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EGFR signalling inhibits Capicua-dependent repression during specification of *Drosophila* wing veins

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SUMMARY

Localised activation of the Ras/Raf pathway by Epidermal Growth Factor Receptor (EGFR) signalling specifies the formation of veins in the *Drosophila* wing. However, little is known about how the EGFR signal regulates transcriptional responses during the vein/intervein cell fate decision. We provide evidence that EGFR signalling induces expression of vein-specific genes by inhibiting the Capicua (Cic) HMG-box repressor, a known regulator of embryonic body patterning. Lack of Cic function causes ectopic expression of EGFR targets such as *argos*, *ventral veinless* and *decapentaplegic* and leads to formation of extra vein tissue. In vein cells, EGFR signalling downregulates Cic protein levels in the nucleus and relieves repression of vein-specific genes, whereas intervein cells maintain high levels of Cic throughout larval and pupal development, repressing the expression of vein-specific genes and allowing intervein differentiation. However, regulation of some EGFR targets such as *rhomboid* appears not to be under direct control of Cic, suggesting that EGFR signalling branches out in the nucleus and controls different targets via distinct mediator factors. Our results support the idea that localised inactivation of transcriptional repressors such as Cic is a rather general mechanism for regulation of target gene expression by the Ras/Raf pathway.

Key words: Capicua, Bullwinkle, Ras/Raf pathway, Transcriptional repression, Vein development, *Drosophila*

INTRODUCTION

Activation of receptor tyrosine kinases (RTKs) by secreted growth factors regulates cell proliferation and cell fate determination in many different cellular and developmental contexts. Despite such widespread functions, RTK signalling involves a common cytoplasmic cascade including Ras, and the serine-threonine kinases Raf, MAPKK/MEK and MAPK. Thus, a central issue is how activation of the same cytoplasmic components elicits distinct responses depending on the cell context (Hill and Treisman, 1995; Simon, 2000). The nuclear response to the signal depends in many cases on the availability of different transcription factors for direct phosphorylation by the Ras/Raf pathway. In addition, RTK signalling can trigger mechanisms of either activation or repression of gene expression, adding an extra level of complexity to the final output. Thus, cell responses to RTK signalling are largely determined by the mechanisms and factors that mediate transcriptional regulation downstream of the RTK cascade.

A well-established model of RTK signalling concerns the formation of veins in the *Drosophila* wing. The insect wings consist of a bilayer of epidermal cells closely apposed by their basal sides that secrete a thin and transparent cuticle. In *Drosophila*, wings display a stereotyped pattern of five longitudinal veins and two transverse crossveins. The veins are specialised epithelial cells present in both wing surfaces. During late differentiation, dorsal and ventral veins match accurately providing a hollow space for the passage of axons and trachea (García-Bellido and de Celis, 1992).

Vein tissue specification occurs during larval and pupal stages within the wing imaginal discs, and involves a complex network of gene regulation. Central to this network is the Epidermal Growth Factor Receptor (EGFR) RTK (Díaz-Benjumea and Hafen, 1994), which, in *Drosophila*, functions in a wide spectrum of developmental processes such as oogenesis, patterning of embryonic structures such as the ventral epidermis and trachea, and specification of photoreceptor cells in the compound eye (Schweitzer and Shilo, 1997). In wing discs, activation of EGFR in rows of cells causes their determination as prospective wing veins. Consistent with this function, loss-of-function mutations in components of the EGFR pathway cause loss of veins, whereas gain-of-function alleles and/or overexpression of those components result in ectopic vein development (Díaz-Benjumea and Hafen, 1994).

The role of EGFR signalling in vein patterning involves complex molecular interactions that (1) restrict its activity to appropriate cells, and (2) impose cell fate changes in response to signalling. Activation of EGFR in precise rows of cells

depends on several signalling pathways that pattern the wing disc (de Celis and Barrio, 2000), and relies ultimately on the localised activity in the veins of different EGFR ligands and the Rhomboid (Rho) transmembrane protein, a mediator of EGFR activation (Schnepp et al., 1996; Sturtevant et al., 1993). Regulated EGFR activation specifies vein fates by both activation and repression of several downstream target genes. These encode transcription factors and also signalling molecules, such as the Decapentaplegic (Dpp) TGFβ fly homologue, that have to be activated in the veins for their proper differentiation (de Celis, 1997).

Although a broad picture of how veins are specified is emerging, little is known about how the EGFR cascade regulates target gene expression in the nucleus of developing vein cells. In this work, we present evidence that the EGFR pathway functions, at least in part, by promoting destabilisation of the Capicua (Cic) protein in vein nuclei. Cic is an HMG-box transcription factor that controls early embryonic patterning in response to Torso RTK signalling. Cic functions as a repressor of two genes expressed in the terminal regions of the embryo, *tailless* (*tll*) and *huckebein* (*hkb*). Cic accumulates in central regions of the embryo, but is under negative post-transcriptional control by Torso signalling at the termini, thus permitting expression of *tll* and *hkb* at the embryonic poles (Jiménez et al., 2000).

Our results support a similar regulation of the Cic protein by EGFR signalling in the wing. We have found that Cic activity prevents differentiation of vein tissue in the wing pouch. In turn, restricted activation of the Ras/Raf pathway in the presumptive veins antagonises Cic repressor activity in this tissue, thus allowing localised expression of genes mediating vein differentiation. We discuss the role of Cic as a general transcriptional repressor regulated by the Ras/Raf pathway.

MATERIALS AND METHODS

Fly stocks

We have used the following genetic variants: the cic^2 allele (formerly *E(Dl)D49*) (Klein and Campos-Ortega, 1992), cic^1, $bwk^{\Delta 4}$ and $bwk^{\Delta 81}$(Jiménez et al., 2000), bwk^{8482}, $bwk^{\Delta 14}$ $bwk^{\Delta 11}$ mutants (Rittenhouse and Berg, 1995). We also used the second chromosome insertion $P[w^+; cic{:}flu]60$, a *flu*-tagged version of the rescuing construct containing the *cic* complete coding sequence (Jiménez et al., 2000) (see Fig. 1). As loss of function alleles in the Ras/Raf pathway, we used the *Ras-1* null allele $Ras^{\Delta C40b}$ (Hou et al., 1995). EGFR ectopic activation was achieved by overexpressing the protein Vein (Vn) with an UAS Vn (Schnepp et al., 1996) and the EngrailedGAL4^{e21A} line (Brand, 1997). Other mutants used were the rho^{ve} vn^1 combination (Díaz-Benjumea and García-Bellido, 1990) and the $argos^{w11}$ *lacZ* reporter (Freeman et al., 1992). To counterstain the cell nuclei in some preparations, staining was carried out in flies expressing ubiquitous nuclear GFP (Davis et al., 1995).

Characterisation of the *cic²* allele

The lethal mutation cic^2 was crossed with several deficiencies of the third chromosome deficiency kit (kindly provided by C. Balayo), covering the 92B3-95D6 interval. Of these, *Df(3R)Dl-BX12* and *Df(3R)HB-79* were found to uncover cic^2, mapping the mutation to region 92B-D. Further crosses with *cic* and *bwk* alleles confirmed that cic^2 belongs to these related complementation groups. To characterise cic^2 molecularly, genomic fragments corresponding to the mutant gene were amplified by PCR and sequenced directly.

