours APF (at 22°C) have refined their *rho* expression from proveins to the narrow veins (C) shortly before expression in crossveins appears. At an earlier time (24-28 hours APF at 22°C), pupal wings express *net* along the boundary regions between veins and interveins (most prominently along L1 and L3-L6, more weakly along L2) and along the wing margin, but *net* is repressed in proveins and the wing margin (D). At a slightly later time (28-32 hours APF at 22°C), *net* continues to be expressed weakly in all intervein regions, but remains repressed in all proveins (E). (F-H) *net* (G) and *rho* (F,H) expression patterns in late third instar wing discs (G,H) and pupal wings (F) of *In(2L)TE99(Z)XR93/Df(2L)net*$^{18}$ animals. Note that no expression of *net* is detectable in the entire wing pouch of these animals (G, label marked by asterisk is located in ventral pleura and hinge region below the plane of focus), but *net* is expressed ectopically in notum (n), dorsal hinge (h), and ventral pleura and hinge regions (arrow and asterisk). Pupal wing blades of these *net* mutants also completely lack staining for the DIG-labelled *net* probe (not shown). *rho* expression expands into intervein regions of wing discs (H), with the exception of that between L3 and L4 (arrow). In pupal wings (26-30 hours APF at 22°C), *rho* expression is confined to veins in proximal and medial regions, but still expands into intervein regions in the distal parts of the wing except between L3 and L4 (e.g. into a region delimited by the broken line in F). (I) Endogenous and ectopic expression of *net* under the control of *MS1096* in late third instar *MS1096*/+; *UAS-net*/+ wing disc. Note that *net* is ubiquitously expressed in the wing pouch with the exception of the wing margin (M). The same pattern is observed for *rho* transcripts, when *rho* is expressed under the control of *MS1096*. Arrows in C,D,F mark tracheae. In C-F,H, the position of longitudinal veins L1 to L6 are indicated. Wing discs are shown with their ventral side to the left and anterior upwards (A,B) or with their dorsal side upwards and anterior to the left (G-I). Pupal wings are shown with anterior upwards and proximal to the left.

5′ end, and that *net* codes for a single 2.185 kb mRNA (Fig. 2A). Sequencing the corresponding genomic DNA revealed that the *net* transcript consists of two exons that are separated by a 2.75 kb intron and include one large open reading frame of 1095 bp. All isolated *net*-cDNAs terminate at one of three different positions within the 3′ untranslated region of the transcript and include a short polyA track at their 3′ ends. As most of them end at the same position shortly after a putative polyA addition signal that deviates from the canonical sequence, it appears that this signal is functional (Fig. 2A).

The open reading frame encodes a polypeptide of 365 amino acids including a basic helix-loop-helix (bHLH) domain, which suggests that the Net protein is a DNA-binding transcription factor. The large superfamily of bHLH transcriptional regulators has been subdivided into three different classes (Ohsako et al., 1994). Members that belong to different classes bind to similar but distinct DNA recognition sites, thought to reflect characteristic differences in amino acids at specific positions of their bHLH domains. The Net protein is most closely related to bHLH proteins of class A, encoded by proneural genes like *atonal* (*ato*) and those of the *Achaete-scute Complex* (*ASC*) (Fig. 2B). However, in contrast to class A proteins, which act as activators, Net contains a proline-rich domain in its N-terminal moiety (20% Pro between amino acids 100 and 164; Fig. 2A), which is characteristic of several transcriptional repressors (Cowell, 1994). Diagnostic domains or motifs other than the bHLH and proline-rich domains have not been found.

For a more detailed analysis of the molecular lesions in mutant *net* alleles, we amplified genomic DNA from homozygous *net*$^{K1}$ and *net*$^{1}$ flies by PCR. Sequencing revealed that *net*$^{K1}$ was an insertion, associated with a 5 bp target site duplication, of the B104 transposable element at position 1041 of the spliced *net* transcript, whereas *net*$^{1}$ was a small duplication after nucleotide 1105 of the preceding 244 bp (Fig. 2A). The B104 insertion interrupted the *net* open reading frame

