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RESEARCH ARTICLE

# Patterning of the *Drosophila* L2 vein is driven by regulatory interactions between region-specific transcription factors expressed in response to Dpp signalling

**Mercedes Martín, Cristina M. Ostalé and Jose F. de Celis***

## ABSTRACT

Pattern formation relies on the generation of transcriptional landscapes regulated by signalling pathways. A paradigm of epithelial patterning is the distribution of vein territories in the *Drosophila* wing disc. In this tissue, Decapentaplegic signalling regulates its target genes at different distances from the source of the ligand. The transformation of signalling into coherent territories of gene expression requires regulatory cross-interactions between these target genes. Here, we analyse the mechanisms generating the domain of *knirps* expression in the presumptive L2 vein of the wing imaginal disc. We find that *knirps* is regulated by four Decapentaplegic target genes encoding the transcription factors *aristaless*, *spalt major*, *spalt-related* and *optix*. The expression of *optix* is activated by Dpp and repressed by the Spalt proteins, becoming restricted to the most anterior region of the wing blade. In turn, the expression of *knirps* is activated by Aristaless and repressed by Optix and the Spalt proteins. In this manner, the expression of *knirps* becomes restricted to those cells where Spalt levels are sufficient to repress *optix*, but not sufficient to repress *knirps*.

**KEY WORDS: Optix, Spalt, Vein patterning, *Drosophila* wing, Transcriptional regulation**

## INTRODUCTION

Pattern formation is a developmental operation that generates bi-dimensional arrays of cells with particular differentiation characteristics (Martin et al., 2016). Classical examples of pattern formation processes that have been subject to intensive genetic and developmental analysis are the distribution of bristles and veins in the epidermis of *Drosophila* (Campuzano and Modolell, 1992; de Celis, 2003). Both processes occur in the wing imaginal disc, an epithelial tissue that proliferates during the larval stages and differentiates the thorax and wing during metamorphosis. The distribution of bristles in the thorax and the ordered array of veins in the wing share the involvement of a variety of transcription factors, which function is to subdivide the epithelium into progressively smaller domains of gene expression. In the case of the bristles, this subdivision culminates with the expression of the proneural genes in clusters of cells (Campuzano and Modolell, 1992), whereas in the case of the veins, the 'pre-patterning' stage terminates with the establishment of longitudinal domains of Epidermal growth factor receptor (EGFR) signalling activity (Sturtevant et al., 1993; Sturtevant and Bier, 1995; de Celis, 2003).

The veins are linear structures that develop in the wings of all arthropods, forming species-specific patterns (de Celis and Diaz-Benjumea, 2003). Each vein differentiates a cuticle that is thicker and more pigmented that the cuticle of intervein cells. The differentiation of the veins in *Drosophila* is driven by signalling through the EGFR and Decapentaplegic (Dpp) pathways, the activities of which are restricted to the developing veins during imaginal and pupal development (EGFR) or only during pupal development (Dpp) (de Celis, 2003). Although all veins differentiate using the same signals and transcription factors, they differ from each other in the wing surface in which the vein characteristics are more prominent ('corrugation'). In the case of *Drosophila*, the veins L2 and proximal L4 corrugate in the ventral surface, whereas the veins L3, distal L4 and L5 do so in the dorsal wing surface. Vein corrugation allows the tracking of each vein in different species, and constitutes an invariant phylogenetic feature (Garcia-Bellido and de Celis, 1992).

Apart from their specific corrugation, vein differentiation is governed by a common developmental program, the main components of which are signalling pathways (Notch, EGFR and Dpp) and transcription factors (Ventral veinless). Furthermore, the morphological appearance of veins is similar. However, superimposed on a shared developmental program, each vein expresses vein-specific transcription factors that might dictate their specific characteristics. These transcription factors have a diversity of functions during development, and include *abrupt*, which is expressed in the L5 vein (Cook et al., 2004), the genes of the Knirps complex [*knirps* (*kni*) and *knirps-like*], which are expressed in the L2 provein (Lunde et al., 1998), and two genes of the Iroquois complex (*araucan* and *caupolican*), the expression of which is restricted to the developing L1, L3 and L5 veins (Gómez-Skarmeta and Modolell, 1996). In this manner, vein formation encompasses a general vein differentiation program involving EGFR and Dpp signalling, and also a vein-specific program conferred by specific transcription factors. How these two systems interact is still unknown.