Clonal analysis

Adult *cic* mutant FRT/FLP (Xu and Rubin, 1993) M^+ clones marked with the *forked* mutation were induced in males of the following genotypes: $yhsFLPf^{36a;}$ *FRT82B UbqGFP83* f^{+87D} *M(3)95A/FRT82B* $bwk^{\Delta 11}$ *e* and $yhsFLPf^{36a;}$ *FRT82B UbqGFP83* f^{+87D} *M(3)95A/FRT82B sr* cic^2 *e*. Clones mutant for *Ras-1* and the double mutant clones *Ras-1/cic* were induced, respectively, in males of the genotype $yhsFLPf^{36a}$*; FRT82B UbqGFP83* f^{+87D} *M(3)95A/FRT82B* $Ras^{\Delta C40b}$ and $yhsFLPf^{36a;}$ *FRT82B UbqGFP83* f^{+87D} *M(3)95A/FRT82B* $Ras^{\Delta C40b}$*sr* cic^2 *e.* The expression of the *argos lacZ* reporter was monitored in *cic* mutant clones generated in flies with the genotype $yhsFLPf^{36a;}$ *FRT82B UbqGFP83* f^{+87D} *M(3)95A/aos*^{w11} *FRT82B sr* cic^2 *e*. Clones were generated at 60±12 hours AEL with a 5 minutes heat shock pulse at 37°C.

Staining procedures

Immunostaining was performed according to standard protocols. For staining of Cic protein we used a polyclonal antibody from rat (1/300). Other primary antibodies were: mouse anti-Bs (1/600) (kindly provided by E. Martín-Blanco), rat anti-Vvl (1/300) (Llimargas and Casanova, 1997), mouse anti-β-gal (1/100) (Promega) and rabbit anti-β-gal (1/1000) (Promega). Secondary antibodies include anti-rat and anti-mouse conjugated to fluorescein and Texas Red and anti-rabbit and anti-rat conjugated to Cy5, all of them diluted 1/200 (Jackson Laboratories). Fluorescence was visualised in a Leica TCS confocal microscope. In situ hybridisation was carried out according to Sturtevant et al. (Sturtevant et al., 1993) with DIG-labelled antisense riboprobes made with the full cDNAs of the *dpp*, *rho* and *cic* genes.

RESULTS

capicua and *bullwinke* mutations affect a single gene

The *E(Dl)D49* mutation was identified in a screen for dominant enhancers of the wing vein phenotype of *Dl/+* flies (Klein and Campos-Ortega, 1992). *E(Dl)D49* is a recessive lethal mutation, but the heterozygous flies present with variable penetrance small patches of extravein tissue in the distal part of the L5 wing vein. Using standard recombination and deficiency tests, *E(Dl)D49* was mapped to the 92B-D cytological region, in the right arm of the third autosome (see Materials and Methods). Crosses with known mutations in the same region showed that *E(Dl)D49* is allelic to two sets of mutations: *bullwinkle* (*bwk*) and *capicua* (*cic*) (Rittenhouse and Berg, 1995; Jiménez et al., 2000). For convenience, we renamed the *E(Dl)D49* allele cic^2 .

The original *bwk* allele, bwk^{8482}, is a P-element-induced female sterile mutation that affects the patterning of the ovary follicle cells and the early embryo, giving rise to embryos with abnormal eggshells that also show a bicaudal phenotype (Rittenhouse and Berg, 1995). bwk^{8482} also has zygotic effects: homozygous adult flies display ectopic vein tissue in their wings (Rittenhouse and Berg, 1995). In contrast, the original cic^1 allele is a female sterile mutation caused by the insertion of a hobo transposon 300 bp away from the bwk^{8482} insertion (Fig. 1). Homozygous cic^1 females produce embryos with strongly suppressed trunk regions that are replaced by terminal structures (Jiménez et al., 2000).

The genetic relationship between *cic* and *bwk* mutations is unclear. Although cic^1 and bwk^{8482} complement each other, both mutations are uncovered by specific small deficiencies (less than 500 bp) in the region (Jiménez et al., 2000). To help

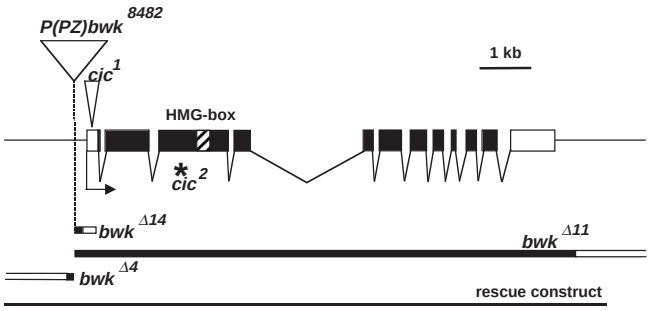

Fig. 1. Diagram of the *cic* locus and several mutations affecting the gene. The organisation of the major maternal transcript is shown; coding and untranslated regions are depicted by black and white boxes, respectively. The *P(PZ)bwk*^{8482} (P-element) and *cic*^{1} (hobo) transposon insertions are indicated by triangles. The *cic*^{2} allele maps ~380 bp upstream of the HMG-box region (hatched) and consists of a deletion of 13 nucleotides, tctgattgtgtcc. Fragments deleted by three independent *P(PZ)bwk*^{8482}-derived excisions are indicated by bars (open ends denote uncertainties in their limits). The genomic region included in the *cic*$^{+}$ rescuing construct is also indicated.

clarify this issue, we analysed the phenotypes observed in heteroallelic combinations of several *cic*- and *bwk*-related alleles. This analysis shows that both *cic*^{1} and *bwk*^{8482} mutations are not complemented by *cic*^{2} and specific P-element excision alleles obtained by mobilisation of *bwk*^{8482} (e.g. *bwk*$^{\Delta 11}$ and *bwk*$^{\Delta 14}$) (Fig. 1), resulting in *cic*- and *bwk*-associated phenotypes, respectively (Table 1). Thus, *cic*^{2}, *bwk*$^{\Delta 11}$ and *bwk*$^{\Delta 14}$ behave as strong hypomorphs or nulls for both *cic* and *bwk* genetic functions. These observations, together with the proximity of the *bwk*^{8482} and *cic*^{1} transposon insertions, raise the possibility that *cic* and *bwk* mutations disrupt different functions of a single gene. Alternatively, *bwk* and *cic* may be adjacent genes that are simultaneously affected by *cic*^{2} and some *bwk*^{8482} derived excisions.