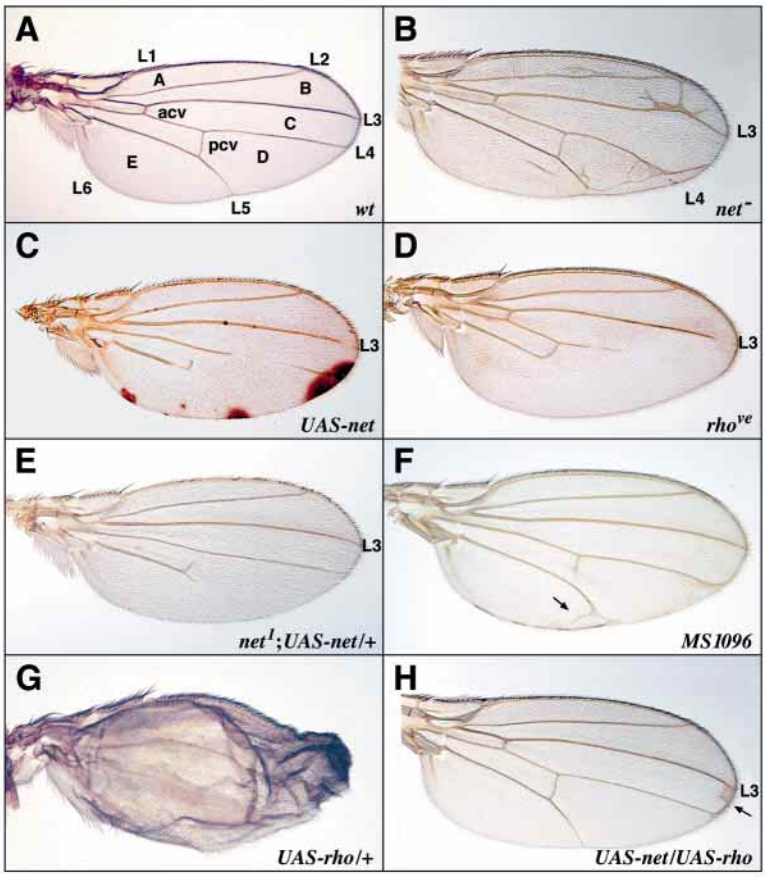


**Fig. 4.** Wing phenotypes of *net* gain-of-function and *rho* loss-of-function mutants are similar, and the dramatic wing phenotype caused by ubiquitous *rho* activity is suppressed by ubiquitous co-expression of *net*. (A) Wild-type wing with designations of longitudinal veins (L1-L6), anterior (acv) and posterior crossveins (pcv), and intervein sectors (A-E). (B) Wing of a *net* null mutant, *In(2L)TE99(Z)XR93/Df(2L)net*$^{18}$, in which ectopic veins and plexi are most prominent in distal portions of sectors B and D and slightly less abundant in sectors A and E. This phenotype is indistinguishable from that of homozygous *net*$^{1}$ and *net*$^{K1}$ mutants. (C,D) Wing of a *MS1096*/Y; *UAS-net/UAS-net* male (C) is compared to that of a *rho*$^{ve}$ mutant (D). Two doses of the *UAS-net* gain-of-function transgene, expressed ectopically under the control of *MS1096*, suppress the distal portions of L3-L5 and produce a wing phenotype very similar to that of the *rho* loss-of-function mutant *rho*$^{ve}$. (E,F) Wing of a *MS1096*/+; *net*$^{1}$; *UAS-net/+* female (E) is compared to that of the GAL4 driver line *MS1096/MS1096* (F). One dose of the *net* gain-of-function transgene still suppresses the distal part of L5, is able to completely suppress the *net* null phenotype of *net*$^{1}$ and the weak vein phenotype of the MS1096 driver line (except its loss of the anterior crossvein), which lacks the anterior crossvein, affects the posterior crossvein, and displays few ectopic veins (arrow in F; Milán et al., 1998), but hardly suppresses the distal half of L4 as observed for two doses of the *UAS-net* transgene (C). (G,H) Wings of *MS1096*/+; *UAS-rho/+* (G) and of *MS1096*/+; *UAS-net/UAS-rho* mutants (H) are compared. The dramatic wing phenotype resulting from the ubiquitous expression of *UAS-rho* under the control of *MS1096*, which transforms the entire wing blade into vein-like tissue of dorsal and ventral epithelia that fail to adhere (G), is suppressed to give rise to a nearly wild-type wing phenotype (H; only deltas between L3 and L4 (arrow) remain), when *UAS-net* is co-expressed with *UAS-rho* during wing development. All wings are oriented with anterior upwards and proximal to the left.

by several inframe stop codons and thus produced a Net protein truncated just before its bHLH domain (Fig. 2A). The 244 bp *net*$^{1}$ duplication also generated several inframe stop codons, truncating Net in the basic region of the bHLH domain (Fig. 2A). Assuming that bHLH-dependent DNA binding is essential for Net function, we conclude that both *net*$^{K1}$ and *net*$^{1}$ are lack-of-function alleles. These findings and the complete rescue by the RIIb transgene of the net mutant phenotype demonstrate unequivocally that the 2.2 kb transcript encodes the Net functions.

### *net* expression in intervein regions of the developing wing disc and pupal wing

Northern blot analysis showed that the 2.2 kb *net* transcript was expressed during all stages of the *Drosophila* life cycle (not shown). In situ hybridization of a *net* cDNA probe to whole-mount embryos (not shown) and imaginal discs revealed complex expression patterns. As, during development, the putative *net* lack-of-function alleles cause discernible defects only in the wing, the specific expression of *net* in the embryo may serve a redundant function. Therefore, the *net* function was analysed only during wing development.

