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Relationships between *extramacrochaetae* and *Notch* signalling in *Drosophila* wing development

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SUMMARY

The function of *extramacrochaetae* is required during the development of the *Drosophila* wing in processes such as cell proliferation and vein differentiation. *extramacrochaetae* encodes a transcription factor of the HLH family, but unlike other members of this family, Extramacrochaetae lacks the basic region that is involved in interaction with DNA. Some phenotypes caused by *extramacrochaetae* in the wing are similar to those observed when *Notch* signalling is compromised. Furthermore, maximal levels of *extramacrochaetae* expression in the wing disc are restricted to places where *Notch* activity is higher, suggesting that *extramacrochaetae* could mediate some aspects of *Notch* signalling during wing development. We have studied the relationships between *extramacrochaetae* and *Notch* in wing development, with emphasis on the processes of vein formation and cell proliferation. We observe strong genetic interaction between *extramacrochaetae* and different components of the *Notch* signalling pathway, suggesting a functional relationship between them. We show that the higher level of *extramacrochaetae* expression coincides with the domain of expression of *Notch* and its downstream gene *Enhancer of split-mβ*. The expression of *extramacrochaetae* at the dorso/ventral boundary and in boundary cells between veins and interveins depends on *Notch* activity. We propose that at least during vein differentiation and wing margin formation, *extramacrochaetae* is regulated by *Notch* and collaborates with other *Notch*-downstream genes such as *Enhancer of split-mβ*.

Key words: *extramacrochaetae*, *Notch* signalling, Vein differentiation, *Enhancer of split*, *Drosphila melanogaster*

INTRODUCTION

The wing of *Drosophila melanogaster* provides a useful model system to analyze the morphogenetic processes that occur during the development of a multicellular organism. The wing derives from an anlage of ectodermal cells specified in early embryogenesis that proliferates during the larval stages and the first hours of pupal development (García-Bellido and Merriam, 1971; Madhavan and Schneidermann, 1977; Bate and Martínez-Arias, 1991; Milán et al., 1996a,b). During metamorphosis the wing disc evaginates and the two surfaces of the wing, dorsal and ventral, which have been separated throughout larval development, become apposed. The adult wing is characterized by a pattern of four longitudinal veins, formed by stripes of cells that appear more compacted and pigmented than intervein cells. Veins have dorsal and ventral components that are independently specified during imaginal development, but for each vein only the dorsal or ventral component protrudes from the wing surface (García-Bellido and de Celis, 1992).

The venation pattern is defined during the larval stage, as shown by the existence of clonal restrictions along veins and by the localized expression of several genes in presumptive veins in the third instar wing disc (González-Gaitán et al., 1994; Sturtevant et al., 1993, 1997). One of these genes, *veinlet* (*ve*), encodes a membrane protein with seven transmembrane domains (Bier et al., 1990), which collaborates in the activation of the Ras signalling pathway (Sturtevant et al., 1993; Sturtevant and Bier, 1995; Perrimon and Perkins, 1997). The Ras signalling pathway is locally activated in presumptive vein territories during the third larval instar (Gabay et al., 1997), and has a determining influence in promoting vein formation (Díaz-Benjumea and Hafen, 1994). The restricted expression of *ve* in presumptive veins is generated in part by repression mediated by the *Notch* signalling pathway. Loss-of-function alleles of *Notch* cause the formation of thicker veins, whereas *Notch* gain-of-function alleles cause the lack of veins, and these phenotypes are associated with an expansion or suppression of *ve* expression, respectively (de Celis and García-Bellido, 1994a; Sturtevant and Bier, 1995; de Celis et al., 1997). *Notch* encodes a transmembrane protein that acts as a receptor in multiple developmental processes (Artavanis-Tsakonas et al., 1995). The activation of *Notch* during vein development depends on interactions with the transmembrane protein *Delta* (*Dl*), and occurs specifically in the cells that separate

each vein from the adjacent interveins (boundary cells; de Celis et al., 1997). The analysis of the phenotypes produced by temperature-sensitive alleles of *N* and *Dl* indicates that they are required in vein differentiation at least until 24 hours after puparium formation (APF) (Shellenbarger and Mohler, 1978; Parody and Muskavitch, 1993). Other intracellular components of the *Notch* signalling pathway, such as *Supressor of Hairless* (*Su* (*H*)), *Hairless* and the basic helix-loop-helix (bHLH) protein *E(spl)mβ* of the *Enhancer of split complex* (*E(spl)-C*) are also required to regulate the expression of *ve* and the formation of veins of normal thickness (de Celis et al., 1997). During pupal development the expression of *Dl* is localised to the developing veins, whereas both *Notch* and *E(spl)mβ* are expressed at higher levels in the boundary cells (Huppert et al., 1997; de Celis et al., 1997). Interestingly, *E(spl)* deficiencies do not reproduce completely the phenotype caused by *Notch* null alleles in clones, suggesting that other components downstream of *Notch* are required to prevent vein differentiation. Similarly, *Notch* activity, but not *E(spl)mβ*, is also required during the proliferation of imaginal cells (de Celis and García-Bellido, 1994a; de Celis and Bray, 1997), suggesting that the activity of *Notch* in this process also requires additional *Notch* target genes.

The *extramacrochaetae (emc*) gene is a good candidate to mediate some aspects of *Notch* signalling during the proliferation of imaginal wing cells and the differentiation of veins. Null alleles of *emc* are cell lethal, but clones of hypomorph alleles cause phenotypes that are similar to those observed in *Notch* mutant clones. Thus, clones of *emc* mutant cells are smaller and more elongated than control clones, appear more frequently along veins, and can differentiate ectopic veins (García-Alonso and García-Bellido, 1988; de Celis et al., 1995; Baonza and García-Bellido, 1999). Furthermore, *emc* is expressed at higher levels in several places where *Notch* is activated, such as the cells that define the dorsoventral boundary during imaginal development and the boundary intervein cells during pupal development (Cubas and Modolell, 1992; de Celis et al., 1995). The similarities in the phenotype caused by *emc* and *Notch* mutants, and the coincidence between maximal accumulation of *emc* and *Notch* activity, suggest that *emc* could respond to *Notch* activation and mediate some aspects of *Notch* function during wing imaginal development. *emc* encodes a nuclear protein with an HLH domain (Ellis et al., 1990; Garrell and Modolell, 1990). However, the Emc protein does not have the basic region that is involved in interaction with DNA, and consequently it can only interact with and antagonise the activity of other bHLH proteins (Ellis et al., 1990; Garrell and Modolell, 1990; Van Doren et al., 1991, 1992; Cubas and Modolell, 1992).

In this work we have studied the functional relationships between *emc* and *Notch* signalling in both wing discs and pupal wings. We find strong genetic interaction between *emc* and different members of the *Notch* signalling pathway, suggesting that *emc* and *Notch* are functionally related. We show that the expression of *emc* is complementary to the expression of *Dl*, and that in pupal wings maximal expression of *emc* coincides with that of *Notch* and *E(spl)mβ*. Moreover, we show that the expression of *emc* at the dorso/ventral boundary and in boundary cells between veins and interveins depends on *Notch* activity. We propose that in at least two developmental processes, vein differentiation and wing margin formation, *emc* is regulated by *Notch* and collaborates with other *Notch*-downstream genes.