As *cic*^{2} is an EMS-induced mutation, we looked for a point molecular lesion in the *cic*-coding region associated to the *cic*^{2} chromosome, and identified a deletion of 13 bp causing a frameshift upstream of the Cic HMG box (Fig. 1). Thus, a complete absence of *cic* activity recapitulates all known defects of *cic* and *bwk* mutations, strongly indicating that all these mutations affect the *cic* gene. In this context, the *cic*^{1} and *bwk*^{8482} mutations probably represent regulatory alleles that affect only specific *cic* functions. As the hobo element that causes the *cic*^{1} mutation is inserted within the first exon of the gene, we suspect that *bwk* specific functions depend on use of an alternative promoter located upstream of the *bwk*^{8482} insertion (Fig. 1). Consistent with these ideas, a genomic construct containing the complete *cic* transcription unit and 1.2 kb of upstream region rescues the *cic*^{1} related terminal defects, and significantly reduces the extravein phenotypes of *cic*^{2}/*bwk*$^{\Delta 14}$, *cic*^{2}/*bwk*^{8482} and *cic*^{2}/*bwk*$^{\Delta 11}$ flies. However, this genomic construct fails to rescue the chorion phenotype of embryos laid by *bwk*^{8482} females (Jiménez et al., 2000), possibly because it lacks specific regulatory regions required for *bwk*-associated functions (Rittenhouse and Berg, 1995).

Cic is required during wing development for intervein specification

To study the role of *cic* in wing development, we examined the effects of a complete loss of function of the gene in an heteroallelic combination of the *cic*^{2} allele with the deficiency *bwk*$^{\Delta 11}$, which removes the entire *cic*-coding region (Fig. 1). This combination is generally lethal, but adult scapers can be obtained with a low frequency. The most striking feature of these flies is their abnormal wings, which are reduced in size and often show blisters that separate the ventral and dorsal surfaces. The mutant wings display abundant extravein tissue, preferentially close to the normal veins, which appear thickened (Fig. 2A-D). This phenotype is a consequence of intervein cells acquiring morphological features of vein cells, which are typically smaller, more pigmented and with shorter and thicker trichomes than wild-type intervein cells. However, close examination reveals that the transformation to vein tissue is not complete and many intervein cells acquire intermediate morphologies between those shown by typical vein and intervein cells (Fig. 2C,D). In addition, some regions such as the area between veins L3 and L4 never differentiate as vein tissue in mutant wings, indicating that specification of intervein fate in this territory is independent of *cic* (see Discussion). Other structures, such as the wing margin bristles and the campaniformia sensilla located along the L3 vein, appear in their normal positions in the mutant wings, indicating that *cic* absence only affects the process of vein/intervein specification.

We have not detected additional defects in *cic*^{2}/*bwk*$^{\Delta 11}$ adult flies, except in the external parts of the male genitalia. In these males, the cuticle plates forming the genitalia are oriented in

Table 1. Phenotypes of mutant alleles

	Mutant allele						
Mutant allele	*cic*^{1}	*bwk*$^{\Delta 4}$	*bwk*^{8482}	*bwk*$^{\Delta 14}$	*bwk*$^{\Delta 81}$	*cic*^{2}	*bwk*$^{\Delta 11}$
cic^{1}	Terminal	Wild type	Wild type	Terminal	Terminal	Terminal	Terminal
bwk^{8482}	Wild type	Extra veins Bwk	Lethal*	Extra veins Bwk	Extra veins Bwk	Extra veins Bwk	Extra veins Bwk
cic^{2}	Terminal	Extra veins Bwk	Extra veins Bwk	Extra veins Almost lethal	Lethal	Lethal	Extra veins Almost lethal

*Although the original *bwk*^{8482} mutation is homozygous viable (Rittenhouse and Berg, 1995), in our hands, the *bwk*^{8482}-bearing chromosome now behaves as a homozygous lethal.

Wild type, adult flies of the corresponding genotype are morphologically normal and fertile; terminal, adult females morphologically normal but laying embryos with transformations from trunk to terminal parts; extra vein, adult wings differentiating ectopic veins; bwk, adult females lay few eggs displaying eggshell defects; almost lethal, cases where the female progeny could not be scored.

cic^{1}, mutation that affects only the terminal function; *bwk*$^{\Delta 4}$, *bwk*^{8482}, mutations that affect the wing and Bwk functions, but not the terminal; *bwk*$^{\Delta 14}$, *bwk*$^{\Delta 81}$, *cic*^{2}, *bwk*$^{\Delta 11}$, mutations that affect all three functions.

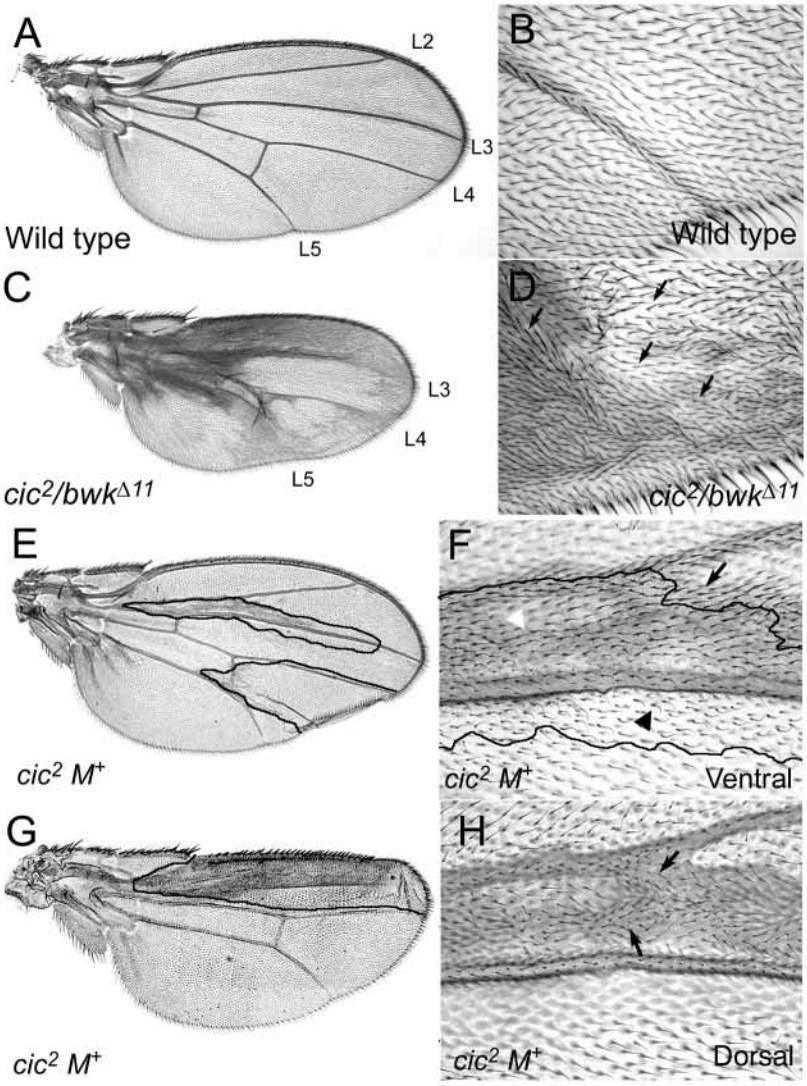

Fig. 2. Mutations in *cic* cause the development of ectopic veins. (A,B) Wild-type and $cic^2/bwk^{\Delta 11}$ (C,D) adult wings showing the L2-L5 longitudinal veins. (B,D) Show a close view of the region corresponding to the L5 tip. Note the presence of cells showing different degrees of pigmentation, and bearing hairs of different length and thickness in D (small arrows). (E,F,H) Adult wing containing two cic^2 M^+ homozygous clones marked with the *forked* hair marker. (F) Close view of the anterior ventral clone shown in E, showing that *cic* mutant cells can differentiate either as ectopic vein tissue (white arrowhead) or as intervein tissue (black arrowhead). Note also a patch of cells outside the clone that differentiate as an ectopic vein (small arrow). (H) Close view of the wild-type dorsal surface opposite the ventral clone shown in F, displaying patches of ectopic veins (small arrows). (G) Adult wing containing a dorsal and ventral cic^2 M^+ clone occupying all the anterior compartment. In this mosaic wing, all the region between the margin and the L3 differentiates as vein tissue, whereas the region between L3 and the AP boundary differentiates as intervein, exactly as in the $cic^2/bwk^{\Delta 11}$ mutants.