Vein patterning is linked to the progressive subdivision of the wing blade region of the wing imaginal disc in domains of gene expression along the anteroposterior axis. This subdivision occurs in an epithelium undergoing extensive cell proliferation with a consequent increase in size, and culminates in the determination of alternate provein and intervein territories. The Hedgehog (Hh) and Dpp signalling pathways initiate the regionalization on the wing blade. These pathways regulate the expression in broad domains of *knot* (Hh) (Vervoort et al., 1999; Mohler et al., 2000; Crozatier et al., 2002), *spalt major* and *spalt-related* (Dpp) (de Celis and Barrio, 2000), and *bifid/optomotor blind* (Dpp) (Grimm and Pflugfelder,

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1996), and these transcription factors regulate the expression of the vein-specific genes *abrupt*, *kni* and *iroquois* in individual veins (Gómez-Skarmeta and Modolell, 1996; Lunde et al., 1998; Cook et al., 2004) and of *blistered* (*bs*) in the interveins (Fristrom et al., 1994; Montagne et al., 1996; Nussbaumer et al., 2000).

The formation of the L2 vein requires the function of the Knirps genes (*kni* and *knirps-like*), the expression of which is restricted to the presumptive region of the L2 vein in third instar wing discs (Lunde et al., 2003). The region of *kni* expression is included within the most anterior territory of *spalt major* (*salm*) and *spalt-related* (*salr*) expression, corresponding to a subset of cells expressing low levels of Spalt proteins (Sal) (de Celis and Barrio, 2000). Two alternative models had been proposed to account for the expression of *kni* in the primordium of the L2 vein. In one model, the expression of Sal activates non-autonomously the expression of *kni* in more anterior cells (Lunde et al., 2003). In the other model, high levels of Sal repress *kni* expression, whereas low levels of Salm and Salr would be required to promote this expression (de Celis and Barrio, 2000). Both models are unsatisfactory, because no inducer of *kni* acting non-autonomously has been identified so far, and because the activity of Sal as a dose-dependent transcriptional activator or repressor has not been proved.

In this work we have identified and characterised a novel component of the genetic machinery that patterns the wing blade and positions the veins. This gene, named *optix*, encodes a sequence-specific transcription factor of the Six family, a group of proteins containing a homeodomain (Seimiya and Gehring, 2000; Anderson et al., 2012). We show that Optix is a region-specific transcription factor, the expression of which is restricted to the most anterior region of the wing blade primordium. We also find that Sal represses the expression of *optix*, and that Optix is a strong repressor of *kni* transcription. We propose that the domain of *kni* expression is stabilised by the combined repressive activities of Optix and Sal, which define the most anterior and posterior boundaries of *kni* expression. We also show that Aristaless (*al*), another homeobox transcription factor (Campbell et al., 1993; Schneitz et al., 1993), is necessary to activate the expression of *kni* in the anterior compartment. Interestingly, Dpp signalling regulates the expression domains of *al*, *salm*/*salr* and *optix* at different thresholds of activation. The mechanism combining activation and repression of *kni* by a set of three transcription factors engaged in cross-regulatory interactions, and acting downstream of Dpp signalling, ensures that the L2 primordium (*kni* expression domain) is permanently and dynamically established with reference to the source of Dpp signalling in the growing epithelium.