MATERIALS AND METHODS

Genetic strains

We have used in the *emc* gene the loss-of-function allele emc^{1} (García-Alonso and García-Bellido, 1988), the deficiency $Df(3L)emc^{E12}$, the duplication *Dp(3;Y;1)M2, mwh*$^{+}$, *emc*$^{+}$ and the gain-of-function emc^{Ach} (García-Alonso and García-Bellido, 1988; Garrell and Modolell, 1990). The cell markers used for clonal analyses were *multiple wing hairs* (*mwh*) and *forked* (*f*), and the *Minute* (M) allele used to generate *M*$^{+}$ clones was *M(3) 65F* (Lindsley and Zimm, 1992). At the *Notch* locus (*N*) we used the null allele N^{55e11}, the temperature-sensitive allele $l(1)N^{ts}$ and the gain-of-function allele Ax^{M3} and Ax^{16172} (de Celis and García-Bellido, 1994b); at the *Dl* locus we used the allele Dl^{M1} (Díaz-Benjumea and García-Bellido, 1990). We also used two reporter lines, a PlacZ insertion in *emc* (emc^{P5C}; Garrell and Modolell, 1990) and an *E(spl)mβ-CD2* reporter construct (de Celis et al., 1998), the UAS lines UAS-*E(spl)mβ*, UAS-N^{intra}, UAS-N^{ecd}, UAS-*Ser* (de Celis and Bray, 1997), UAS-*Dl* and UAS-*DlD* (a negative form of *Dl*; Huppert et al., 1997), and the GAL4 lines GAL4-MS1096 (Capdevila and Guerrero, 1994) and GAL4-c719 (kindly provided by E. Martín-Blanco).

Generation of UAS-*emc*

A full-length *emc* cDNA was cloned into the *Eco*RI site of pUAST (Brand and Perrimon, 1993). pUAST-*emc* was injected into embryos following conventional protocols, and several independent lines with insertions in the second and third chromosome were established.

Generation of mosaics

Mitotic recombination clones

Mitotic recombination was induced by X-rays (dose 1000 R; 300 R/min, 100 Kv, 15 mA and 2 mm Al filter). Irradiated larvae were timed in hours after egg laying (AEL). Adult flies of the appropriate genetic constitution were dissected and their wings mounted in lactic acid-ethanol (1:1) for microscopic examination. emc^{1} *M*$^{+}$ clones were induced in flies of the following genotypes: *mwh* emc^{1}*/M(3)65F*, $l(1)N^{ts}$/+; *mwh* emc^{1}*/M(3)65F*, N^{55e11}/+; *mwh* emc^{1}*/M(3)65F*, Ax^{M3}/+; *mwh* emc^{1}*/M(3)65F* and *mwh* emc^{1} Dl^{M1}*/M(3)65F*. Mitotic recombination proximal to the *Minute* mutation results in emc^{1} clones labeled with *mwh*. emc^{1} clones in $l(1)N^{ts}$/+; *mwh* emc^{1}*/M(3)65F* larvae were induced at 60±12 hours and 84±12 hours AEL. Larvae were grown at 25°C and a temperature pulse of 60 hours was applied before or after puparium formation. $emc^{1}/Dfemc^{E12}$ clones were induced in flies of the following genotypes: (1) *Dp(3;Y;1)M2, mwh*$^{+}$, *emc*$^{+}$ f^{36a}/+; *mwh* emc^{1}*/ mwh* $Df(3L)emc^{E12}$, (2) *Dp(3;y;1)M2, mwh*$^{+}$, *emc*$^{+}$ f^{36a}/N^{55e11}; *mwh* emc^{1}*/mwh* $Df(3L)emc^{E12}$ and (3)*Dp(3;Y;1)M2, mwh*$^{+}$, *emc*$^{+}$ f^{36a}/Ax^{M3}; *mwh* emc^{1}*/ mwh* $Df(3L)emc^{E12}$. Mitotic recombination proximal to *forked* results in *mwh/f* twin clones labeling $emc^{1}/Df(3L)emc^{E12}$ and *emc*$^{+}$ cells, respectively. In addition *mwh* $emc^{1}/Df(3L)emc^{E12}$ cells will also be homozygous for N^{55e11} (2) or Ax^{M3} (3).

Clones of cells expressing GAL4 were induced 48-72 hours after egg laying by 7-minute heat shocks at 37°C in flies of the following genotypes: (1) f^{36a} *FLP1.22; P[abx/Ubx<FRT* f^{+}*FRT>Gal4-lacZ]/UAS-*N^{intra}. The flip-out of the *<FRT* f^{+}*FRT>* cassette results in the expression of a *GAL4-lacZ* hybrid gene under the control of the *abx/Ubx* promoter. Clones were detected by the expression of β-gal (de Celis and Bray, 1997). (2) *y w FLP1.22; Act5C<FRT yellow*$^{+}$ *FRT> GAL4 UAS–LacZ* or *UAS-GFP*/ *UAS-X*, where X is *UAS-*N^{ecd}, *UAS-Dl, UAS-DlD* or *UAS-Ser*. The flip-out of the *<FRT yellow*$^{+}$ *FRT>* cassette results in the expression of the transcriptional activator

GAL4 gene under the control of the *Act5C* promoter (Ito et al., 1997). Clones were detected by expression of β-gal or GFP, and were analysed in third instar larvae. Clones were also induced 0-6 hours after puparium formation in *hsFLP1.22; Act5C<FRT yellow$^+$ FRT> GAL4 UAS-GFP/UAS-Dl.* These clones were visualised 24-30 hours after puparium formation.

In situ hybridisation and immunocytochemistry

Whole-mount in situ hybridisation with digoxigenin-labelled DNA probes in imaginal discs was performed as described previously for both imaginal discs (Cubas et al., 1991) and pupal wings (Sturtevant et al., 1993). For immunocytochemistry we used rabbit anti-β-galactosidase (Cappel), mouse monoclonal anti-*Dl* (Fehon et al., 1991), rabbit monoclonal anti-Emc and mouse anti-CD2 (Serotec). For nuclear staining we used oligogreen at 1/5000 dilution. Secondary antibodies were from Jackson Immunological Laboratories (used at 1/200 dilution).

Two-hybrid system

We have followed the system developed by Brent (Gyuris et al., 1993). We used the high copy plasmid pSH18-34 as *LacZ* reporter. This plasmid contains four high affinity overlapping type colE1 LexA operator, which bind two LexA dimmers (Finley and Brent, 1994). pEG202 was used as the LexA fusion vector (Finley and Brent, 1994). Proteins expressed from this plasmid contain amino acids 1-202 of LexA, which include the DNA binding and dimerization domains. Fusion of *emc* was produced by ligating PCR amplification product into pEG202 as a 5′ *Eco*RI-3′*Xho*I fragment containing the sequence that encodes amino acids 1-76. This region includes the HLH domain. The fusion was sequenced to detect possible PCR-induced mutations.

PJG4-5 plasmid containing the B42 activation domain, was used as an activation vector. The pJG4-5 with the insertion of *E(spl)mβ*, *E(spl)mδ* and *da* were kindly provided by C. Delidakis (Alifragis et al., 1997). The yeast strain used was EGY 48.

RESULTS

emc interacts genetically with genes of the *Notch* signalling pathway

Loss-of-function alleles of *emc* and *Notch* have similar behavior in mitotic recombination clones, causing reduced cell viability and growing preferentially along the veins (García-Alonso and García-Bellido, 1988; de Celis and García-Bellido, 1994a; de Celis et al., 1995). To explore the possibility that *emc* and *Notch* are related during cell proliferation, we studied the behavior of *emc* mutant cells that are also mutant for *Notch* loss- or gain-of-function alleles (Table 1). In these experiments, mitotic recombination was induced in larvae of three different genotypes (see Materials and Methods), and *emc* mutant cells were also homozygous for a *Notch* null allele or a *Notch* gain-of-function allele. Cells doubly mutant for *emc* and *Notch* have extremely poor viability (Table 1), indicating that *emc* and *Notch* cooperate to promote cell proliferation in the wing. The failure to form clones of normal size by *emc/Df(emc)* mutant cells is not rescued by the homozygosity of a *Notch* gain of function allele (Ax^{M3}; Table 1), suggesting that *emc* is required downstream or in parallel to *Notch* during cell proliferation.