opposite direction to those of wild-type males, as if the whole structure was rotated 180° (not shown). This phenotype could result from defects in the musculature associated to the male genitalia, but we have not studied it in detail.

To analyse the effects of *cic* in vein development further, we generated clones homozygous for either cic^2 or $bwk^{\Delta 11}$ mutations with the *Minute* technique, which allows the generation of clones that cover large territories (Morata and Ripoll, 1975) (see Materials and Methods). Both sets of experiments gave similar results; we describe below those corresponding to cic^2. In our analysis, we studied 45 clones, covering the complete wing surface. The mutant clones are consistently smaller than their respective controls, suggesting that proliferation of cic^2 mutant cells is impaired. Mutant cells often differentiate as vein tissue in ectopic positions, particularly if they are close to normal veins (Fig. 2E-G). However, these changes in cell fate are not strictly cell autonomous, as many intervein cells within the mutant clones differentiate normally. This could be due in part to a rescue of the phenotype by wild-type cells situated in the other surface of the wing, because mutant clones that occupy only one wing surface display always less extravein tissue than clones occupying both dorsal and ventral surfaces (Fig. 2G). In addition, mutant clones can also induce neighbouring wild-type cells to differentiate as vein tissue, either in the same wing surface or in the opposite one (Fig. 2F,H). We conclude that *cic* functions as a negative regulator of vein differentiation in intervein territories, probably as part of a network involving cell interactions.

Post-transcriptional regulation of Cic protein in the wing disc

We next examined the expression pattern of *cic* in wild-type wing discs. In situ hybridisation of third instar discs showed uniform distribution of *cic* transcripts (Fig. 3A). By contrast, staining of similar discs with a specific Cic antibody revealed a complex pattern of protein accumulation: Cic accumulates at high levels in the wing pouch and in the primordial hinge region, but not in the notum region (Fig. 3B). At this stage, Cic levels begin to drop in the presumptive third longitudinal vein and in two rows of cells running along the D/V boundary, which correspond to the future wing margin (Fig. 3B,C). Moreover, the remaining Cic protein in those cells is cytoplasmic, whereas in other regions of the wing pouch (and in the adjacent peripodial cells), Cic is clearly nuclear (Fig. 3C,D). During pupariation [from 6 to 34 hours after puparium formation (APF)], Cic levels also decline in all presumptive longitudinal wing veins and crossveins (Fig. 3E-G). This specific accumulation of Cic in intervein sectors is consistent with its role as a negative regulator of vein differentiation.

The Cic protein is downregulated by the Ras/Raf pathway in the wing disc

In the early embryo, Cic is under negative post-transcriptional regulation by the Ras/Raf signalling pathway (see Introduction). Several lines of evidence are consistent with a similar mechanism of Cic inactivation in the wing. First, Ras/Raf signalling in response to EGFR activation is required for vein differentiation (Díaz-Benjumea and Hafen, 1994), and levels of