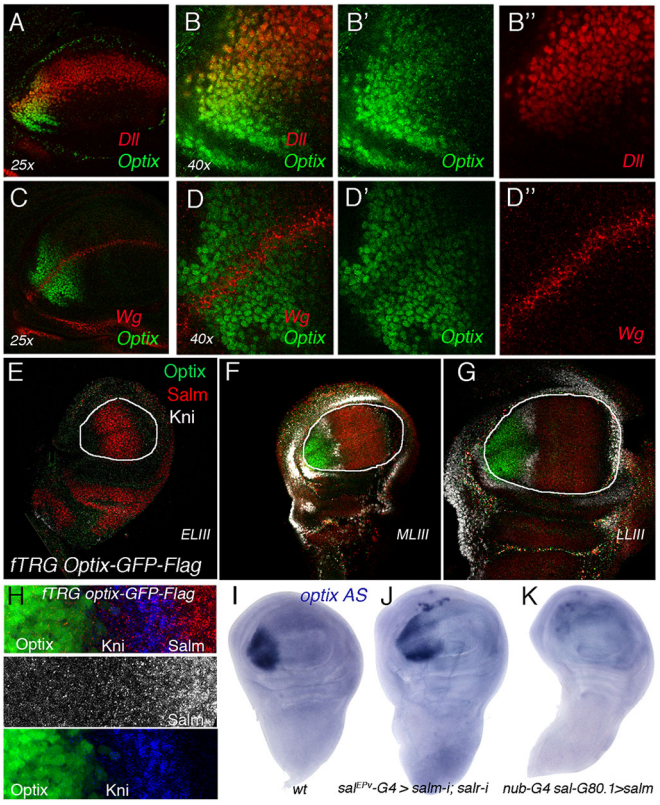


**Fig. 1. *optix* expression in the wing imaginal disc.** (A) Expression of *fTRG optix-GFP-Flag* (Optix, green) and Distalless (Dll, red) in late third instar wing disc. (B-B″) Higher magnification of the Optix domain of expression showing partial localisation with Dll. The split green and red channels are shown in B′ and B″. (C) Expression of *fTRG optix-GFP-Flag* (Optix, green) and Wingless (Wg, red) in late third instar wing disc. (D-D″) Higher magnification of the Optix domain showing its symmetric disposition with respect to the dorsoventral boundary. (E-G) Wing imaginal discs of *fTRG optix-GFP-Flag* genotype in early (ELIII; E), mid (MLIII; F) and late (LLIII; H) third instar larvae, showing the expression of FLAG (Optix; green), Salm (red) and Kni (white). Optix expression appears in mid third instar wing discs in an anterior sector of the wing blade complementary to the domain of Sal localisation. (H) Higher magnification of the Optix-Sal border (63×, zoom 2×). The Sal and Optix-Kni channels are shown below (Salm and Optix-Kni). The Kni domain (blue) is localised in the region of low levels of Sal (red and white), and does not overlap with the Optix domain (green). (I-K) Expression of *optix* mRNA in wild-type third instar wing disc (I), and in wing discs with reduced expression of *salm* and *salr* (*sal$^{EPv}$-Gal4/UAS-salm-i; UAS-salr-i*; J) or with ectopic expression of *salm* (*nub-Gal4 sal$^{EPv}$ -Gal80.1; UAS salm$^{14B}$/+*; K). The expression of *optix* is extended posteriorly upon loss of *salm*/*salr* (J) and lost when *salm* is ectopically expressed (K).

## RESULTS

### Optix is expressed in an anterior domain of the wing blade region, and Sal represses its expression

The gene *optix* encodes a nuclear protein of the Six family, and contains a Six-domain and a homeodomain (Toy et al., 1998). The function of *optix* has been characterised mainly during eye development, where *optix* is a component of the genetic network regulating the initiation of the eye field (Seimiya and Gehring, 2000). The expression of *optix* is also present in the wing disc, appearing to be restricted to an anterior sector of the wing pouch (Fig. 1). To monitor the localisation of Optix, we used a GFP-FLAG-tagged Optix protein expressed under the regulation of *optix* regulatory sequences (Sarov et al., 2016) (Fig. 1). The expression of Optix-*GFP-FLAG* includes wing blade cells expressing Distalless (Dll) (Fig. 1A-B″) and also more anterior-lateral cells where Dll is not detected (Fig. 1B-B″). The domain of Optix expression is localised symmetrically with respect to the dorsoventral compartment boundary (Fig. 1C-D″). The expression of Optix-*GFP-FLAG* is not detected in early third instar discs (ELIII; Fig. 1E), but accumulates in the anterior region of the wing from mid third instar onwards (MLIII and LLIII; Fig. 1F,G). The localisation of *optix* appears in a domain that is complementary to the domain of Sal accumulation throughout development (Fig. 1F-H). The expression of *kni* is also detected in mid to late third instar discs, and appears inserted between the Optix and Sal domains. (Fig. 1F-H). The gene *optix* was identified as a candidate Sal target gene because its expression levels increase in *salm*/*salr* mutant discs compared with wild-type discs (Organista et al., 2015). We confirmed this result by *in situ* hybridisation of *optix* in *salm*/*salr*

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knockdown and *salm*/*salr* overexpression backgrounds. In the first case (*sal$^{EPv}$-Gal4/UAS-salm-i; UAS-salr-i/+*) we found a posterior expansion of the *optix* expression domain (Fig. 1I,J). Complementary to this, the expression of *optix* is reduced when *salm* is ectopically expressed in anterior cells (*nub-Gal4 sal$^{EPv}$-Gal80; UAS-salm/+*; Fig. 1K). Taken together, these results indicate that the posterior boundary of *optix* expression is defined by transcriptional repression mediated by Sal proteins.