The consequences of reducing *emc* or *Notch* function during vein differentiation are different. Whereas *emc* mutant clones show ectopic veins in specific positions, and only occasionally cause the differentiation of thicker veins (Fig. 1B), *Notch, Su(H)* and *E(spl)-C* mutant clones always produce the formation of thicker veins, but they do not cause the appearance of ectopic veins (de Celis et al., 1997). To analyse possible interactions between *emc* and *Notch* signalling in vein differentiation, we compared the behavior of homozygous emc^1 clones induced in two different *Notch* mutant backgrounds (see Materials and Methods). The width of the veins formed by emc^1 mutant cells in N^{55e11} heterozygous females is much greater than that of normal veins (Fig. 1D,E; compare with A,B). This phenotype is very similar to that produced by N^{55e11} homozygous clones (de Celis and García-Bellido, 1994a). In contrast, emc^1 clones induced in heterozygous Ax^{M3} wings never cause the formation of thicker veins (Fig. 1C), causing phenotypes similar to emc^1 clones induced in wild-type control wings. *emc* clones were also induced in *Dl* (Dl^{M1}) heterozygous wings, which have a dominant vein thickening phenotype affecting mainly the distal part of the veins. The phenotype of the emc^1 clones in these wings is similar to that found in N^{55e11} heterozygous wings, with many more cells differentiating as vein in the position of the normal veins (Fig. 1F). Thus, when *Notch* signalling is reduced, vein differentiation in normal vein territories becomes very sensitive to reductions in *emc* function.

The requirement of *Notch* in vein formation occurs during both larval and pupal development (de Celis et al., 1997). In addition *emc* is required to position the veins during larval development (de Celis et al., 1995), but it is not known if *emc* activity is also needed during the latest stages of vein differentiation. Therefore the effects of *emc* clones on vein thickness observed in different *Notch* mutant backgrounds could be due to a sequential requirement of both proteins occurring at different stages of vein formation. To characterise the most likely time window when *Notch* and *emc* interact in the determination of vein thickness, we induced emc^1 clones in $l(1)N^{ts}$ heterozygous females that were grown at the restrictive temperature during larval or pupal development (see

Table 1. Clonal analysis of *emc* in *Notch* mutant backgrounds

Genotype	Hours AEL	Number of wings	*f/mwh* clones	*f/mwh* cells	*f* clones	*f* cells
(1) *Dp(3;1)M2, mwh$^+$, emc$^+$f^{36a}/+; emc^1/Df(3L)emc^{E12}*	48-72	732	73	286/44	105	474
	72-96	82	67	56/13	24	66
(2) *Dp(3;1)M2, mwh$^+$, emc$^+$f^{36a}/N^{55e11}; emc^1/Df(3L)emc^{E12}*	48-72	279	0	–	42	590
	72-96	173	27	33/5	119	117
(3) *Dp(3;1)M2, mwh$^+$, emc$^+$f^{36a}/Ax^{M3}; emc^1/Df(3L)emc^{E12}*	48-72	68	14	143/34	18	176

AEL, after egg-laying; *f, forked*; *mwh, multiple wing hairs*.
For details of mutants, see Materials and Methods.

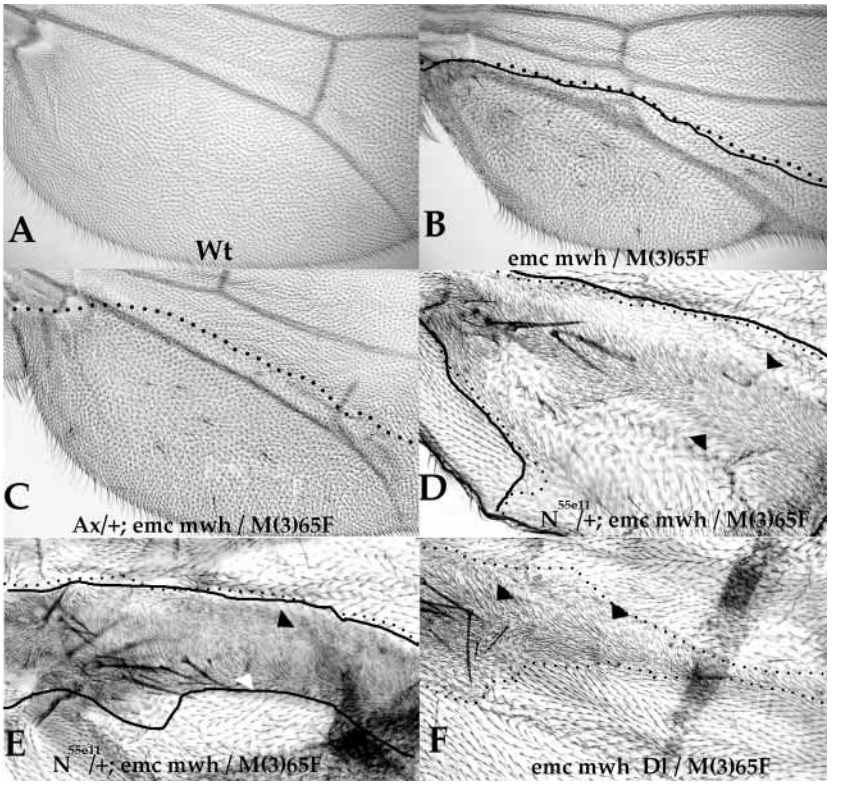

Fig. 1. Vein differentiation defects caused by *emc*^{1} mutant clones induced in different mutant backgrounds affecting *Notch* signalling. (A) L5 vein in a wild-type (Wt) wing. (B) Large *emc*^{1} *M*$^{+}$ clone covering L5 and causing the thickening of this vein. (C) Dorsal *emc*^{1} *M*$^{+}$ clone induced in an *Ax*^{M3}/+ wing. The width of the veins is not affected. (D,E) *emc*^{1} *M*$^{+}$ clones induced in *N*^{55e11}/+ mutant wings. Dorsal and ventral *emc*^{1} *M*$^{+}$ clones differentiate a L5 vein thicker than a normal vein and than veins of *N*^{55e11}/+ mutant wings (D). The intervein region between L4 and L5 is obliterated by a dorsal and ventral *emc*^{1} *M*$^{+}$ clones in an *N*^{55e11}/+wing. The fused veins L4 and L5 are much thicker than normal veins (E) (arrowheads indicate the width of the fused veins). (F) *emc*^{1} *M*$^{+}$ clone induced in a *Dl*^{M1}/+ mutant wing, differentiating a thicker dorsal L5. The effects produced by this and similar clones are identical to those observed in *N*^{55e11}/+ wings. Dotted and solid lines indicate the extension of each clone in the dorsal and ventral surfaces, respectively.

Materials and Methods). The phenotypes of increased vein thickness characteristic of *emc* clones induced in *Notch* backgrounds were observed mainly when the pupae were grown at the restrictive temperature. Thus 20 out of 22 *emc*^{1} clones localised in vein regions caused thickening of the veins when pupal development takes place at 29°C, and only 1 out of 10 similar clones caused thickening when imaginal development takes place at 29°C (Fig. 2D; compare with C). Although this result does not discard interactions between *Notch* and *emc* occurring during larval development, it indicates that *emc* and *Notch* interact to determine the correct width of the veins at least during pupal development.

Coincidence between maximal Emc accumulation and expression of *E(spl)mβ*

The expression of *emc* during imaginal development is detected in most cells of the wing disc, but maximal accumulation of both *emc* RNA and protein is localised to particular places. Maximal levels of *emc* in the prospective wing blade are restricted to the dorsal and ventral cells that form the dorsoventral (d/v) boundary and to a wide stripe of cells straddling the anterior-posterior compartment boundary (Cubas and Modolell, 1992; de Celis et al., 1995). Immediately adjacent to the d/v boundary, cells express lower levels of *emc* than other cells in the wing pouch.