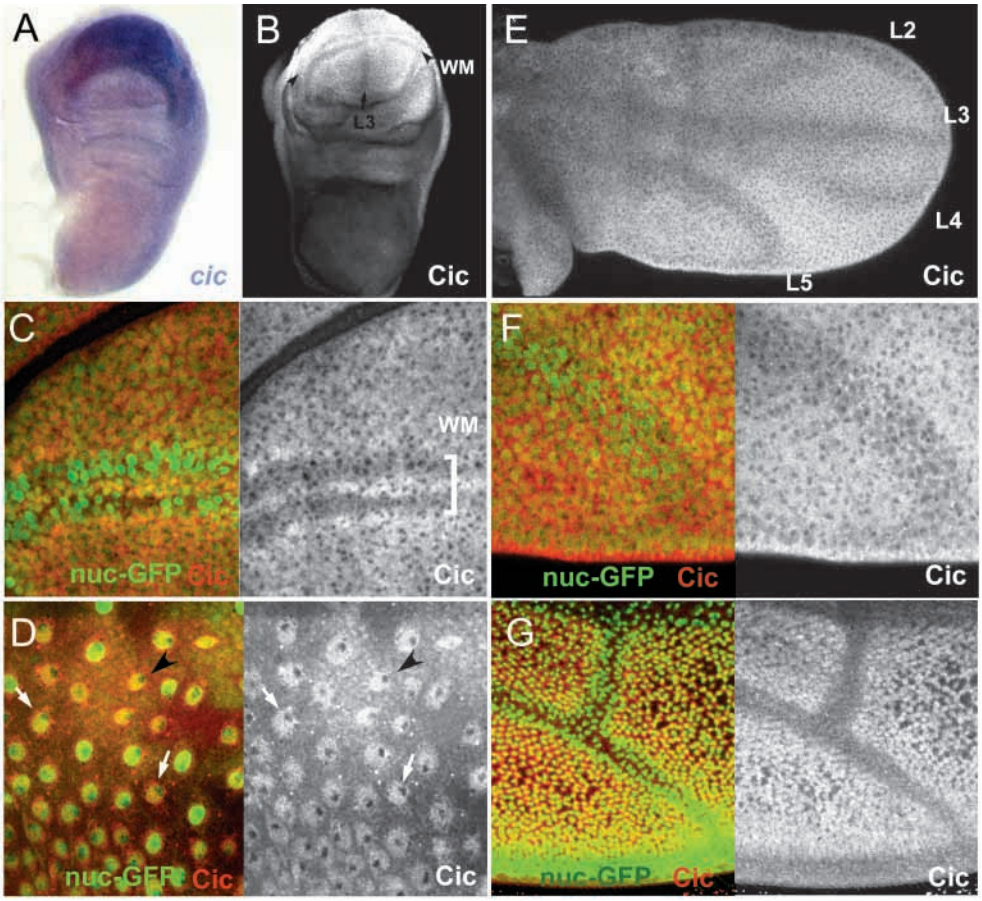

Fig. 3. The levels of Cic protein are regulated post-transcriptionally in the wing imaginal disc. (A) Third larval instar imaginal wing disc stained with a *cic* antisense riboprobe. The *cic* transcript is uniformly distributed throughout the disc. (B-D) Third larval instar imaginal disc stained with an anti-Cic antibody; (B,C) a view of the main disc epithelium; (D) the peripodial membrane. (B) The Cic protein is expressed in the wing pouch but is absent in the notum region. Low Cic levels are observed in the prospective wing margin and along the L3 longitudinal vein. (C) A close view of the anterior D/V boundary region stained with anti-Cic (red) shows that Cic is clearly downregulated in the prospective wing margin. The cell nuclei are counterstained with nuclear GFP (green). The Cic protein is present in both the nucleus and the cytoplasm of most wing pouch cells, but is not present in the cell nucleolus that appears as a small black area. (D) Close view of the wing peripodial membrane stained as in C. In these large cells, Cic protein also accumulates in the nucleus (except the nucleolus, as in the wing epidermis, black arrowhead) and can be also observed in the perinuclear cytoplasm (white arrows). (E,F) Six hours APF wing discs stained as in B,C. At this stage Cic levels are decreasing in all the presumptive longitudinal veins. (F) Detail that includes the tip of the L5 vein. (G) Detail of the L5 vein tip in a 28-32 hours APF wing disc stained as in C. Cic is present at high levels in the intervein cells and expressed at much lower levels in the L5 and the posterior crossvein.

active, diphosphorylated MAPK are maximal in presumptive veins (Gabay et al., 1997), where Cic protein is downregulated. Furthermore, both ectopic activation of the Ras/Raf pathway and loss of *cic* function cause similar extravein phenotypes (Brunner et al., 1994). Thus, it is conceivable that Ras/Raf signalling induces vein differentiation by downregulating Cic protein in prospective vein cells.

If this hypothesis is correct, Ras/Raf signalling should be dispensable, at least in part, for vein differentiation in the absence of *cic* activity. We tested this idea in two different ways. In one case, we eliminated Ras/Raf signalling in the wing by mutating factors required for EGFR activation. The hypomorphic viable combination rho^{ve} vn^{1} affects the activity in the wing of both the *vein* (*vn*) locus, which encodes a putative EGFR ligand (Schnepp et al., 1996), and *rho*, which encodes a transmembrane protein involved in EGFR activation (Sturtevant et al., 1993). In flies mutant for this combination, the activation of MAPK in the presumptive vein cells is prevented (Martín-Blanco et al., 1999) and as a consequence, veins fail to differentiate (Fig. 4A). By contrast, flies with the triple mutant combination rho^{ve} vn^{1} cic^{2}/rho^{ve} vn^{1} $bwk^{\Delta 11}$ display the same extravein phenotypes as $cic^{2}/bwk^{\Delta 11}$ mutant wings (Fig. 4B,C), suggesting that Cic functions downstream of EGFR activation. We generated large M^{+} clones homozygous for the $Ras^{\Delta C40b}$ and cic^{2} null alleles. Mutant clones lacking only the *Ras-1* (*Ras85D* –FlyBase) function show the expected loss of vein tissue (Fig. 4D), but this effect is suppressed in clones lacking both *Ras-1* and *cic* activities, which show ectopic vein development (Fig. 4E,F). These results support the notion that Ras/Raf signalling inhibits *cic* function during vein differentiation.

To test directly if activity of the Ras/Raf pathway mediates downregulation of Cic protein in veins, we monitored its distribution in clones homozygous for $Ras^{\Delta C40b}$. Mutant clones induced in second larval instar were examined in early pupal discs, where Cic protein levels begin to decline in all the differentiating veins. In 0-6 hours APF mosaic wings, Cic is autonomously expressed at high levels within the *Ras* mutant clones, even when they occupy positions corresponding to the normal veins (Fig. 4G,H). Furthermore, Cic levels are higher in the mutant clones than in the rest of the wing, suggesting that *Ras* activity also exerts basal downregulation on Cic in intervein cells. We also performed the converse experiment and tested the effect of the hyperactivation of EGFR signalling on Cic accumulation. We used the GAL4/UAS system to overexpress the EGFR activator Vein in the posterior compartment of pupal wings (Materials and Methods). As shown in Fig. 4I, this caused a significant reduction of Cic protein levels in the posterior compartment of mutant wings by 0-6 hours APF. Altogether, these genetic and