### Optix is required for the correct development of the anterior wing and for the positioning of the L2 primordium

To define the functional requirements of *optix* during wing development, we expressed *optix* RNAi in the entire wing pouch (*nub-Gal4/UAS-optix-i*). These wings display a consistent phenotype restricted to the most anterior part of the wing (Fig. 2A,B), the region where *optix* is expressed in the wing disc. The most remarkable effect of *optix-i* is an anterior displacement in the position of the L2 vein (Fig. 2B). The L2 vein originates from its normal location at the base of the wing, but shifts anteriorly after entering into the wing blade and becomes fused to the anterior wing margin (Fig. 2A-E). This displacement is accompanied by a strong reduction in the size of the most anterior intervein (L2-anterior wing margin; region A in Fig. 2A,C). Because the size of the adjacent intervein (L2-L3 intervein; region B in Fig. 2C) is not modified, we interpret that the *optix-i* phenotype is mostly the consequence of a failure of the A intervein to grow, more than a mere anterior displacement of the L2 vein. In addition, *optix-i* wings also have minor defects in the development of the anterior costal cell (region 1 in Fig. 2A,C), which appears slightly enlarged in size. Additional defects in the development of the anterior wing are also manifested in the triple row, which is formed by a lower number of bristles than in normal wings (Fig. 2F). To further characterise the defects of *optix-i* wings, we studied the two parameters with a major influence on