The expression of *emc* evolves during pupal development, with low levels of *emc* being detected in the presumptive veins L3 and L4 first (at 0-4 hours (APF; Fig. 3A) and also L5 later (4-8 hours and 8-10 hours APF; Fig. 3B,C). At 18-21 hours APF the expression of *emc* is increased in a 2-3 cell-wide stripe at both sides of each vein (not shown). This pattern of accumulation of *emc* at vein/intervein boundaries is maintained

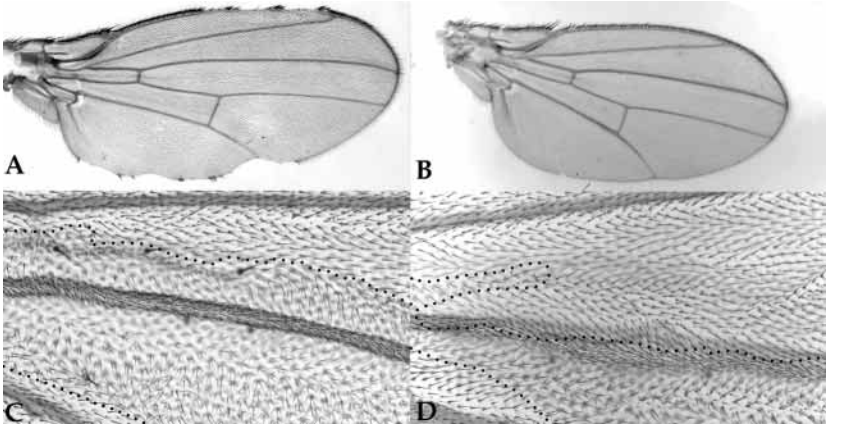

Fig. 2. Phenotype of *emc*^{1} *M*$^{+}$ clones in *l(1)N*^{ts}/+ mutant background. (A,B) Wing phenotype caused by a pulse of 60 hours before (A) or 60 hours after (B) puparium formation in an *l(1)N*^{ts}/+; *emc*^{1} *M*$^{+}$ background. The pulse of restrictive temperature during larval development causes nicks in the wing margin (A), and during pupal development produces a weak thickening of the veins L3 and L5. (C,D) Dorsal *emc*^{1} *M*$^{+}$ clones induced in *l(1)N*^{ts}/+ mutant wings grown at the restrictive temperature during larval (C) or pupal (D) development. Only when pupal development takes place at the restrictive temperature is the thickness of the vein increased (D). Dotted lines indicate the extension of each clone in the dorsal surface.

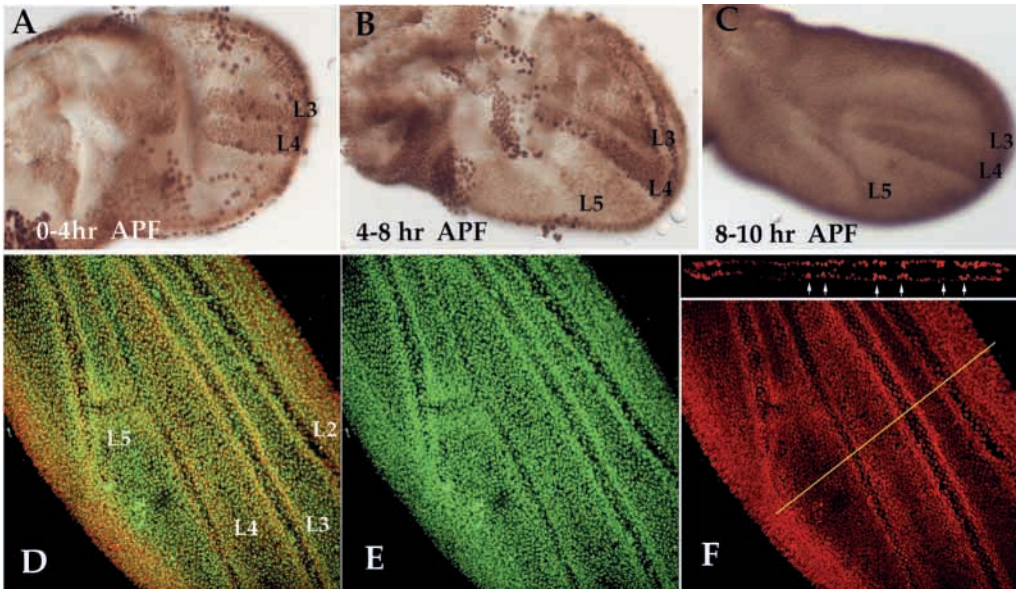

Fig. 3. Expression of *emc* during pupal development. (A-C) β-Gal expression in the *emc*^{P5C} strain at 0-4 hours (A), 4-8 hours (B) and 8-10 hours (C) after puparium formation (APF). (D-F) Expression of *emc* (*emc*^{P5C}; red in D,F) and distribution of nuclei (oligogreen, green in D,E) in pupal wings 24 hours APF. The yellow line in F indicates the position in the wing of the transversal section shown in the upper panel. High levels of *emc* expression are observed at vein/intervein boundaries (arrows). L3, L4 and L5 indicate the longitudinal veins 3, 4 and 5, respectively.

and refined during later stages of pupal development. Thus, in pupae of 24-30 hours APF the levels of *emc* in these stripes are higher and the expression of *emc* in the vein regions is very reduced (Fig. 3D,F). The dorsoventral and vein/intervein boundaries in the wing disc in pupal wings correspond to places where high levels of *Notch* activation occur, as monitored by the preferential accumulation of the *Notch*-downstream gene *E(spl)mβ* (de Celis et al., 1996b).

To compare the expression of *emc* with that of genes of the *Notch* signalling pathway, we studied the pattern of expression of *Dl* and *E(spl)mβ* in third instar wing discs and pupal wings of the strain *emc*^{P5c}, which includes a Plac-Z insertion within the *emc* gene (Garrell and Modolell, 1990). The expression of *Dl* in third instar imaginal wing discs is maximal in the presumptive veins, and also in two stripes of cells abutting the dorsoventral boundary (Kooh et al., 1993; Fig. 4B). In pupal wings, Dl and Notch proteins are expressed