In wing discs, *net* expression was first observed in early third instar larvae. *net* transcripts were confined throughout the third instar to prospective intervein sectors and to a narrow region straddling the wing margin (Fig. 3A), but were excluded from primordial wing veins expressing *rho* (Fig. 3B; Sturtevant et al., 1993). The complementary expression patterns of *net* and *rho* were maintained until the pupal wings had developed and expression of *rho* was being restricted from seven- to eight-cell wide stripes of vein-competent provein cells to the narrow two to three cell wide stripes of future vein cells (Fig. 3C; Sturtevant et al., 1994) in P1 pupae (Yu et al., 1996). Shortly before this P1 pupal stage, *net* expression was enhanced along the wing margin and in regions flanking proveins (Fig. 3D). During P1 and the subsequent P2 pupal stage, when crossveins are recognisable, *net* was expressed in the intervein regions, but continued to be repressed in the broad provein stripes (Fig. 3E). After 30 hours APF (after puparium formation) at 22°C, *net* remained repressed in proveins while its expression in intervein regions was considerably reduced (not shown). Thus, *net* and *rho* expression appeared to be mutually exclusive during the entire development of the wing although *net* expression did not expand when *rho* expression was reduced to the narrow stripes of vein cells in P1 pupae.

### *net* activity represses *rho* transcription

In homozygous *net*$^{1}$ wing discs, *rho* is ectopically expressed in broad domains, alternating with areas devoid of *rho* expression (Sturtevant and Bier, 1995; Sturtevant et al., 1997). To determine if this pattern of *rho* expression in wing discs reflects the null phenotype of *net* mutants, we examined *rho* expression in *In(2L)TE99(Z)XR93/Df(2L)net*$^{18}$ wing discs, which fail to express *net* in the wing pouch (Fig. 3G). These

discs displayed the same *rho* expression during larval and pupal stages as described for $net^1$ mutants, i.e. *rho* expanded into all intervein regions with the exception of the sector between veins L3 and L4, but the effect was most pronounced in the distal intervein regions B (between L2 and L3) and D (between L4 and L5) (Fig. 3F,H). Hence, it appears that *net* represses *rho* in intervein regions of the wild-type wing, except in the region between L3 and L4. These effects of *net* mutations on *rho* expression are consistent with the null phenotype in the wing blade of $In(2L)TE99(Z)XR93/Df(2L)net^{18}$ flies, which show ectopic veins most frequently in the distal intervein sectors B and D (Fig. 4B). This *net* null phenotype is indistinguishable from that of $net^1$ and $net^{K1}$ mutants (not shown), which confirms our assumption that the bHLH domain of Net is indispensable for its function.

To test if Net acts as a repressor of *rho* transcription, we expressed *net* ectopically in wing discs by means of the GAL4/UAS system (Brand and Perrimon, 1993). When activated by the GAL4 driver line MS1096, a GAL4 P-element insertion at the *Beadex* locus (Rørth, 1996; Milán et al., 1998), *UAS-net* was expressed nearly ubiquitously in the wing pouch (Fig. 3I) and widely repressed *rho* transcription (Fig. 5A), which displays a pattern similar to that in homozygous $rho^{ve}$ discs (Sturtevant et al., 1993; D. B., H.-P. L., H. J. and M. N., unpublished). Consistent with this effect on *rho* expression, the wing phenotype of *MS1096/Y*; *UAS-net* flies was almost identical to that of homozygous $rho^{ve}$ flies (Fig. 4D): the most distal part of L3 and the distal half of L5 were missing, while the distal part of L4 was also absent or interrupted by gaps (Fig. 4C). In contrast, L2 was wild type (Fig. 4C) except in occasional flies, in which its most distal part was missing, although *rho* expression was clearly absent from L2 proveins (Fig. 5A). In addition, wings in which *net* was ectopically expressed during development were smaller (Fig. 4C). Interestingly, when the dosage of *MS1096* and *UAS-net* was reduced to one copy in $net^1$ females, the $net^1$ phenotype was rescued (Fig. 4E). The suppression of distal L5 formation was independent of $net^1$ (not shown), while L4 was no longer suppressed at the reduced dosage. The absence of the anterior crossvein (Fig. 4C,E) was not caused by the ectopic *net* expression, but induced by the *MS1096* driver in the absence of its *UAS-net* target (Fig. 4F; Milán et al., 1998).

We conclude that Net is able to repress the transcription of *rho* in the wing pouch, but *net* activity is crucial for the repression of *rho* in wild-type discs mainly in the distal intervein sectors A, B, D and E. Repression of *rho* by ectopic Net has no effect on the proximal portions of wing veins and, in most flies, on L2, which is consistent with the phenotype of $rho^{ve}$ mutants, demonstrating that vein formation completely depends on *rho* activity only in the distal portions of longitudinal veins L3 to L5 (Sturtevant et al., 1993; Fig. 4D).