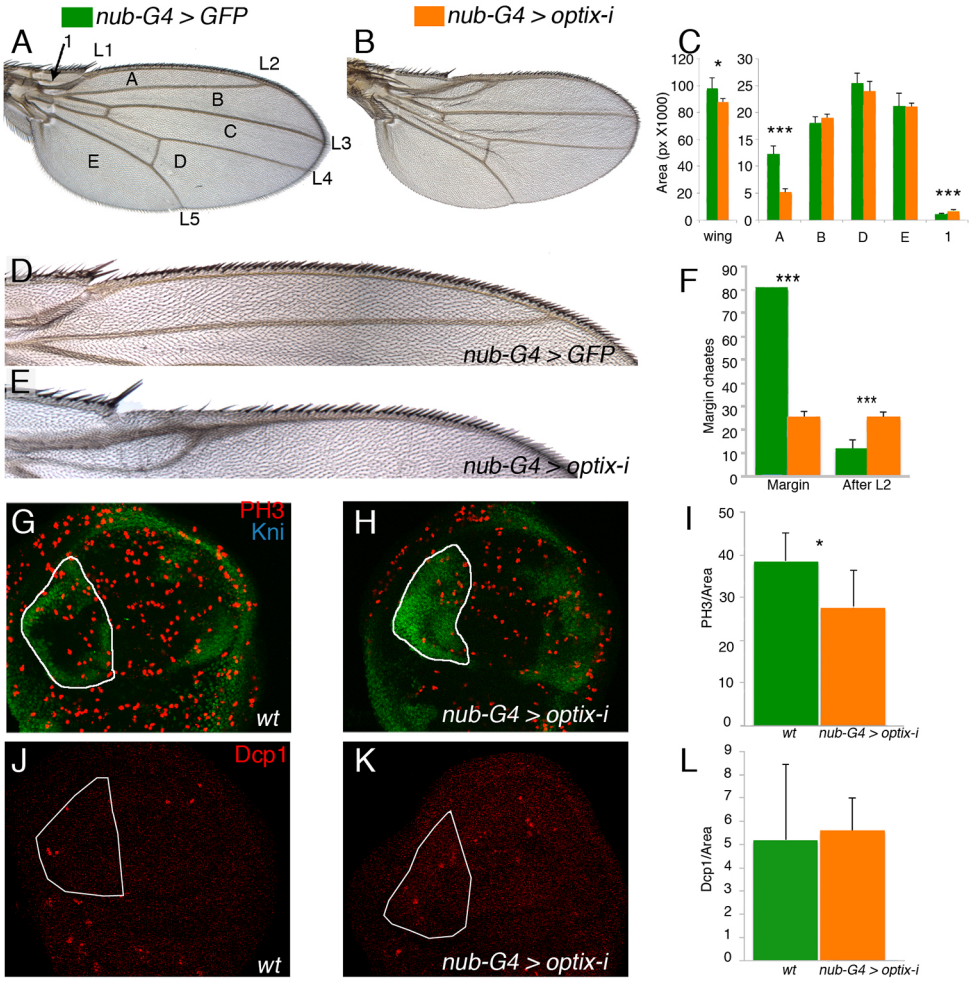


**Fig. 2. Functional requirements of Optix during wing development.** (A) Wild-type adult wing showing the A to E intervein regions, the L1 to L5 veins and the anterior costa (1). (B) *nub-Gal4*/*UAS-optix-i* wing showing an anterior displacement of the L2 vein. (C) Quantification of *optix* knockdown and wild-type control wings. *optix* knockdown wings are smaller than control wings (orange bar compared with green bar in the left graph), and this reduction is due to the smaller size of the A region (right graph, first two columns). In addition, *optix* knockdown wings show an extension of the costal region (1 in A). (D,E) Higher magnification of the L2 vein and A intervein in control (*nub-Gal4*/*UAS-GFP*) and *optix* knockdown (*nub-Gal4*/*UAS-optix*-i) wings. (F) Quantification of the L2 vein displacement phenotype measured by the number of triple row bristles located anterior and posterior to the position where the L2 vein contacts the anterior wing margin in control (green column) and *optix* knockdown (orange column) wings. (G,H) Representative examples of control (G) and *optix* (H) late third instar wing discs showing the localisation of cells in mitosis (PH3 in red). The expression of Kni is shown in green. (I) Mitotic index of the anterior wing blade region (outlined in G,H) in control (green) and *optix-i* (orange) wing disc. (J,K) Representative examples of control (J) and *optix* (K) late third instar wing discs showing the localisation of apoptotic cells (Dcp1 in red). (L) Apoptotic cell quantification in the anterior wing blade region (outlined in J,K) in control (green) and *optix* (orange) wing discs. **P*<0.05, ****P*<0.001. Ten discs of each genotype were analysed.

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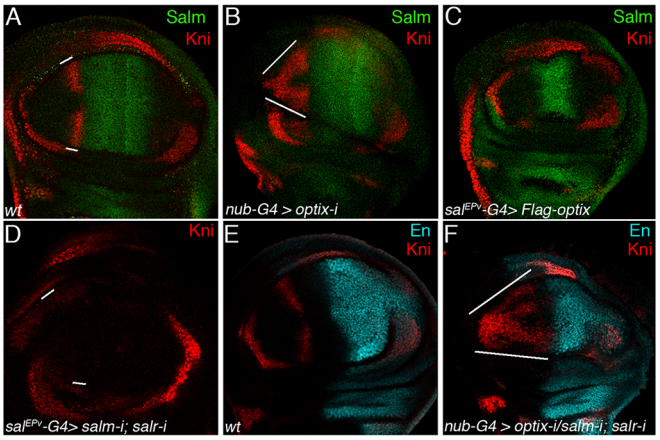


**Fig. 3. Regulation of *kni* expression in larval development.** Late third instar wing discs showing the expression of Kni (red), Sal (green) and En (cyan). The expression domain of Kni is marked with white lines. (A-C) Wild-type (A), *nub-Gal4*/*UAS-optix-i* (B) and *sal$^{EPv}$-Gal4 UAS-FLAG-optix* (C) wing discs. Kni expression expands anteriorly when *optix* expression is reduced (B), and is lost in the presumptive L2 vein when Optix is misexpressed in the domain of Sal (C). (D) Kni expression in the L2 region is lost in *sal$^{EPv}$-Gal4*/*UAS-salm-i; UAS-salr-i*/+ wing discs. (E) Wild-type control disc showing the expression of Kni and En. (F) *nub-Gal4 UAS-optix-i*/*UAS-salm-i; UASsalr-i*/+ wing disc showing an extension of the Kni domain to the anterior-posterior compartment boundary.