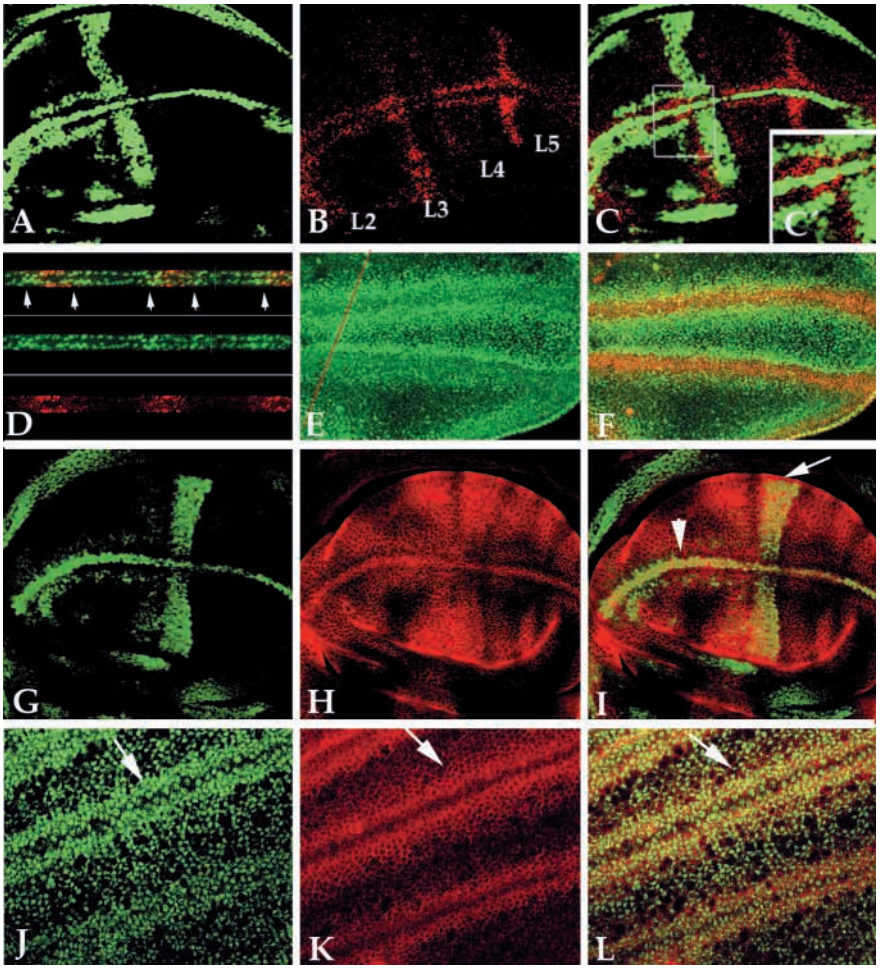

Fig. 4. Expression of *Dl*, *E(spl)mβ* and *emc* in third instar wing discs and pupal wings. (A-F) Expression of *emc* (*emc-lacZ* green) relative to Dl (red) in wing discs (A-C) and pupal wings (D-F). The expression of both genes is complementary at both stages, with maximal expression of *emc-lacZ* in the cells that form the dorsoventral (d/v) boundary and the L3/L4 intervein (A-C, higher magnification C′) and at both sides of each vein in pupal wings (D-F). At this stage one or two cells coexpress *emc* and Dl. (D) Horizontal section corresponding to the red line in E. Vein/intervein boundaries are indicated by arrows. (G-L) Expression of *emc* (*emc-lacZ*, green) related to the expression of *E(spl)mβ* (*E(spl)mβ-CD2*, red). The merged the pictures (I, wing disc and L, pupal wing) demonstrate that highest levels of *emc* (G,J) and *E(spl)mβ* expression (H,K) occur in the same places. These are the d/v boundary cells (arrowhead, I) and cells of the intervein region between veins 3 and 4 (arrow, I) in wing discs, and the vein/intervein boundary cells in pupal wing (arrows in J-L).

in complementary regions, with Dl restricted to the veins and Notch restricted to 2- to 3-cell-wide stripes localized at vein/intervein boundaries (de Celis et al., 1997). We find that maximal accumulation of *emc* is complementary to *Dl* expression both during imaginal and pupal development (Fig. 4A-F). The expression of *E(spl)mβ* coincides with the maximal accumulation of *emc*, both at the dorsoventral boundary in the imaginal disc and at vein/intervein boundaries during pupal development (Fig. 4G-L). The coincidence between the maximal accumulation of *emc* and *E(spl)mβ*, as well as the complementary pattern of *emc* and *Dl*, suggest that *emc* transcription could be in part regulated by *Notch* signalling in a *Dl*-dependant manner. This possibility was studied by analysing the effects of alterations in *Notch* activity on the expression of *emc*.

Consequences of changes of *Notch* activity in the expression of *emc* in imaginal wing discs

Using a combined Flip out/Gal4 system (see Materials and Methods) we induced clones of cells expressing either a dominant negative form of *Notch* (N^{ecd}) or the intracellular part of *Notch* (N^{intra}), which corresponds to a ligand-independent activated form of the protein. If *Notch* signalling regulates *emc* expression, we expect to find opposite changes in *emc* expression in N^{ecd} and N^{intra} clones. Clones of cells expressing high levels of N^{ecd} do not affect the expression of *emc* in most parts of the wing disc (Fig. 5A-C), indicating that both the basal expression of *emc* and the preferential accumulation of *emc* in intervein regions in the wing pouch are not regulated by *Notch*. However, N^{ecd} clones straddling the dorsoventral boundary autonomously fail to express *emc* at the d/v border (Fig. 5A-E). The converse result was observed in clones of N^{intra}-expressing cells, which were always associated with higher levels of *emc* expression anywhere in the wing disc, although not in all cells within each clone (Fig. 5F-H). The effects on *emc* expression of modifications in *Notch* activity are independent of the *Notch* target gene *E(spl)mβ*, because cells expressing *E(spl)mβ* do not show any alteration in the levels of *emc* expression (data not shown).

We expect clones of ligand-producing cells to have similar effects to N^{intra} on the expression of *emc*. Clones of cells expressing high levels of *Dl* or *Ser* induce the expression of several target genes in cells adjacent to the clone in the dorsal or ventral compartments, respectively (de Celis and Bray, 1997). However, clones of *Dl*- or *Ser*-expressing cells induced using a weaker promotor (see Materials and Methods) cause the expression of the same markers both within and outside the clone (A. B. and A. G.-B., unpublished). This observation indicates that at this level of expression, *Dl* and *Ser* induce high levels of *Notch* activity both in the cells expressing the ligands and in the adjacent wild-type cells. In these clones we find high levels of *emc* expression both within the clone, and in a row of wild-type cells immediately adjacent to it. Surrounding the wild-type cells with high *emc* levels appears a row of cells with lower *emc* levels (Fig. 5I,J, clones of *Ser*-expressing cells; data not shown). Thus, dorsal clones of *Dl*-expressing cells (and ventral clones of *Ser*-expressing cells) reproduce the same *emc* expression pattern that is observed at the d/v boundary, suggesting that the accumulation of *emc* here is regulated by *Notch*.

Notch signalling regulates the expression of *emc* in pupal wings

emc mutant clones occasionally cause thickening of veins (Fig. 1B). This phenotype is greatly exaggerated in *Notch* mutant wings, suggesting that *emc* and *Notch* interact in the definition of vein width. This interaction could be, in part, due to the regulation of *emc* expression by *Notch* at vein/intervein boundaries. Therefore, we studied the expression of *emc* during pupal development in several *Notch* mutant backgrounds. When *Notch* activity is reduced (fa^{nd} mutants, not shown, or by expressing a dominant negative form of *Notch*, c-719/+; N^{ecd}/emc^{P5c}), the preferential accumulation of *emc* observed in pupal wings at vein/intervein boundaries is affected (Fig. 6C,F,G). Thus, cells with maximal levels of *emc* either disappear or are displaced to new positions that correspond to the borders of the broader mutant veins. Similarly the stripes of cells where *emc* accumulates at high levels adjacent to the

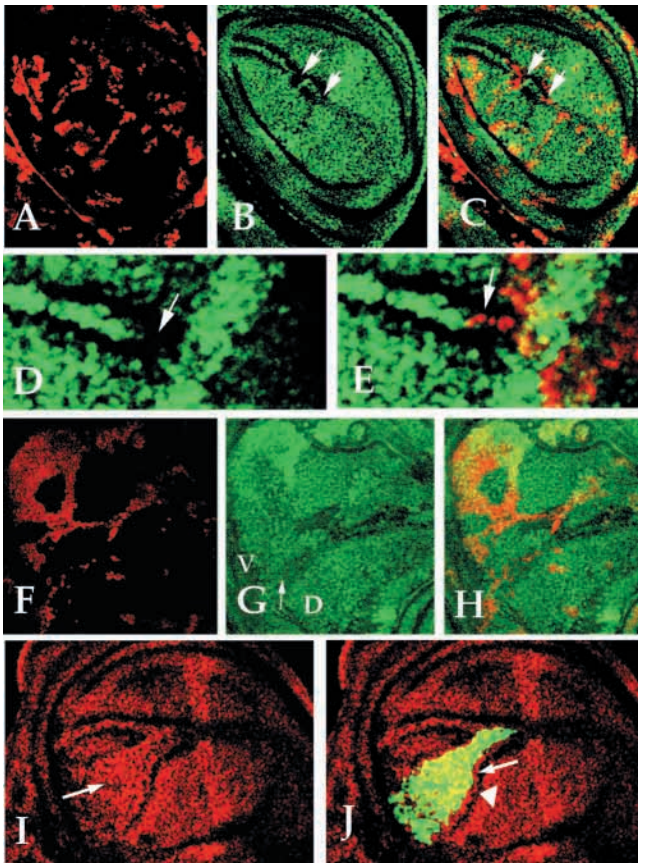

Fig. 5. Effect on the expression of Emc of clones of cells with modified Notch activity. (A-E) Emc expression (green) in clones of N^{ecd}-expressing cells (red). Emc is not expressed in the d/v boundary cells occupied by the clone (arrows in B,C). (D,E) Higher magnification of a clone of N^{ecd}-expressing cells showing that Emc is autonomously removed. (F-H) Clones of N^{intra}-expressing cells (red) cause ectopic expression of Emc (green) in the clone (overlap of expression in yellow). V and D indicate ventral and dorsal compartment, respectively; the arrow shows the d/v boundary. (I,J) Clone of *Dl*-expressing cells (green) inducing high levels of Emc (red) in the clone (arrow, I) as well as in a stripe of cells surrounding the clone (arrow, J). Emc is not expressed in a stripe of cells adjacent to the wild-type cells with high Emc levels (arrowhead, J).