### Rho and Ras activities prevent *net* transcription

Ectopic expression of *rho* under the control of a heat-inducible promoter during late third instar or early pupal development causes broadening of veins and ectopic vein formation, whereas absence or reduction of *rho* activity, or impaired Egfr-dependent signalling, generates the complementary phenotype, i.e. loss of vein structures (Sturtevant et al., 1993; Fig. 4D). To test if the development of ectopic veins is linked to the repression of *net*, *net* transcription was examined in wing discs after ectopic activation of *UAS-rho* by *MS1096*. Indeed, in such discs, which express *rho* strongly in almost the entire disc (not shown, but the pattern is identical to that of *net* transcripts in Fig. 3I), no *net* transcripts were detectable (Fig. 5B), which demonstrates that ectopic *rho* expression represses *net* transcription. It appears that in these wing discs all intervein regions develop as veins, since the resulting adult wings have a tube-like appearance and consist entirely of vein-like tissue (Fig. 4G; Guichard et al., 1999). This phenotype is much stronger than that of *net* null mutants (Fig. 4B). Ectopic Rho protein suppresses intervein fate in regions where such a fate is independent of *net* expression. Hence, we assume that in these regions *rho* represses intervein-promoting and vein-suppression genes that are different from *net*, and thus activates vein-promoting genes. These genes may show partial redundancy with *net* functions in regions in which their expression overlaps with that of *net*.

To investigate further whether *rho* mediates its repression of *net* through activated components of the Egfr signalling pathway, constitutively active Ras protein was expressed in *MS1096/+*; $UAS\text{-}Dras1^{V12}$/+ wing discs. As evident from Fig. 5C, constitutive Ras activity produced overgrown wing discs that fail to express *net*. Although the adult wing phenotype could not be observed in these flies because they die as early pupae, ectopic activation of the Ras/mitogen-activated protein kinase (MAPK) signalling pathway in wing discs has been shown to give rise to ectopic veins (Karim and Rubin, 1998). These results illustrate that ectopic expression of *rho* or activated components of the Egfr signalling pathway represses the transcription of *net* and presumably of additional vein-suppression genes, and suggest that their repression is a prerequisite for vein formation.

Wing discs in which Ras is constitutively active accumulated high levels of *rho* transcripts in intervein regions (Fig. 5D). In contrast, *rho* expression was disrupted in wing discs and pupal wings that are homozygous for $rho^{ve}$ (Sturtevant et al., 1993; D. B., H.-P. L., H. J. and M. N., unpublished, but pattern similar to that in Fig. 5A). In such discs, *net* transcription expanded into the vein primordia of third instar wing discs (compare Fig. 5E with Fig. 3A) and remained expressed ectopically in the distal portions of L3 to L5 of pupal wings (Fig. 5F), where vein development was suppressed (Fig. 4D). This result is consistent with the observation that ectopic expression of *net* was able to repress vein formation only in regions (Fig. 4C) where vein formation depended on *rho* expression (Fig. 4D). These findings show that *net* and *rho* transcripts accumulate in mutually exclusive patterns in developing wings, and specify intervein and vein precursor cells, respectively. Thus, *net* expression is negatively regulated by Egfr signalling and, in turn, represses *rho* expression.

Finally, *UAS-net* and *UAS-rho* were co-expressed ubiquitously under the control of *MS1096*. Surprisingly, while ubiquitous expression of either *net* or *rho* in developing wings generated curved wings with suppressed distal L5 (Fig. 4E) or tube-like wings composed of vein-like tissue (Fig. 4G), ubiquitous co-expression of *net* and *rho* resulted in a nearly wild-type wing blade (Fig. 4H). Since the ubiquitous expression of Rho, which is under the control of *MS1096*, is independent of Net, this result indicates that Net represses, in addition to *rho*, other vein-promoting genes downstream of Rho in Egfr signalling. If this were not the case and the only