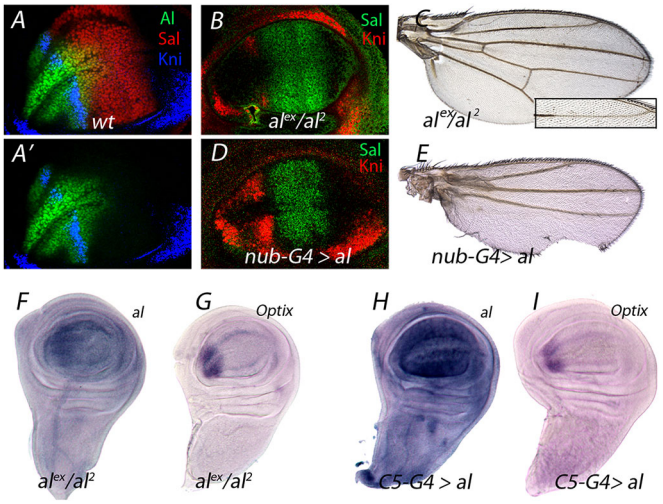


**Fig. 4. Al is needed for Kni expression.** (A,A′) Wing imaginal discs showing the expression of Al (green), Salm (red) and Kni (blue). Kni expression in the L2 territory is included within the Al expression domain. (B,C) Wing disc (B) and adult wing (C) of the hypomorphic *al* heteroallelic combination *al$^{ex}$*/*al$^{2}$*. Kni expression is reduced (B) and the L2 vein is thinner than normal (see also the inset of the distal L2 region in C). (D,E) Wing disc (D) and adult wing (E) resulting from Al overexpression in the entire wing (*nub-Gal4*/*UAS-al*). The Kni expression domain extends anteriorly in when *al* is overexpressed in the wing blade and the resulting wings present several defects, including a change in the position of the L2 vein and the loss of the L5 vein. (F,G) *In situ* hybridisation in *al$^{ex}$*/*al$^{2}$* wing discs with *al* (F) and *optix* (G). (H,I) *In situ* hybridisation in *C5-Gal4*/*UAS-al* wing discs with al (H) and *optix* (I). The expression of *optix* is not modified upon manipulations of *al* levels.

wing disc growth and wing size: cell proliferation and apoptosis of imaginal cells. We found a significant reduction in the mitotic index ($P$<0.05) of the anterior region of *optix-i* wing discs compared with the equivalent region of wild-type discs (Fig. 2G-I). In contrast, we could not find any effect of *optix* knockdown on cell death (Fig. 2J-L), indicating that the reduction in size observed in *optix-i* wings is the result of lower than normal cell proliferation in the *optix* domain of expression.

### Regulation of *kni* expression by Optix and Sal

Because *optix-i* has a dramatic effect in the growth of the A intervein and the positioning of the L2 vein, we studied the localisation of Kni in *optix* loss- and gain-of-function conditions. The expression of Kni is restricted to the L2 vein primordium, and coincides with a region of low levels of Sal expression (Fig. 3A). We found that Kni expression is expanded anteriorly in *optix* knockdown wings (Fig. 3B). Complementary to this, Kni is lost in the wing blade upon *optix* overexpression (Fig. 3C). The expression of Kni is also lost in discs with reduced expression of *salm* and *salr* (Fig. 3D). This effect was interpreted as evidence for a positive regulation of *kni* by Sal proteins (de Celis and Barrio, 2000). However, when we reduced *optix* expression in *salm/salr* knockdown discs, we observed an anterior and posterior expansion of Kni, the domain of expression of which now covers the entire anterior compartment (Fig. 3E,F). These observations indicate that the activation of *kni* by Sal proteins is indirect, and is caused by the repression of *optix* by Salm/Salr. In this manner, the Kni domain in the wing pouch is narrowed to the region where there is enough Salm/Salr to repress *optix* but not enough to repress *kni*.

### Aristaless is required to activate *kni* expression in the anterior compartment

As both Sal and Optix proteins behave as the *kni* repressors setting the posterior and anterior limits to the *kni* domain, some other factor must be involved in the activation of *kni* in the anterior compartment. The domain of *kni* expression is embedded within a larger territory of *aristaless* (*al*) expression (Fig. 4A,A′). Because Al is required for the formation of the L2 vein (Campbell et al., 1993), we wondered whether its function might be related to the regulation of *kni* expression. We found that *kni* expression is strongly reduced in hypomorphic *al* mutant backgrounds (Fig. 4B). The corresponding adult wings still differentiate a normally positioned L2 vein, but this vein is considerable thinner than in wild-type wings (Fig. 4C). Similarly, the overexpression of *al* in the entire wing pouch causes a consistent anterior enlargement of the *kni* domain of expression (Fig. 4D). The phenotype of wings overexpressing *al* varies depending on the strength of the *UAS-al* line used (data not shown), and consists of a strong reduction in the size of the wing and defects in the patterning of all longitudinal veins (Fig. 4E). The effects of Al on *kni* expression do not seem mediated by *optix*, because the expression of *optix* is not modified in loss- or gain-of-function conditions of *al* (Fig. 4F-I).