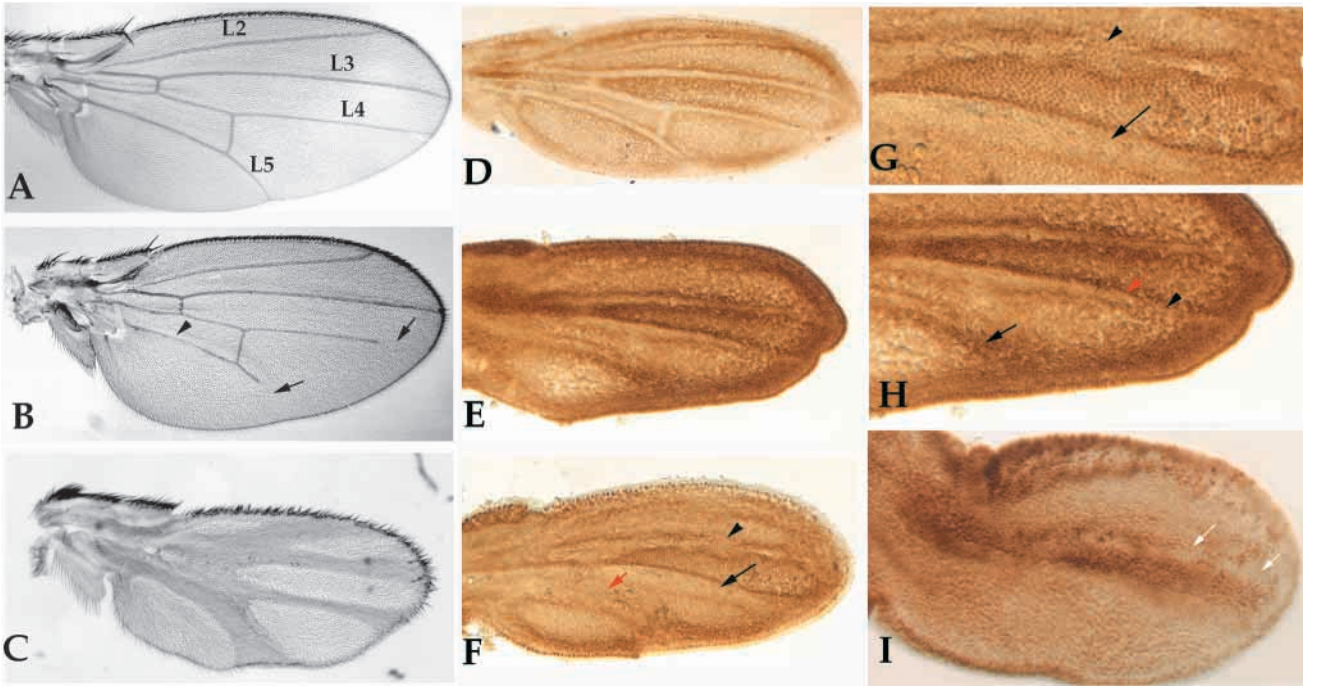

Fig. 6. *emc* expression in *Notch* mutant pupal wings. (A) Wild-type wing. (B) Ax^{16272} wing. (C) *Gal4c719/+; UAS-*N^{ecd}*/*emc^{P5c} wing. (D) *emc-lacZ* expression pattern in an emc^{P5c} pupal wing at 24-30 hours after puparium formation (APF). (E,H) *emc-lacZ* expression pattern in Ax^{16272}*;* emc^{P5c} pupal wing. (F,G) *lacZ* expression pattern in *Gal4c719/+; UAS-*N^{ecd}*/*emc^{P5c} pupal wing. (I) emc^{P5c} expression at 4-8 hours APF in *Gal4c719/+; UAS-*N^{ecd}*/*emc^{P5c} mutant wing. The white arrows indicate regions where the normal expression of *emc* is disrupted. G and H are higher magnifications of F and E, respectively. In Ax^{16172} pupal wings *emc* is misexpressed in the most distal part of L4 (H, arrowhead) and L5 veins (H, arrow). The red arrow (F,H) indicates the normal level of *emc* expression in the stripe of cells adjacent to L4.

veins L3 and L4 are disrupted in early pupae (4-8 hours APF; compare Figs 6I and 3B,C). In contrast, in pupal wings of the *Notch* gain-of-function allele Ax^{16172} *emc* is missexpressed in the regions corresponding to the vein stretches eliminated by this mutation (Fig. 6B,E,H). These results suggest that *Notch* signaling is required since the beginning of pupal development to establish the normal *emc* expression pattern and for increasing the levels of *emc* expression at vein/intervein boundaries.

Clones of cells expressing a negative form of *Notch* (N^{ecd}) confirm a requirement for *Notch* to establish the normal expression of *emc*. Thus, N^{ecd} clones straddling vein/intervein boundaries show a cell-autonomous reduction of *emc* expression in pupal wings (Fig. 7A-D). We have further analyzed whether the regulation of *emc* by *Notch* signalling depends on the *Dl* ligand by generating clones of cells expressing a dominant negative form of *Dl* (UAS-*DlD*; Huppert et al., 1997) in pupal wings. The effects of these clones on *emc* expression are identical to those observed in clones of N^{ecd}-expressing cells (Fig. 7E,F). To exclude the possibility that putative effects of *Notch* and *emc* acting on similar genes during larval development affected *emc* expression in pupae, we induced clones of *DL*-expressing cells at 0-6 hours APF (see Materials and Methods). In these clones the differentiation of some veins (L2, L4 and L5) is prevented, suggesting that *Notch* signalling is increased (Fig. 7K). In the corresponding pupal wings, *emc* is expressed in presumptive vein territories in cells adjacent to those expressing *Dl* ectopically (Fig. 7G-J). Altogether, these results suggest that *Notch* activation by *Dl* during pupal development is responsible for the upregulation of *emc* transcription at vein/intervein boundaries.

emc and *E(spl)mβ* cooperate to repress *ve* expression and vein formation

The expression of *E(spl)mβ* in pupal wings depends on the activity of *Notch* signalling, and it has been shown that *E(spl)mβ* is involved in the definition of the width of the vein (de Celis et al., 1997). Ectopic expression of *E(spl)mβ* (using the GAL4 line MS-1096) prevents the differentiation of all veins except L2 and proximal regions of L4 and L5 (data not shown), and this phenotype is associated with *ve* repression (Fig. 8B). In the corresponding pupal wings *emc* is expressed at basal levels throughout the wing blade, except in two regions that correspond to the presumptive veins L2 and L4, which show lower levels of *emc* (data not shown). This suggests that the expression of *emc* is not regulated by *E(spl)mβ*, and therefore it is likely that both *emc* and *E(spl)mβ* genes act in parallel in response to *Notch* activation. In this model, we expect that the phenotype of loss of vein caused by the ectopic expression of *E(spl)mβ* will be exaggerated when *emc* is simultaneously overexpressed. The ectopic expression of *emc* alone using several GAL4 strains does not affect vein differentiation, although, surprisingly, it occasionally causes ectopic veins (data not shown). However, when *emc* and *E(spl)mβ* are both ectopically coexpressed under the control of the GAL4 line MS1096, the loss of vein phenotype and the repression of *ve* characteristic of *E(spl)mβ* overexpression are exaggerated (Fig. 8A and not shown). These results suggest