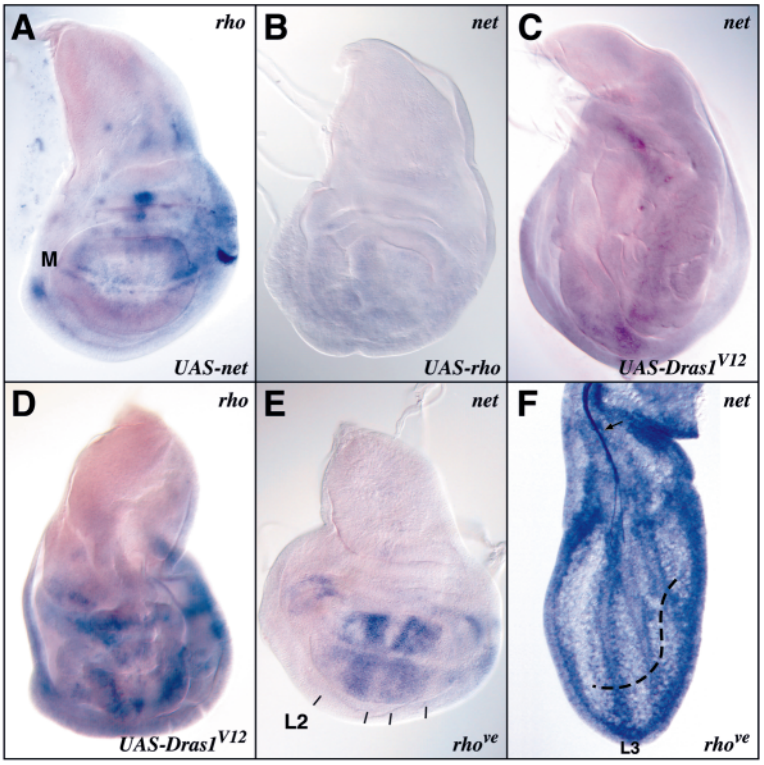


**Fig. 5.** Opposite effects on *net* and *rho* expression in mutant wing discs expressing *net* or *rho* ectopically, and ectopic expression of *net* in the absence of *rho* activity. Expression patterns of *rho* (A,D) and *net* (B,C,E,F) transcripts were analysed by in situ hybridization of DIG-labelled probes to wing discs of MS1096/+; *UAS-net/UAS-net* (A), MS1096/+; *UAS-rho/+* (B), MS1096/+; *UAS-Dras1$^{V12}$*/+ (C,D) and *rho$^{ve}$* (E) late third instar larvae, and to a *rho$^{ve}$* pupal wing (F). (A) *rho* expression is repressed by the ectopic expression of *net* in the wing pouch except in two stripes flanking the wing margin (M). (B-D) *net* expression is repressed in the entire wing pouch by the ectopic expression of *rho* (B) or the constitutive activation of Ras1 (C), which activates *rho* ubiquitously in the wing pouch (D) and generates hyperplastic discs (C,D). (E,F) In *rho$^{ve}$* animals that lack *rho* activity in most of the wing pouch (*rho* expression similar to pattern shown in A), *net* is expressed ectopically in all vein primordia of third instar wing discs, although not at the same level as in intervein regions (E; positions of L2 to L5 are indicated), and remains expressed ectopically 26-28 hours APF (at 22°C) in the distal portions of pupal wings (F; distal to broken line; arrow marks trachea), where vein development is suppressed (Fig. 4D). All wing discs are shown with their dorsal side upwards and anterior to the left (A-E), the pupal wing is shown with anterior to the left and proximal upwards (F).

function of Net was to repress the endogenous *rho* gene, we would expect the same phenotype after ubiquitous co-expression of Net and Rho as after ubiquitous expression of Rho (Fig. 4G) rather than the nearly wild-type wing observed (Fig. 4H). Therefore, Net suppresses vein development in interveins through a control that consists of at least two tiers, one repressing *rho*, the other interfering with the activation of vein-promoting genes downstream of Rho-enhanced Egfr signalling. Curiously, although ubiquitous expression of Net is able to suppress the effect of ubiquitously co-expressed Rho in intervein regions, it is unable to repress vein development in veins. It follows that differences between future vein and intervein regions exist that are sufficient to correctly determine vein versus intervein fates in the presence of an excess of both Net and Rho. Our findings further suggest a considerable redundancy in the gene networks participating in the specification of vein versus intervein fates. This conclusion is also borne out by the observation that if both Rho and Net are reduced or lost in *rho$^{ve}$ net$^{1}$* double mutants, veins are less severely truncated than in wings of *rho$^{ve}$* single mutants (Díaz-Benjumea and García-Bellido, 1990a; Sturtevant and Bier, 1995).

## DISCUSSION

### Mutual repression of *net* and *rho* in wing vein patterning

The specification of vein versus intervein fate in the *Drosophila* wing disc crucially depends on the restriction of Rho expression to the primordial veins (Wasserman and Freeman, 1997; Bier, 1998). Rho enhances Egfr signalling, which generates elevated levels of activated MAPK essential for proper vein development and the suppression of intervein fate in vein cells (Gabay et al., 1997; Guichard et al., 1999). In the absence of Egfr signalling, either in clones mutant for the *Drosophila* Egfr (Díaz-Benjumea and García-Bellido, 1990b) or in wings of *rho$^{ve}$ vn$^{1}$* flies (Díaz-Benjumea and García-Bellido, 1990a; Sturtevant and Bier, 1995), veins fail to develop and assume an intervein fate.