### Molecular structure of the *kni* regulatory region

The genomic structure of *kni* is complex, and includes two related transcripts (*kni* and *knirps-like*) separated by 71.6 kb of DNA containing the coding region for CG13251. The work of Lunde et al. defined a 1.4 kb regulatory region located 5′ to *kni*, named EX (Fig. 5A) (Lunde et al., 2003). This region was further dissected into repressor (0.7 kb) and activator (0.7 kb) regions, and drives reporter expression in the L2 vein primordium (Fig. 5A,B) (Lunde et al., 2003). We found that a reporter containing the EX region (named *kni-EX-GFP*; Fig. 5A,B) responds to *optix* knockdown in the same way as the endogenous *kni* gene (Fig. 5E), driving reporter expression anteriorly in *optix-i* background (Fig. 5E). The repressor region of *kni-EX* contains ten putative consensus binding-sites for Optix (Fig. 5A and Fig. S1). Five of these sites map to a 100 bp sequence conserved in 7 *Drosophila* species (Fig. S1). To evaluate

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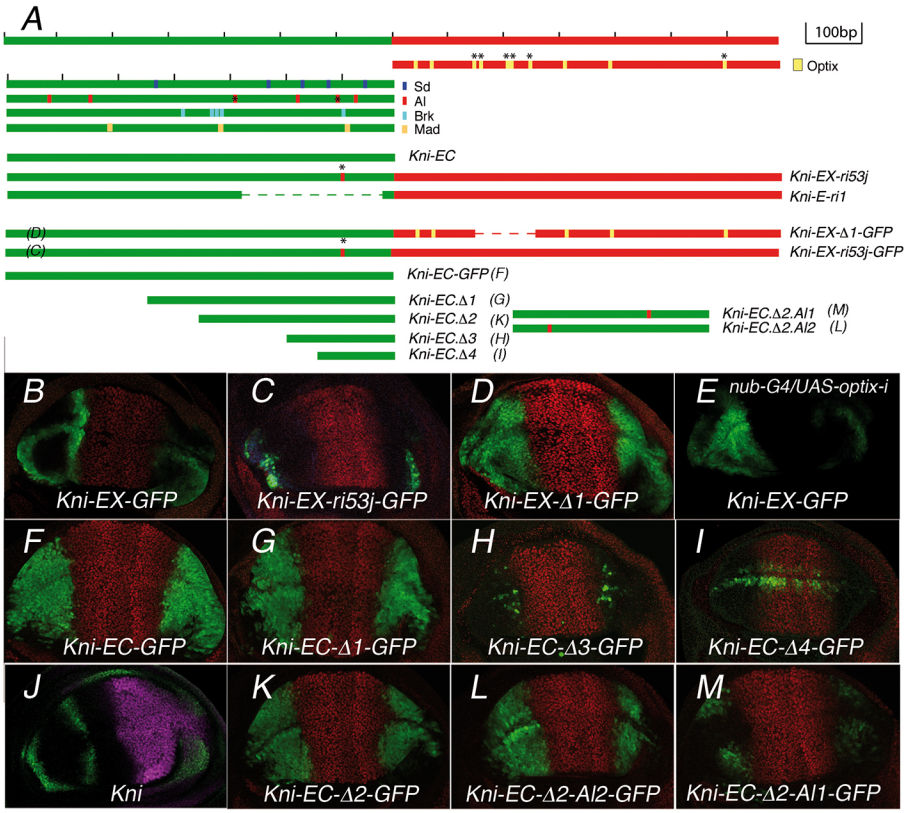