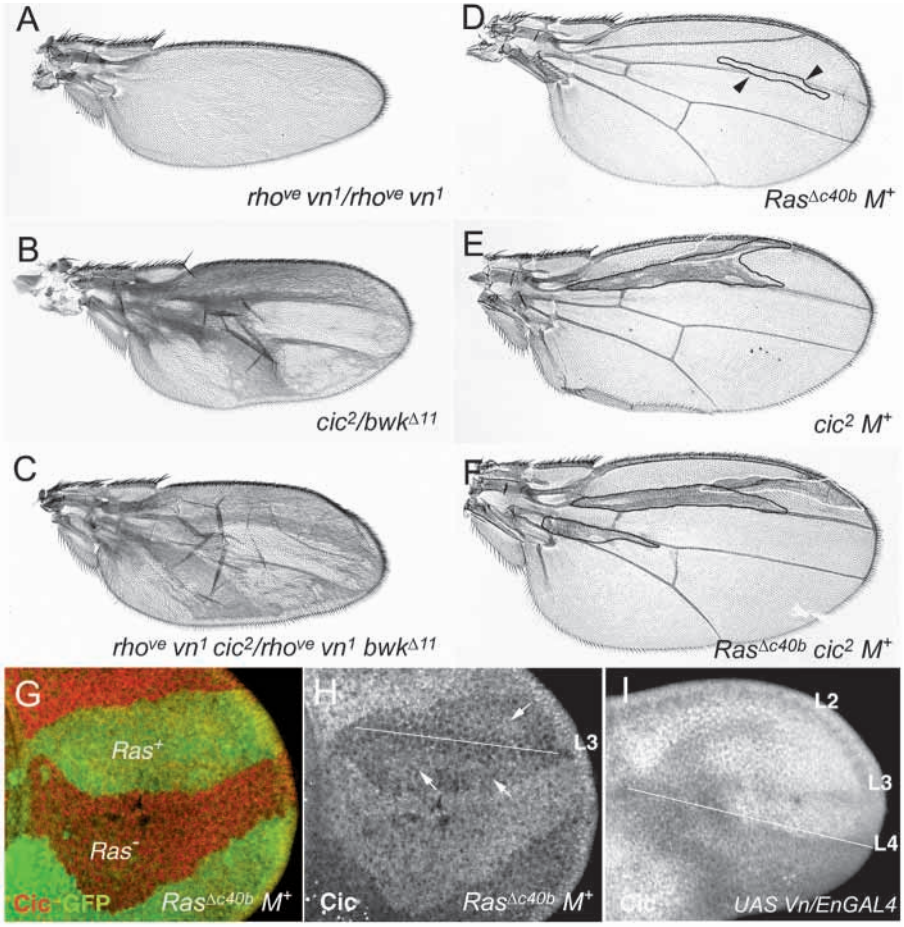

Fig. 4. *cic* acts downstream of the Ras/Raf pathway. (A) The differentiation of all the wing veins is prevented in *rho^{ve} vn^{1}* homozygotes. (B) *cic^{2}/bwk$^{\Delta 11}$* flies differentiate ectopic veins throughout the wing. (C) In the triple mutant *rho^{ve} vn^{1} cic^{2}/ rho^{ve} vn^{1} bwk$^{\Delta 11}$*, the loss of veins phenotype is completely suppressed and the extravein phenotype is completely epistatic. (D) Mutant clones *Ras$^{\Delta c40b}$ M^{+}* autonomously prevent vein differentiation. (E) Two anterior clones *cic^{2} M^{+}* cause ectopic veins (dorsal and ventral regions of the clone outlined in black and white, respectively). (F) Wing containing three *M^{+}* clones mutant for both *cic^{2}* and *Ras$^{\Delta c40b}$*, differentiating extravein tissue. (G) Pupal wing staged 0-6 hours APF containing two large *Ras$^{\Delta c40b}$ M^{+}* clones and stained with anti-Cic (red). Mutant clones are marked as patches free of GFP staining (green). (H) Red channel in G. Cic is autonomously expressed at high levels in all the *Ras$^{\Delta c40b}$* mutant cells. Cic is expressed at normal levels outside the mutant territories (white arrows) and is downregulated in the L3 vein (white line). (I) 0-6 hours APF pupal wing of a UASVn/Engrailed GAL4 fly stained with anti-Cic. Note the low levels of Cic present in the cells posterior to the L4 presumptive region (white line).

immunohistochemical data indicate that the Cic protein is negatively regulated by EGFR signalling during wing vein specification.

Cic acts as a repressor of wing vein-specific genes

The above results suggest that EGFR signalling activates the expression of genes involved in vein patterning by antagonising *cic* repressor activity in presumptive vein cells. To test this hypothesis, we examined the role of *cic* in regulating expression of genes induced by the EGFR pathway. One such gene is *aos*, which encodes a secreted factor that mediates

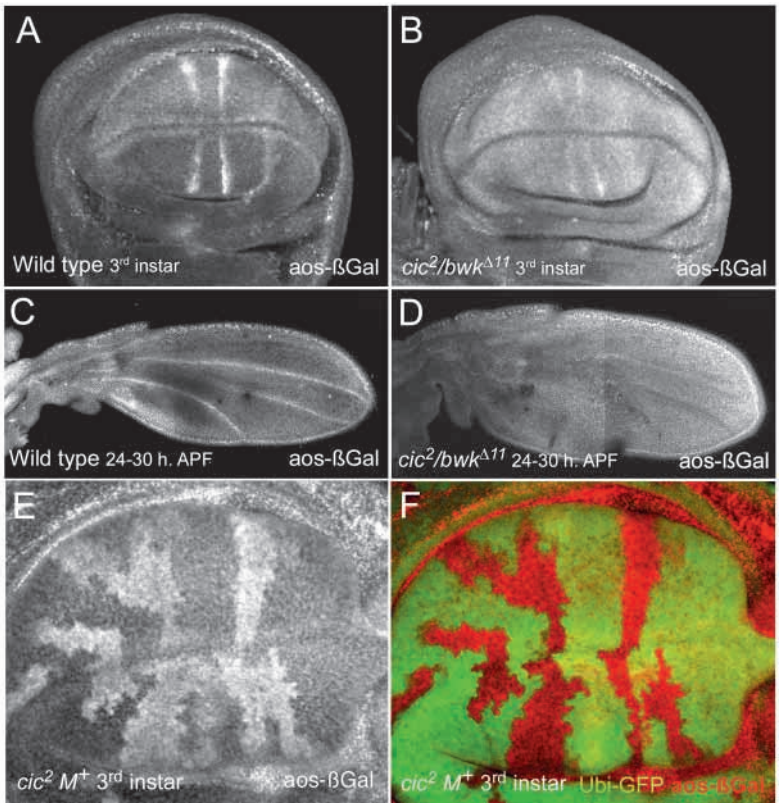

Fig. 5. Aos is repressed in the intervein tissue by the activity of Cic. (A) Wild-type and (B) *cic^{2}/bwk$^{\Delta 11}$* third larval instar wing imaginal discs carrying the *aos-lacZ* reporter stained with anti β-Gal. The aos reporter gene is expressed throughout the wing pouch in the mutant. (C,D) Wild-type and *cic^{2}/bwk$^{\Delta 11}$* wings (24-30 hours APF) carrying the *aos-lacZ* reporter stained as in A,B. Homogeneous levels of the *aos* reporter are found throughout the wing blade in the mutant. (E,F) Third larval instar imaginal wing disc expressing the *aos-lacZ* reporter and containing *cic^{2} M^{+}* mutant clones marked as cells lacking GFP (green in F). Staining with anti-β-gal (red in F) shows that *aos-lacZ* is expressed autonomously in all the *cic* mutant cells.

negative feedback modulation of EGFR signalling in many developmental processes (Golembo et al., 1996; Wasserman and Freeman, 1998). In the wing, *aos* is expressed specifically in presumptive vein cells, where EGFR activity is maximal (Gabay et al., 1997) (Fig. 5A). We analysed expression of an *aos-lacZ* reporter gene in third larval instar and pupal $cic^2/bwk^{\Delta 11}$ mutant discs and found a dramatic derepression compared with the wild-type pattern. Ectopic *aos-lacZ* expression is observed throughout the wing pouch, except in the presumptive wing margin (Fig. 5B). This ectopic expression is maintained in intervein cells until at least 30 hours APF (Fig. 5D). However, we note that in *cic* mutant discs, *aos* is expressed at higher levels in presumptive vein cells than in intervein territories, suggesting that *cic* is not the only factor involved in *aos* regulation (Fig. 5B). We have also analysed whether *aos* expression is repressed autonomously by *cic*. We find that in clones homozygous for the cic^2 mutation, the *aos-lacZ* reporter is expressed by all the cells within the clones (Fig. 5E,F). Thus, *cic* behaves in a cell autonomous way as a repressor of *aos* transcription in intervein cells.

cic could also regulate the expression of *vvl*, which encodes a POU domain transcription factor required for vein differentiation (de Celis et al., 1995). Double mutants for EGFR and *vvl* display synergistic phenotypes (de Celis et al., 1995), indicating that these two elements interact genetically. Indeed, *vvl* expression in the wing is regulated by Ras/Raf signalling (J. F. de Celis, personal communication). In wild-type wing discs, Vvl protein is distributed rather uniformly during larval stages, but it becomes restricted to vein cells during early pupal development (Fig. 6A), so that by 24-30 hours APF, the protein is no longer detectable in intervein cells (Fig. 6C,E). By contrast, Vvl protein persists in intervein cells of $cic^2/bwk^{\Delta 11}$ mutant discs from 6 to 30 hours APF, albeit at variable levels (Fig. 6B,D,F). Thus, *cic* activity is necessary for repression of *vvl* in intervein regions of early pupal discs.