Our results show that a counterpart of *rho* is *net* whose transcription is confined to intervein sectors of third instar wing discs. Absence of *net* activity in third instar wing discs results in derepression of *rho* in all intervein regions except sector C and initiates the formation of ectopic veins. Conversely, in the absence of *rho* activity, *net* is ectopically expressed in vein primordia of wing discs, as well as in distal veins L3 to L5 of pupal wings where differentiation of veins is suppressed. Thus, while *net* is repressed in vein cells by high Egfr signalling dependent on Rho, Net protein suppresses vein fate in intervein cells by repressing *rho* transcription (Sturtevant and Bier, 1995). Repression of *net* in veins by high levels of Rho-dependent Egfr signalling is crucial for vein development, as ectopic expression of *net* is able to repress *rho* transcription and suppress vein fate. However, Egfr signalling in the absence of Rho is sufficient to initiate normal vein development in the proximal and anterior portions of the wing. Consistent with this finding, repression of *rho* by ectopic Net prevents vein formation only in regions where it depends on Rho.

Ubiquitous expression of *rho* or activated Ras represses *net* transcription in the entire wing disc and promotes vein development throughout the wing. Such flies have tube-like wings composed mainly, if not exclusively, of vein cells (Guichard et al., 1999). Since this phenotype is much stronger than that of *net* null mutants, it follows (1) that additional factors that repress *rho* must be present in intervein regions,

and (2) that ectopic Rho is able to suppress intervein fate in regions where such a fate is independent of *net* expression, and hence that in these regions *rho* is able to repress intervein-promoting and vein-suppression genes different from *net*. The first conclusion is also supported by the notion that *rho* does not expand into the intervein region between L3 and L4 in *net* mutants. The simplest explanation for these observations is that *rho* expression is controlled by a set of separate silencers responding to different repressors in different, perhaps overlapping intervein regions and that Net acts as one of the repressors of *rho*.

### *net* encodes a novel member of the bHLH protein family

Net is a member of the bHLH transcription factor family (Horimoto et al., 1994; Murre et al., 1994; Phillips, 1994), which has been subdivided into three structurally and functionally distinct classes (Ohsako et al., 1994). Since Net contains a valine residue in position 13 of the basic region of its bHLH domain, one might surmise that it is a class A protein (Fig. 2B) and acts as a transcriptional activator. This assumption is consistent with the fact that Net has no class C-type repressor domains like the orange domain or the C-terminal WRPW-motif (Dawson et al., 1995; Fisher et al., 1996), which associates with the co-repressor Groucho (Paroush et al., 1994; Alifragis et al., 1997; Jimenez et al., 1997). However, Net includes a proline-rich domain in its N-terminal moiety that might function as a repressor domain as shown for non-bHLH transcriptional regulators such as Even-skipped, Female-specific 1 and RGM1 (Cowell, 1994; Hanna-Rose and Hansen, 1996). Thus, despite the similarity of the Net bHLH domain to that of class A bHLH proteins, Net may act as a transcriptional repressor. This supposition is supported by the observation that Net represses *rho* expression during wing development. Although we cannot exclude that Net activates a gene whose product represses *rho*, we found that Net was also able to repress vein-promoting genes in the presence of activator proteins activated by ectopic Rho through enhanced Egfr signalling in interveins (Fig. 4H; see below). The simplest explanation of these results is that Net is a transcriptional repressor and thus exceptional among class A proteins.

### Tiers of vein versus intervein fate specification

Any attempt to understand the role of *net* in the patterning of wing veins must consider its function in the context of the complex process of vein versus intervein specification, which occurs in several tiers of intimately linked gene regulatory circuits (Biehs et al., 1998; Lunde et al., 1998). The first distinction between vein and intervein anlagen is made during the third larval instar when the wing pouch is subdivided along the anteroposterior axis into sectors by prepattern genes activating morphogenetic signals, such as Hh and Dpp, whose threshold concentrations determine the sector boundaries. Longitudinal veins are first specified by the activity of vein-organising genes that are activated in narrow stripes along these boundaries by short range signals, while intervein genes, like *N* and *net*, are thought to be activated through cues of prepattern genes (Biehs et al., 1998). In the second tier of regulation, Egfr signalling plays a decisive role. Accordingly, one of the first genes to be activated by vein-organising genes in third instar wing discs is *rho* (Sturtevant et al., 1997). During late third instar and early prepupal stages, the membrane proteins Rho and S enhance signalling of the ubiquitously expressed Egfr, activated by the neuregulin-like protein Vn (Schnepp et al., 1996), and possibly additional ligands, in longitudinal veins and adjacent provein cells (Guichard et al., 1999). High Egfr signalling activates *Delta* (*Dl*) as well as *rho*. Because Rho is able to stimulate Egfr signalling in adjacent cells, *rho* and *Dl* expression continue to expand (Sturtevant et al., 1994; de Celis et al., 1997). Net is required to prevent the expansion of *rho* (and *Dl*) expression beyond the proveins into the intervein regions (Sturtevant and Bier, 1995). Our results suggest that Net might act in this process as a repressor of *rho* by binding to its wing enhancer, which is deleted in the $rho^{ve}$ allele (Sturtevant et al., 1993). Conversely, high Egfr signalling stimulated by Rho is essential in proveins to repress *net*, as *net* transcription is no longer confined to intervein cells in $rho^{ve}$ mutant wings.

