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-β. 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-β.

Key words: *extramacrochaetae*, *Notch* signalling, vein differentiation, Enhancer of split, *Drosophila 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, 1994; 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 DI 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 Suppressor 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 DI 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 Garcia-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 (Garcia-Alonso and Garcia-Bellido, 1988; de Celis et al., 1995; Baonza and Garcia-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 and the basic helix-loop-helix (bHLH) proteins (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 DI, 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 dorsoventral 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<sup>-1</sup> (Garcia-Alonso and Garcia-Bellido, 1988), the deficiency Dp(3L)emc<sup>E12</sup>, the duplication Dp(3;Y)M2, mwh<sup>+</sup>, emc<sup>+</sup> and the gain-of-function emc<sup>ach</sup> (Garcia-Alonso and Garcia-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<sup>+</sup> clones was M(3)65F (Lindsley and Zimm, 1992). At the Notch locus we used the null allele N<sup>55e11</sup>, the temperature-sensitive allele l(1)N<sup>T</sup> and the gain-of-function allele Ax<sup>M3</sup> and Ax<sup>16172</sup> (de Celis and Garcia-Bellido, 1994b); at the DI locus we used the allele Dp<sup>DI</sup> (Diaz-Benjumea and Garcia-Bellido, 1990). We also used two reporter lines, a PlacZ insertion in emc (emc<sup>BSC</sup>; 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<sup>extra</sup>, UAS-N<sup>end</sup>, UAS-Scr (de Celis and Bray, 1997), UAS-DI and UAS-DAD (a negative form of DI; Huppert et al., 1997), and the GAL4 lines GAL4-MS1096 (Capdevila and Guerrero, 1994) and GAL4-c719 (kindly provided by E. Martin-Blanco).

#### Generation of UAS-emc

A full-length emc cDNA was cloned into the EcoRI 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 Kυ, 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<sup>-</sup> M<sup>+</sup> clones were induced in flies of the following genotypes: mwh emc<sup>-</sup>Ma/M(3)65F, l(1)N<sup>T</sup>+</sup>; mwh emc<sup>-</sup>Ma/M(3)65F, N<sup>55e11</sup>+; mwh emc<sup>-</sup>Ma/M(3)65F, Ax<sup>M3</sup>+</sup>; and mwh emc<sup>-</sup>Ma/M(3)65F and mwh emc<sup>-</sup>Dp<sup>DI</sup>/M(3)65F. Mitotic recombination proximal to the Minute mutation results in emc<sup>-</sup> clones labeled with mwh. emc<sup>-</sup> clones in l(1)N<sup>T</sup>+/+; mwh emc<sup>-</sup>Ma/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<sup>-</sup>/emc<sup>-</sup> clones were induced in flies of the following genotypes: (1) Dp<sup>3;Y</sup>1M2, mwh<sup>+</sup>; emc<sup>-</sup>/emc<sup>-</sup> mwh emc<sup>-</sup>/mwh [3L]emc<sup>12</sup>, (2) Dp<sup>3;Y</sup>1M2, mwh<sup>+</sup>; emc<sup>-</sup>/emc<sup>-</sup> mwh [3L]emc<sup>12</sup>. Mitotic recombination proximal to forked results in mwhf clones labeling emc<sup>-</sup>/Df(3L)emc<sup>12</sup> and emc<sup>-</sup> cells, respectively. In addition mwh<sup>-</sup>/Df(3L)emc<sup>12</sup> cells will also be homozygous for N<sup>55e11</sup> (2) or Ax<sup>M3</sup> (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<sup>69a</sup> FLp1.22; P(abx/Ubx<sup>FRT</sup> f<sup>FRT</sup>) GAL4-lacz/UAS-N<sup>extra</sup>. The flip-out of the <sup>FRT</sup> f<sup>FRT</sup> cassette results in the expression of a GAL4-lacz-tpUAS hybrid gene under the control of the abo/Ubx promoter. Clones were detected by the expression of β-gal (de Celis and Bray, 1997). (2) y w FLp1.22; Act5C<sup>FRT</sup> yellow<sup>FRT</sup> GAL4 UAS-lacZ or UAS-GFP/UAS-X, where X is UAS-N<sup>end</sup>, UAS-DI, UAS-DAD or UAS-Scr. The flip-out of the <sup>FRT</sup> yellow<sup>FRT</sup> 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-DI. 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-DI (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 collE1 LexA operator, which bind two LexA dimers (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′ EcoRI-3′XhoI 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. pEG4-5 plasmid containing the B42 activation domain, was used as an activation vector. The pG4-5 with the insertion of E(spl)m7, E(spl)mδ and da were kindly provided by C. Delidakis (Alfrigas 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 (Garçia-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/DI(emc) mutant cells is not rescued by the homozygosity of a Notch gain of function allele (AxM3; 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 emc1 clones induced in two different Notch mutant backgrounds (see Materials and Methods). The width of the veins formed by emc1 mutant cells in N55e11 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 N55e11 homozygous clones (de Celis and Garcia-Bellido, 1994a). In contrast, emc1 clones induced in heterozygous AxM3 wings never cause the formation of thicker veins (Fig. 1C), causing phenotypes similar to emc1 clones induced in wild-type control wings. emc clones were also induced in DI (DpM1) heterozygous wings, which have a dominant vein thickening phenotype affecting mainly the distal part of the veins. The phenotype of the emc1 clones in these wings is similar to that found in N55e11 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 emc1 clones in l(1)N55 heterozygous females that were grown at the restrictive temperature during larval or pupal development (see