Another gene activated in the veins by the Ras/Raf signalling is *rho* (Sturtevant et al., 1993; Martín-Blanco et al., 1999). In the wing, *rho* activity participates in a positive feedback loop that boosts EGFR activation in vein cells (Martín-Blanco et al., 1999). We examined *rho* expression in $cic^2/bwk^{\Delta 11}$ wing discs at different stages of development. A normal *rho* pattern was observed during late larval and early pupal stages (not shown), but clear ectopic expression was detected in intervein regions by 24-30 hours APF. This ectopic expression precedes the differentiation of extraveins in the adult mutant wings (Fig. 6G,H). Thus, *cic* is required, either directly or indirectly, for *rho* repression in intervein cells during late pupal stages.

The differentiation of vein cells during pupal stages also requires the function of the secreted Dpp factor, that is implicated in the refinement of vein patterning during pupal development (de Celis, 1997). In wild-type pupal wings, *dpp* transcripts accumulate in precise stripes corresponding to the developing veins (Fig. 6I). By contrast, such transcripts are clearly observed throughout the wings of $cic^2/bwk^{\Delta 11}$ mutants by 24-30 hours APF (Fig. 6J). Dpp levels are still higher in regions that will give rise to vein tissue in the mutant, but there is ectopic *dpp* expression in most of the future intervein cells, indicating that *cic* affects *dpp* expression in late pupal wings. This effect on a secreted factor that diffuses from its source may explain, at least in part, the non autonomous phenotypes of *cic* mutant clones (see Discussion).

The above results indicate that Cic is required during larval and pupal stages for repression in intervein cells of several vein-specific genes. We also checked if the expression of intervein genes is affected in the absence of Cic. One such gene is *blistered* (*bs*), which encodes a homologue of the

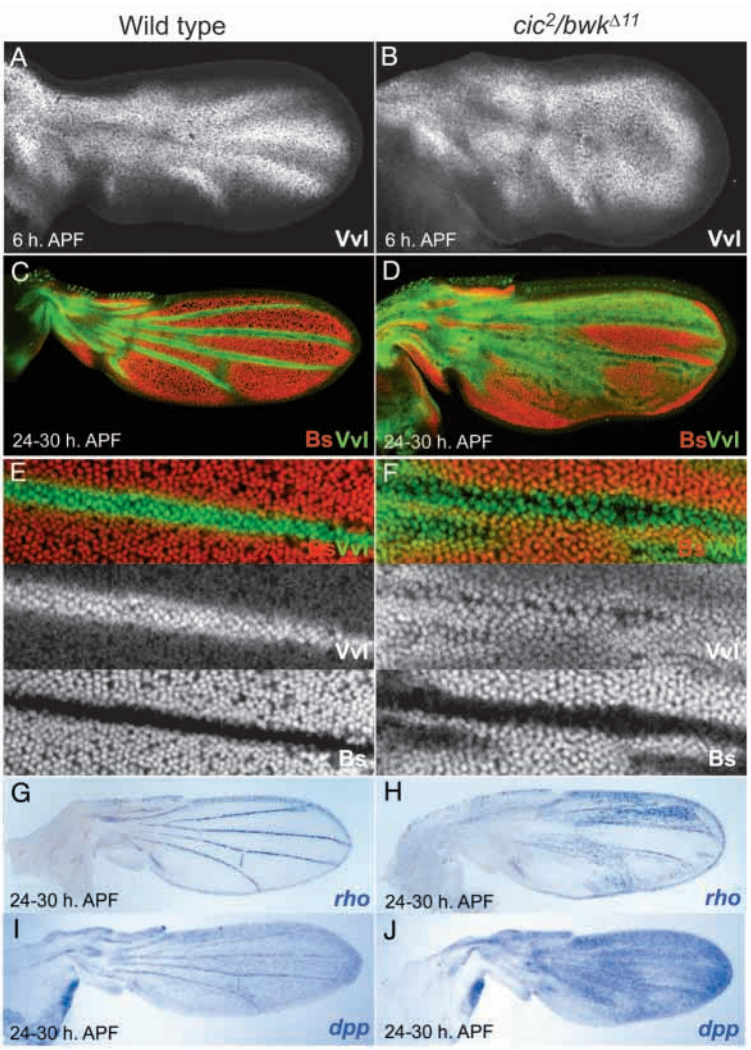

Fig. 6. (A,C,E,G,I) Wild-type wing and (B,D,F,H,J) $cic^2/bwk^{\Delta 11}$ wings stained for various vein and intervein markers. (A,B) 6 hours APF wings stained with a Vvl antibody. At this stage, Vvl begins to fade away from the intervein tissue in the wild type. In the mutants, Vvl expression is maintained in the intervein tissue. (C-F) 24-30 hours APF, wings stained with antibodies against Vvl (green) and Bs (red). (E,F) A close view of the L4 vein. In the wild type, the patterns of Vvl and Bs are almost complementary, only a few nuclei abutting the vein tissue co-express both markers. In the mutant, most cells co-express variable levels of both proteins. (G,H) Pupal wings staged 24-30 hours APF stained with an antisense *rho* riboprobe. In the mutant, *rho* is expressed ectopically in the intervein tissue. (I,J) Pupal wings of the same age stained with a *dpp* antisense riboprobe. In the mutant, *dpp* is expressed at high levels in the future vein regions and at lower levels in the rest of the wing pouch.

mammalian SRF transcription factor (Montagne et al., 1996) and is autonomously required for intervein differentiation (Fristrom et al., 1994). In wild-type discs, Bs and the Cic repressor are co-expressed in all intervein cells throughout late larval and early pupal stages (not shown). We find that Bs expression is normal in third larval instar and 6 hours APF pupal discs of $cic^2/bwk^{\Delta 11}$ mutant flies (not shown), indicating that *cic* is not required for *bs* regulation at these stages. However, Bs levels are clearly reduced in intervein cells at 24-30 hours APF (Fig. 6D). As Cic appears to be a dedicated repressor, it seems unlikely that it could contribute directly to the maintenance of Bs expression in the same cells; rather, we interpret this late repression of *bs* as an indirect effect caused by ectopic expression of vein-specific genes in intervein territories of these mutant discs. Indeed, it has been shown that ectopic expression of *rho* in intervein cells causes downregulation of *bs* during pupal stages (Roch et al., 1998).

The analysis of Bs and Vvl expression led also to the interesting observation that both proteins are co-expressed in many cells throughout the wing pouch of $cic^2/bwk^{\Delta 11}$ mutants (Fig. 6D,F), whereas they show almost perfect complementary expression patterns in wild-type discs (Fig. 6C,E). The co-expression of vein and intervein markers, such as Dpp, Vvl and Bs could explain the combination of vein and intervein cell morphologies observed in $cic^2/bwk^{\Delta 11}$ adult wings.