*Dl* expression in proveins initiates the third tier in the regulation of wing vein patterning. At the same time, Rho-dependent Egfr signalling begins to downregulate *egfr* mRNA in proveins (Sturtevant et al., 1994), yet MAPK activity remains high up to the early P2 pupal stage when crossveins become visible (Guichard et al., 1999), presumably because of the continued stimulation of Egfr signalling through Rho. During this third tier of regulation, Dl activates N signalling in the lateral provein cells flanking the narrow central stripes of vein cells (de Celis et al., 1997). N expression, which has been activated in a pattern complementary to that of Dl in third instar wing discs, is now enhanced by a positive feedback loop of N signalling in lateral provein cells. In addition, since N signalling represses *rho* transcription through the proline-bHLH protein E(spl)mβ, expression of *rho* is again confined to the narrow stripes of vein cells by the early P2 pupal stage (de Celis et al., 1997). This regulatory loop of N signalling appears to be analogous to that operating during lateral inhibition in neurogenesis, by which a neural precursor cell, stochastically selected from a group of proneural cells, prevents its neighbours from adopting a neural fate (Artavanis-Tsakonas and Simpson, 1991). However, in contrast to lateral inhibition, vein cells are not selected stochastically from provein cells, as longitudinal veins are straight, which suggests that expression of *rho* is always restricted to the central cells of the provein stripe. This is achieved by a fourth tier of vein fate regulation through Dpp signalling (Yu et al., 1996). Expression of Dpp is activated by high Egfr signalling in provein cells at the same time as Dl expression. The effect of Dpp, however, is confined to the middle of the provein stripes by the secreted product of the *short gastrulation* (*sog*) gene, an antagonist of Dpp expressed in the adjacent intervein regions. If we assume that Dpp signalling inhibits expression of *N*, N signalling would be blocked only in the central cells of proveins, which are thus determined to become vein cells. Consistent with this last tier of regulation, veins in $dpp^{-}$ clones are no longer straight, but follow an irregular path confined to the wider provein stripe (Yu et al., 1996). Dpp signalling maintains both its own expression and that of Rho in vein cells and determines their differentiation (Yu et al., 1996).

Finally, the refinement of proveins to veins further depends on less well understood mutually inductive processes, in which predetermined vein cells of the dorsal wing surface signal to the underlying ventral cells to maintain vein cell identity

(García-Bellido, 1977; García-Bellido and de Celis, 1992; Milán et al., 1997).

### Redundancy in wing vein patterning

Vein cells are not only specified for their specific fate, but are also prevented from adopting the alternative intervein fate. This is achieved at all regulatory tiers by Rho-stimulated Egfr signalling, which represses, initially in proveins and later in veins, not only *net*, as shown here, but also *blistered* (*bs*; Roch et al., 1998). *bs* encodes a transcription factor, the *Drosophila* homologue of the mammalian serum response factor (DSRF; Montagne et al., 1996), which is instrumental in the specification of intervein fate (Fristrom et al., 1994; Roch et al., 1998). Like *net* and *rho*, activities of *bs* and *rho* repress each other and hence are mutually exclusive and expressed in complementary patterns of intervein and vein territories. However, whereas *net* and *rho* mutually depend on each other already from the onset of vein versus intervein development in third instar wing discs, *bs* and *rho* begin to restrict each other's expression only during prepupal development (Roch et al., 1998). Similar to *net* and *rho*, this mutual repression is not apparent in the proximal parts of the wing because of a redundancy inherent in this regulation. Although *rho* and *net* (or *bs*) are able to repress each other when expressed ectopically, no ectopic expression occurs in the absence of one of these genes in the proximal wing regions.

The activities of Rho and Net are not restricted to the mutual repression of their genes. This conclusion follows from the surprising observation that their ubiquitous co-expression in the developing wing generates a nearly wild-type wing. Since ubiquitous expression of Rho produces wings composed entirely of vein-like tissue, Net is able to repress vein-promoting genes in intervein regions, despite the presence of an activating signal stimulated by the ubiquitous Rho, while the situation is reversed in veins where Net is unable to repress vein-promoting genes in the presence of Net-independent Rho expression. Similarly, Rho-dependent signalling represses intervein-promoting genes, like *bs*, in veins, despite the presence of Net, but does not have this capability in interveins in the presence of Rho-independent Net expression. It follows that even in the absence of endogenous Net and Rho vein and intervein primordia are in different states upon which ubiquitous Net and Rho can act to correctly determine their fates, which implies a considerable redundancy in the specification of vein versus intervein fate.