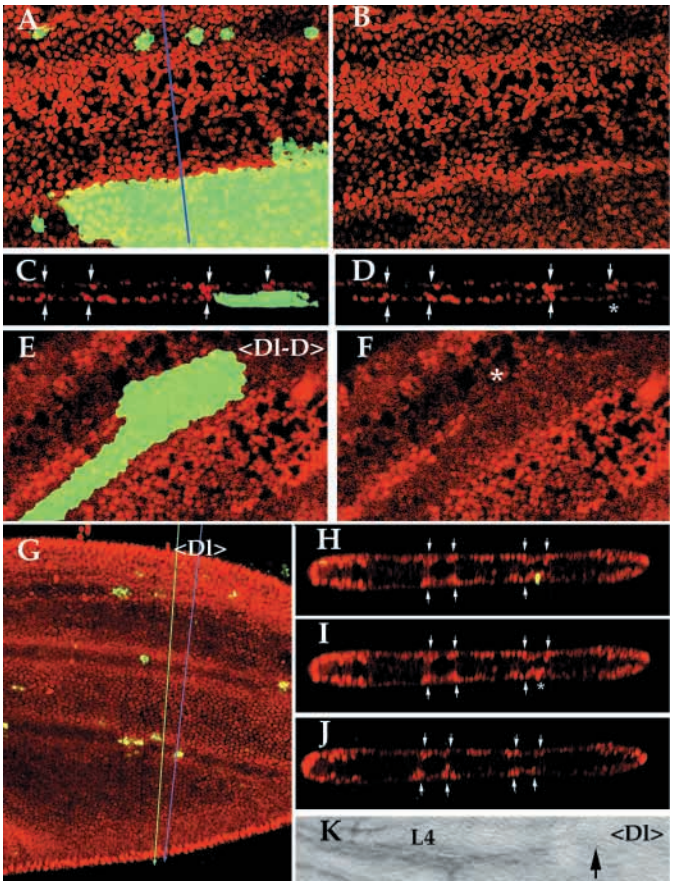

Fig. 7. Expression of *emc* in clones of cells with modified *Notch* activity. (A-D) Clones of N^{ecd}-expressing cells (green) autonomously prevent the accumulation of *emc* (red) in the cells flanking the veins. (C,D) Horizontal section of the same clone shown in A and B (corresponding to the blue line in A) showing the elimination of *emc* accumulation in the vein/intervein boundary affected by the clone (asterisk). Arrows show the remaining vein/intervein boundaries. (E,F) Clone of *DlD*-expressing cells preventing *emc* accumulation in the vein/intervein boundary cells (asterisk in F). (G-K) Effect on *emc* expression and vein differentiation of *Dl*-expressing clones induced during pupal development. (G-J) Position of two horizontal sections, indicated by blue (H,I) and green (J) lines, in a pupal wing with *Dl*-expressing clones. Arrows indicate vein/intervein boundaries (veins L3 and L4, from left to right). Observe the increased *emc* expression associated to a *Dl*-expressing clone within the vein L4 (asterisk in I) compared with a control region (J). (K) Failures on vein differentiation in distal L4 (arrow) caused by clones of *Dl*-expressing cells induced during pupal development.

that *emc* and *E(spl)mβ* act synergistically in vein differentiation during pupal development.

The possibility of direct protein-protein interaction between Emc and E(spl)Mβ was analysed using the yeast two-hybrid system. Interactions were assayed as the level of β-galactosidase activity obtained from a yeast strain bearing three constructs: emc-LexA DNA binding domain (pEG202-*emc*), lacZ reporter (pSH18-34) and the activation domain B42 on vector pJG4-5 fusion to the ORF of the genes *E(spl)mβ*, *E(spl)mδ* or *da* (Alifragis et al., 1997; see Materials and Methods). In these assays we have used *da* as a positive control and *E(spl)mδ* as a negative control (Alifragis et al., 1997). Whereas we observed strong interaction between Emc and Da, the interaction between Emc and E(spl)Mβ occurs at the same level as the negative control E(spl)Mδ (Fig. 8C), suggesting that the genetic interaction found between *emc* and *E(spl)m*β is not a consequence of interactions between the two proteins.

DISCUSSION

The functions of *emc* and *Notch* are required for cell proliferation and vein differentiation during the development of the *Drosophila* wing (de Celis and García-Bellido, 1994a; de Celis et al., 1995, 1997; Go et al., 1998; Baonza and García-Bellido, 1999), but it is not clear whether *emc* and *Notch* signalling are related to each other. The observed interactions between mutant alleles of *emc* and *Notch*, as well as the dependence of *emc* expression on *Notch* activity, suggest that *emc* acts as a downstream component of *Notch*, at least in wing margin formation and vein differentiation.

emc interacts with *Notch* signalling during wing disc proliferation

Notch mutant cells show reduced viability, whereas activation of *Notch* signalling causes strong mitotic activity in the wing disc, independently of the activation of *vestigial* and *wingless* (de Celis and Garcia-Bellido, 1994; Go et al., 1998). These observations suggest that *Notch*, in addition to its function in the establishment of the d/v boundary (Kim et al., 1995, 1996; Rulifson and Blair, 1995; Couso et al., 1995; Neumann and Cohen, 1996; de Celis and Bray, 1997) is also directly involved in the control of cell proliferation. In this function of *Notch* the genes of the *E(spl)* complex are not required (de Celis et al., 1996a,b). *emc* is also involved in regulating cell proliferation during wing disc development, because *emc* mutant cells do not proliferate at all, and clones of cells of strong *emc* hypomorphic alleles reduce cell proliferation in intervein territories (García-Alonso and García-Bellido, 1988; de Celis

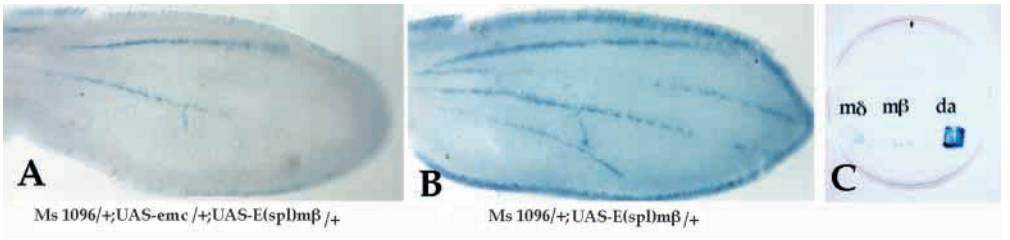

Fig. 8. Interactions between *emc* and *E(spl)mβ*. Expression of *veinlet* in (A) Gal4-MS1096/+; UAS-*E(spl)mβ*/UAS-*emc* and (B) Gal4-MS1096/+; UAS-*E(spl)mβ*. Ectopic coexpression of *E(spl)mβ* and *emc* under the control of the Gal4 MS1096 has stronger effects on suppressing *veinlet* expression than expression of only *E(spl)mβ*. (C) Results of the two-hybrid assay. *emc* and *da* show a strong interaction visualized by the intense blue color of the yeast colonies, whereas the interactions between *emc* and *E(spl)m*β or E(spl)mδ produce a weak coloration.

et al., 1995; Baonza and García-Bellido, 1999). Mutant cells for both *emc* and *Notch* have extremely poor viability, indicating that *emc* and *Notch* cooperate to promote cell proliferation. However, this interaction does not rely on *Notch* controlling *emc* transcription, as the basal level of Emc expression in the wing pouch is not affected in *Notch* mutant cells. Thus, we propose that during imaginal cell proliferation *emc* and *Notch* signalling act in parallel, possibly on the same set of downstream genes, to promote cell proliferation.

The expression of *emc* at the dorso/ventral border depends on the activity of the *Notch* signalling pathway

The activity of *Notch* is necessary for the formation and maintenance of the d/v boundary (de Celis and Garcia-Bellido, 1994; Kim et al., 1995, 1996; Rulifson and Blair, 1995; Couso et al., 1995; Díaz-Benjumea and Cohen, 1995; de Celis et al., 1996a; Neumann and Cohen, 1996; de Celis and Bray, 1997). Thus, loss of *Notch* prevents the formation of the wing margin and, conversely, ectopic *Notch* activity results in the formation of novel margin structures and wing outgrowths. During the third instar, *Notch* expression is maximal in the dorsal and ventral cells that form the d/v boundary (Fehon et al., 1991; Kooh et al., 1993). These cells also correspond to the places where *E(spl)mβ*, a *Notch*-downstream gene, is expressed, indicating high levels of *Notch* signalling here (Jennings et al., 1994; de Celis et al., 1996b, 1997; de Celis and Bray, 1997). The expression of *emc* at the d/v boundary is maximal in the same cells where *Notch* and *E(spl)mβ* genes are expressed, suggesting that *Notch* signalling could regulate *emc* expression. In fact, the expression of *emc* at the d/v border is eliminated in cells lacking *Notch* activity, whereas clones of cells expressing an activated form of *N* express ectopically high levels of Emc. Increased levels of Emc expression are also induced by the *Notch* ligands Dl and Ser in the dorsal and ventral compartments, respectively.