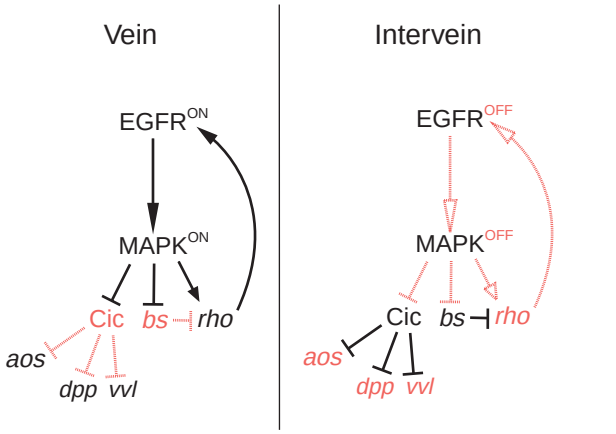

Fig. 7. The role of *cic* in the specification of *Drosophila* wing veins. Active genes and proteins are shown in black, whereas inactive genes and proteins are in red. EGFR and MAPK are active in vein cells and inactive in the intervein tissue from third larval instar onwards (see text). In vein cells, EGFR signalling downregulates Cic protein levels in the nucleus, thus relieving repression of vein-specific genes such as *aos*, *vvl* and *dpp*. In addition, EGFR signalling is required in the veins for the activation of *rho* and the repression of *bs*, in a *cic*-independent manner. *rho* expression in the veins further increases EGFR activation in a paracrine loop. Conversely, absence of EGFR signalling in the intervein territories allows these cells to maintain high levels of Cic throughout larval and pupal development, which represses the expression of vein-specific genes. In addition, these cells accumulate Bs protein that represses *rho* and promotes intervein differentiation.

DISCUSSION

The Cic HMG-box repressor plays a key role in regulation of embryonic terminal patterning under the control of Torso RTK signalling (Jiménez et al., 2000). We have shown that Cic participates also in other developmental processes, such as specification of vein cell fates in the wing. Our genetic data indicate that *cic* terminal-class alleles and the *bwk* group of mutations are hypomorphic alleles disrupting specific aspects of *cic* function, whereas the novel null allele cic^2 and several available deletions affect all functions of the gene. We provide evidence that Cic mediates wing vein patterning acting as a repressor of vein-specific genes downstream of the EGFR signalling pathway.

Role of Cic in wing vein development

There are two key aspects of Cic function as a developmental regulator: its ability to repress specific target genes in defined territories, and its inhibition by the Ras/Raf pathway to allow expression of those targets in complementary positions. In the blastoderm embryo, Cic is required for development of trunk body regions and represses genes mediating differentiation of terminal structures. Torso RTK activation at each pole of the embryo alleviates Cic-dependent repression and initiates the terminal gene expression program (Jiménez et al., 2000). Our results support a similar model for *cic* function during specification of vein versus intervein fate in the wing. Loss of *cic* function in the wing causes formation of ectopic vein tissue, implying that Cic mediates intervein specification by restricting vein formation to appropriate regions. In intervein territories, Cic behaves as a repressor of vein-specific genes such as *aos* and *vvl* but does not seem to affect directly the expression of *bs*, which is required for the specification of intervein fates. Finally, EGFR signalling leads to downregulation of Cic protein levels in vein nuclei, thus relieving Cic-mediated repression and promoting vein development (Fig. 7).

Nevertheless, several data suggest a more complex regulation of vein specification compared to terminal patterning. First, we have shown that expression of *rho*, a positive target of EGFR signalling in the wing and other tissues (Martín-Blanco et al., 1999; Hsu et al., 1996), is not affected by *cic* during third larval instar and early pupariation (Fig. 7). This suggests that EGFR signalling can mediate activation of some targets in the wing disc by mechanisms other than Cic inhibition. Similarly, the EGFR pathway has been shown to repress *bs* expression in presumptive vein cells (Roch et al., 1998), a process that is independent of Cic (Fig. 7). These results imply that different transcription factors act downstream of the EGFR cascade to direct changes in gene expression during patterning of wing veins. Indeed, recent results indicate that EGFR signalling activates certain target genes via direct phosphorylation of Fos protein (Ciapponi et al., 2001).

Moreover, vein differentiation is not a mere result of EGFR activation but depends on other signals such as Dpp and Notch (de Celis, 1997; de Celis et al., 1997), and on the distribution of additional transcription factors that contribute to wing patterning. For example, the Collier/Knot nuclear factor has been shown to induce high levels of Bs expression between veins L3 and L4, promoting intervein development in this region (Vervoort et al., 1999). All these inputs are linked in a complex circuit of intercellular signalling and gene regulation that progressively refines vein determination during late larval and pupal development. This signalling network could provide an explanation for the observed non-autonomy of *cic* phenotypes

during vein specification. Thus, although *cic* represses *aos* expression in a cell-autonomous manner, this and other *cic* targets are likely to participate in signalling mechanisms that affect adjacent cells. Consistent with this idea, we find that *cic* mutant cells express ectopic Dpp product, a diffusible factor that promotes vein differentiation (de Celis, 1997).

We have shown that in *cic* mutant wings, many cells differentiate, acquiring morphological features that are intermediate between those observed in either vein and intervein cells. In these wings, most cells co-express Bs and Vvl proteins, which are normally restricted to vein and intervein cells, respectively, suggesting that vein/intervein fate specification may result from a balance of these factors rather than on a simple binary switch. In this context, the concerted activities of signalling cascades such as Dpp, Notch and Ras/Raf pathways may regulate cell differentiation by modulating the balance of nuclear factors that act in a dose-dependent way. This hypothesis provides a mechanism that could explain the enormous variability observed in the cell morphologies of different insect wings.

Role of Cic as a general repressor downstream of RTK signalling

In this work, we have shown that Cic acts in wing development in a similar way to that previously described in the early embryo. Moreover, the fact that mutant clones for the Groucho repressor display extraveins, similarly to *cic* clones (de Celis and Ruíz-Gómez, 1995), indicates that these two proteins could interact as partners during wing development, as it is the case during embryonic development (Jiménez et al., 2000). Indeed, we have observed weak genetic interactions between different *cic* and *gro* alleles during wing development (F. R., unpublished). Thus, Cic and Gro could be part of a conserved repressor complex downregulated by the Ras/Raf molecular cassette in different cellular contexts. In this regard, the phenotype of *bwk* mutations suggests that Cic may also function as a target of other RTK signals during patterning of the eggshell in the ovary (Rittenhouse and Berg, 1995). However, it should be noted that *cic* does not seem to act in all developmental processes mediated by Ras/Raf signalling in *Drosophila*. For example, the eyes of *cic* mutant flies appear normal (F. R., unpublished), even though the Ras/Raf pathway controls several aspects of cell fate specification and patterning in this tissue (Freeman, 1997). These observations support the idea that the Ras/Raf pathway can regulate cell specification in a *cic*-independent way depending on the cell context.

Previous work has shown that during patterning of ovary follicle cells, the expression of *rho* is controlled by the Ras/Raf pathway via another transcriptional repressor, the CF2 protein (Hsu et al., 1996). CF2 is tagged for cytoplasmic retention and degradation after direct phosphorylation by MAPK (Mantrova and Hsu, 1998). The Cic protein has also consensus sites for phosphorylation by MAPK (Jiménez et al., 2000), suggesting that Cic levels could be regulated post-transcriptionally in a similar way to CF2. This indicates that localised downregulation of specific repressors is a common mechanism for the activation of target genes by the Ras/Raf pathway. The identification of highly conserved *cic* homologues in mice and humans suggests that regulation of gene expression by RTK signalling in vertebrates may also involve relief of Cic-dependent repression.

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