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hours AEL</th>
<th>Number of wings</th>
<th>f/mwh clones</th>
<th>f/mwh cells</th>
<th>f clones</th>
<th>f cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Dp(3;1)M2, mwh+, emc+/f66A/s; emc/Df(3L)emcF12</td>
<td>48-72</td>
<td>732</td>
<td>73</td>
<td>286/44</td>
<td>105</td>
<td>474</td>
</tr>
<tr>
<td></td>
<td>72-96</td>
<td>82</td>
<td>67</td>
<td>56/13</td>
<td>24</td>
<td>66</td>
</tr>
<tr>
<td>(2) Dp(3;1)M2, mwh+, emc+/f66A/N55e11; emc/Df(3L)emcF12</td>
<td>48-72</td>
<td>279</td>
<td>0</td>
<td>–</td>
<td>42</td>
<td>590</td>
</tr>
<tr>
<td></td>
<td>72-96</td>
<td>173</td>
<td>27</td>
<td>33/5</td>
<td>119</td>
<td>117</td>
</tr>
<tr>
<td>(3) Dp(3;1)M2, mwh+, emc+/f66A/AxM3; emc/Df(3L)emcF12</td>
<td>48-72</td>
<td>68</td>
<td>14</td>
<td>143/34</td>
<td>18</td>
<td>176</td>
</tr>
</tbody>
</table>

AEL, after egg-laying; f, forked; mwh, multiple wing hairs.

For details of mutants, see Materials and Methods.
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 emc1 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 emc1 M+ clones in an N55e11+/+ wing. The fused veins L4 and L5 are much thicker than normal veins (E) (arrowheads indicate the width of the fused veins). (F) emc1 M+ clone induced in a Dp1/M+ mutant wing, differentiating a thicker dorsal L5. The effects produced by this and similar clones are identical to those observed in N55e11+/+ wings. Dotted and solid lines indicate the extension of each clone in the dorsal and ventral surfaces, respectively.

Fig. 1. Vein differentiation defects caused by emc1 mutant clones induced in different mutant backgrounds affecting Notch signalling. (A) L5 vein in a wild-type (Wt) wing. (B) Large emc1 M+ clone covering L5 and causing the thickening of this vein. (C) Dorsal emc1 M+ clone induced in an Ax/M+/+ wing. The width of the veins is not affected. (D,E) emc1 M+ clones induced in N55e11+/+ mutant wings. Dorsal and ventral emc1 M+ clones differentiate a L5 vein thicker than a normal vein and than veins of N55e11+/+ mutant wings (D). The intervein region between L4 and L5 is obliterated by a dorsal and ventral emc1 M+ clones in an N55e11+/+ wing. The fused veins L4 and L5 are much thicker than normal veins (E) (arrowheads indicate the width of the fused veins). (F) emc1 M+ clone induced in a Dp1/M+ mutant wing, differentiating a thicker dorsal L5. The effects produced by this and similar clones are identical to those observed in N55e11+/+ wings. Dotted and solid lines indicate the extension of each clone in the dorsal and ventral surfaces, respectively.

Fig. 2. Phenotype of emc1 M+ clones in l(1)N55e11+/+ 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)N55e11+/+ emc1 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 emc1 M+ clones induced in l(1)N55e11+/+ 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.
emc is downstream of Notch signalling 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<sup>P5C</sup>, 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...
in complementary regions, with DI 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 DI 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 DI suggest that emc transcription could be in part regulated by Notch signalling in a DI-dependent 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 (Necd) 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 Necd and N intra clones. Clones of cells expressing high levels of Necd 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, Necd 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 DI or Ser induce the expression of several target genes in cells adjacent to the clone in the dorsal or venial compartments, respectively (de Celis and Bray, 1997). However, clones of DI- or Ser-expressing cells induced using a weaker promoter (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, DI 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 DI-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 (fa1d mutants, not shown, or by expressing a dominant negative form of Notch, c-719/+; Necd/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
emc is downstream of Notch signalling

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 Ax16172 emc is misexpressed in the regions corresponding to the vein stretches eliminated by this mutation (Fig. 6B,E,H). These results suggest that Notch signalling 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 (Necd) confirm a requirement for Notch to establish the normal expression of emc. Thus, Necd 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-DID; Huppert et al., 1997) in pupal wings. The effects of these clones on emc expression are identical to those observed in clones of Necd-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
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)mβ 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 García-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 et al., 1996a,b).
Elimination of same cells where Notch and emc (1994; de Celis et al., 1996b, 1997; de Celis and Bray, 1997). The expression of several genes such as blistered and ve establish the final width of the veins. Notch and emc function in the establishment of the final width of the veins. Both emc and Notch signalling and emc function in the establishment of the final width of the veins.

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., 1995b, 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 DI 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 signalling 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](image)
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 $E(spl)mB$ in these cells, as a consequence of $DI$-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 $DI$ activity results in the formation of thicker veins than elimination of $E(spl)mB$, 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 $E(spl)mB$, 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 $DI$ 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 $l(l)N^{2}$ heterozygotes indicates that during the pupal stage cells are particularly sensitive to reduction in emc and Notch activities. In addition, clones of $DI$-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. We suggest that Emc and E(spl)m contribute to the regulation of the activity and expression of a vein-promoting protein and gene, respectively, thus explaining the observed synergy between Notch signalling and emc function in vein formation (Fig. 9B,C).

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