Because of the necessity for the concomitant suppression of the alternative fate when vein or intervein fates are specified, a system evolved in which Rho determined vein development by repressing *net* and later *bs*, which in turn specify intervein fate by repressing vein development in intervein regions. Such a balanced system is intrinsically labile unless it is stabilised through feedback loops (see above). Multiple feedback loops operate at all tiers of vein fate regulation, and tiers are closely linked and overlap in time, which further enhances the stability of the system because it generates redundancy. We propose that the functions of Net and Bs are partially redundant because they both repress *rho* in intervein regions during overlapping, though not identical, developmental periods. Thus, while Net represses *rho* in all intervein sectors of third instar wing discs except sector C, Bs begins to repress *rho* in these regions only in early prepupal wings (Roch et al., 1998). In view of this hypothesis, it might be less surprising that the wing phenotype of *net* null mutants is much weaker than that resulting from ubiquitous expression of *rho*, which also represses *net* completely, but converts almost the entire wing into vein material (Guichard et al., 1999). We assume that the lack of Net function in $net^{-}$ wing discs is partially compensated by the activation of *bs*, whose product represses *rho* in most of the intervein regions during the prepupal and pupal stage. This assumption is consistent with our observations (Fig. 3F) and with the earlier finding that *bs* null mutants exhibit a wing phenotype very similar to that resulting from ubiquitous expression of *rho* in the developing wing (Fristrom et al., 1994; Roch et al., 1998). The $rho^{ve}$-like phenotype obtained after ubiquitous expression of Net during wing development is largely explained by the ability of Net to repress *rho*. The partial redundancy of *net* and *bs* functions in wing discs is supported by experiments in which ubiquitous expression of Net was still able to suppress the strong ectopic vein formation phenotype of $bs^{2}/bs^{P1292}$ mutants (not shown, but phenotype is indistinguishable from that produced in a $net^{1}$ mutant background shown in Fig. 4E). In addition, *bs* expression is reduced in distal portions of $net^{1}$ wing discs and hence appears to depend partially on Net (Biehs et al., 1998), a finding that is consistent with our observation that LacZ expression of the *bs* enhancer trap line $bs^{P1292}$ is ectopically activated and enhanced after ectopic expression of Net in *MS1096/+*; *UAS-net/*$bs^{P1292}$ wing discs (D. B., H.-P. L., H. J. and M. N., unpublished).

Since *net* and *plexus* (*px*) mutant alleles interact genetically and exhibit indistinguishable genetic behaviours and wing phenotypes (Díaz-Benjumea and García-Bellido, 1990), one might surmise that they are part of the same developmental pathway. Indeed, *px*, which encodes a ubiquitously expressed nuclear matrix protein, like Net is required for the repression of *rho* (Matakatsu et al., 1999). Our finding that *net* expression is normal in $px^{1}$ or $px^{72}$ mutant wing discs (D. B., H.-P. L., H. J. and M. N., unpublished) is unable to support a role for Px in the regulation of *net* activity. However, although $px^{72}$ is the strongest known *px* allele, at present no *px* null alleles are available (García-Bellido, 1977; Matakatsu et al., 1999) to subject this possibility to a conclusive test.

The partial redundancy of *net* and *bs* might have evolved in more advanced insects like Diptera by the acquisition of vein-suppressing genes that reduced the much larger number of wing veins characteristic of more primitive insects. It has been proposed that such cryptic paraveins are still detectable in extravein mutants of *Drosophila* that display a strong tendency to misexpress *rho* and form ectopic veins between and parallel to L1 to L6 (Biehs et al., 1998). According to this hypothesis, *net* may have evolved to suppress paraveins P2 and P6, while *hairy* (*h*) acquired the ability to suppress paraveins P4 and P5 (Biehs et al., 1998). It is attractive to speculate that the paravein-suppressing function of Net may have evolved as an ability to repress *rho* in paraveins, but not in vein primordia where *net* was not expressed, and thus entailed a general vein-suppressing function, which initially may have been shared with *bs*. While *bs* later lost its early vein-suppressing function, its prepupal vein-suppressing function and pupal intervein differentiation function were retained, which resulted in a temporal overlap of *rho* repression by Net and Bs and thus led to a temporal redundancy of *net* and *bs* functions. It is

interesting to note that the *rho* repressing functions of *net* and *h* as well as that of *E(spl)mβ* (de Celis et al., 1997) all encode bHLH proteins, a property that is entirely consistent with a general hypothesis of how gene networks evolve (Noll, 1993).

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