The regulation of *emc* expression at the d/v boundary by *Notch* is not mediated by *E(spl)mβ*, since clones of *E(spl)mβ*-expressing cells do not affect the expression of *emc*. Elimination of *E(spl)mβ* or *emc* does not affect the formation of the wing margin, indicating that these *Notch* targets are not required for *Notch* activity in the formation of this structure (de Celis et al., 1995; de Celis and Bray, 1997). However, *emc* and *E(spl)* are required during the formation of the sensory organs characteristic of the wing margin. Thus, ectopic expression of *emc* (or *E(spl)*) throughout the wing pouch eliminates most of the sensory elements of the anterior wing margin (data not shown). It is likely that this function of *emc* and *E(spl)* relies on the repression of the activity and expression of the Achaete and Scute proteins (Ellis et al., 1990; Garrell and Modolell, 1990; Van Doren et al., 1991, 1992; Cubas and Modolell, 1992).

Notch signalling and *emc* function in the establishment of the final width of the veins

The expression of several genes such as *ve* and *blistered* is restricted to either vein or intervein regions during imaginal development, indicating that at this stage the veins are being specified. A key component of vein specification is the activity of the DER signaling pathway, although it is not known which genes localise DER activation to vein territories. Both *emc* and *Notch* are required at this early stage to position vein territories and to define their extent (de Celis and García-Bellido, 1994a; Celis et al., 1995), respectively, and it is likely that *Notch* and *emc* interact during the definition of vein territories in third instar wing discs. This interaction could be based in the regulation by Notch and Emc of similar target genes controlling the appearance and extent of vein-competent territories (Fig. 9A). However, our results suggest that in this initial establishment of vein territories the expression of *emc* and the activity of *Notch* are independent of each other, because the heterogeneity in *emc* expression related to developing veins observed in third instar discs is not modified in *Notch* mutant backgrounds. Furthermore, some characteristic phenotypes of *emc* clones, such as the appearance of ectopic veins of normal thickness, are never observed in *Notch* clones, indicating that *emc* and *Notch* are affecting independent processes during the initiation of vein development (Fig. 9A).

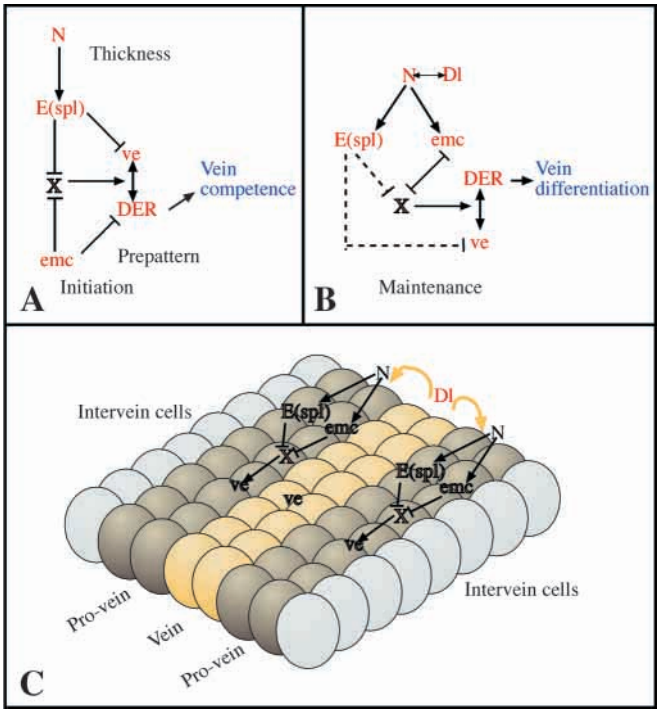

Fig. 9. Model for *Notch* and *emc* function during vein development. (A) Initiation. The functions of *emc* and *Notch* are required from early stages to define the position (Prepattern) and width (Thickness) of vein territories, respectively. These activities can be mediated by affecting DER signalling (DER and *ve*), which is a key component in the specification of veins. In addition, we propose the existence of other genes directing vein formation and encoding transcription factors (X), expression and activity of which are suppressed by E(spl)mβ and Emc, respectively. The activity and expression of *emc* at this stage is independent of Notch signalling. (B) Maintenance. The expression of *E(spl)mβ* and *emc* is activated by *Notch* signalling in the boundary cells that separate each vein from the adjacent interveins during pupal development. In these cells *E(spl)mβ* and *emc* repress and antagonise the expression and activity, respectively, of the gene X, which in turn would be involved in the maintenance of *veinlet* expression. In addition it is also possible that *E(spl)mβ* represses directly the expression of *ve* (dotted line). (C) Genetic interactions in vein/intervein boundaries

After puparium formation the activity of *Notch* is continuously required to maintain the correct width of the vein, and at this stage *Notch* activation occurs in two stripes of cells adjacent to each vein. The accumulation of *E(spl)mβ* in these cells, as a consequence of *Dl*-mediated *Notch* activation, contributes to the restriction of *ve* expression to the vein, and prevents the differentiation as vein of the flanking pro-vein cells (de Celis et al., 1997). Interestingly, the elimination of *Notch* or *Dl* activity results in the formation of thicker veins than elimination of *E(spl)mβ*, suggesting that additional elements are activated in response to *Notch* and participate in the repression of vein differentiation (de Celis et al., 1997). Several arguments suggest that *emc* is one of these components that mediate *Notch* signalling during the pupal development of veins. First, the expression of *emc* in pupal wings is maximal in the same cells that express *E(spl)mβ*, suggesting that *Notch* activity is responsible for the preferential accumulation of *emc* expression. This expression is modified when *Notch* activity is compromised, being detected in the novel flanking cells associated with the thickened *Notch* mutant veins. Second, clones of *emc* mutant cells occasionally cause vein thickening, and this phenotype is greatly exaggerated in *Notch* and *Dl* mutant backgrounds, suggesting that in a situation of insufficient *Notch* activity, the levels of *emc* are critical to repress vein formation. The analysis of *emc* clones in $l(1)N^{ts}$ heterozygotes indicates that during the pupal stage cells are particularly sensitive to reduction in *emc* and *Notch* activities. In addition, clones of *Dl*-expressing cells induced during pupal development cause ectopic expression of *emc*, indicating that during this stage the activity of *Notch* is enough to increase the levels of *emc*. These results do not discard an earlier requirement for both genes in vein determination, but show that during pupal development *emc* and *Notch* do interact in the definition of vein thickness.

The molecular basis of this interaction is unclear; so far there is no *emc*-target gene identified affecting vein formation. By analogy to the function of *emc* in antagonising the activity of proneural proteins, we postulate that Emc modulates the function of some protein involved in promoting vein formation. Interestingly, when both *emc* and *E(spl)mβ* are overexpressed, we observe an enhancement of the *E(spl)mβ* overexpression phenotype of loss of veins, suggesting that the combination of high levels of both *emc* and *E(spl)mβ* results in more effective repression of vein differentiation. Thus, we propose that *Notch* signaling, in addition to activating the expression of *E(spl)mβ*, induces high levels of *emc* expression in flanking cells, and that the combination of *emc* and *E(spl)mβ* is more efficient in suppressing vein formation than *E(spl)mβ* alone. Emc and E(spl) do not interact with each other, and therefore it is unlikely that *emc* contributes to E(spl)mβ activity. Therefore we suggest that Emc and E(spl)mβ contribute to the regulation of the activity and expression of a vein-promoting protein and gene, respectively, thus explaining the observed synergy between *Notch* signaling and *emc* function in vein formation (Fig. 9B,C).

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