**Fig. 5. Regulation of *kni* expression in the L2 vein.** (A) Schematic representation of the 1.4 kb *kni* EX fragment (Lunde et al., 2003), indicating the 0.69 kb activating region (left, green) and the 0.71 repressor region (right, red). Consensus binding sites identified for Optix (yellow squares), Sd (blue squares), Al (red squares), Brk (light blue squares) and Mad (orange squares). The extent of the regulatory region cloned in front of GFP is shown underneath. *Kni-EC-lacZ*, *Kni-EX-ri53j* and *kni-EX-ri1* were characterised previously (Lunde et al., 2003). All other constructs are from this work. (B-E) Third instar wing discs showing the expression of GFP driven by the EX regulatory fragment and its derivate: *kni-EX-GFP* (B), *kni-EX-ri53j-GFP* (C), *kni-EX-Δ1-GFP* (D) and *kni-EX-GFP* in *nub-Gal4/UAS-optix-RNAi* discs (E). *kni-EX-Δ1* lacks six conserved Optix putative binding sites and *kni-EX-ri53j* carries the same nucleotide substitution found in the allele $ri^{53j}$. (F-I) Third instar wing discs showing the expression of GFP driven by the EC regulatory fragment and its derivate: *kni-EC-GFP* (F), *kni-EC-Δ1-GFP* (G), *kni-EC-Δ3-GFP* (H) and *kni-EC-Δ4-GFP* (I). (J) Control disc showing the expression of Kni (green) and En (magenta). (K-M) Expression of *kni-EC-Δ2-GFP* (K), and *kni-EC-Δ2-GFP* carrying mutations in the Al sites 2 (*kni-EC-Δ1-GFP-al2*; L) and 1 (*kni-EC-Δ1-GFP-al1*; M).

the contribution of this region to *kni* regulation, we generated the deletion *kni-EX-Δ1-GFP* in the context of the *kni-EX* enhancer (Fig. 5A). The expression of GFP driven by *kni-EX-Δ1-GFP* is now detected in an expanded anterior sector of the wing pouch (Fig. 5D), in a pattern indistinguishable to that of *kni-EX-GFP* in *optix-i* discs (Fig. 5E). We wanted to further dissect the 700 bp activating region of *kni* (Kni-EC-GFP; Fig. 5A) (Lunde et al., 2003). We confirmed that this region is able to drive reporter gene expression in expanded anterior and posterior domains, in a pattern complementary to that of Sal (Fig. 5F). We infer that the EC region still contains the regulatory region mediating repression by Sal, but that has lost the regions mediating repression by Optix (identified by the deletion generated in *kni-EX-Δ1-GFP*, see Fig. 5D). The EC region, and to a lesser extent the *kni-EX-Δ1* fragment, also lost repression in posterior cells, suggesting that regulatory regions mediating the repression by the posterior determinant Engrailed have being lost in the *Kni-EC-GFP* construct (Fig. 5F).

To further dissect the *kni*-EC activator fragment, we made four consecutive deletions (*kni-EC-Δ1* to *kni-EC-Δ4*; Fig. 5A,G-I,K). Deletions 1 (Fig. 5A,G) and 2 (Fig. 5A,K) still retain full enhancer activity and Sal repression. In contrast, deletion 3 shows strongly reduced activity in the wing blade, but still retains Sal repression (Fig. 5A,H). The smaller fragment we analysed, *kni-EC-Δ4*, shows a distinct expression pattern restricted to two stripes of cells adjacent to the dorsoventral boundary (Fig. 5A,I). In this manner, we were able to further delimit the activating region of *kni* to a 330 bp fragment (*kni-EC-Δ2*) that also includes sequences mediating repression by Sal. These sequences must be in part included in the 200 bp stretch included in *kni-EC-Δ2* but lost in *kni-EC-Δ4*. The activator region *kni-EC* contains one putative Al binding site (TAATTAA; Noyes et al., 2008; see Fig. S1) at position 587 that we named Al1. In addition, this fragment contains a less-conserved putative Al binding site at position 416, named Al2 (GTAATTAT; Fig. S1). To analyse the contribution of these Al consensus-binding regions to the expression of *kni*, we generated mutations in Al1 (*kni-EC-Δ1-Al1*; Fig. 5M) or Al2 (*kni-EC-Δ1-Al2*) and studied their impact in the context of the minimal activating fragment: *kni-D2*. We found that mutating the Al1-binding size strongly reduces but does not abolish, the activity of *kni-EC-Δ1* (Fig. 5M). In contrast, the mutation in Al2 has no effect on reporter activity (Fig. 5L). These results match very well with the effects of two *kni* alleles that were mapped to the *kni-EC* fragment (Lunde et al., 2003). Thus, part of the region that distinguishes our deletions D2 and D3 is included in the region deleted in the *kni* allele $kni^{ri1}$, which lacks all *kni* expression in the L2 primordium (Lunde et al., 2003). In addition, the hypomorphic allele $kni^{ri53j}$ maps two base pairs adjacent to the Al1 putative binding site (Lunde et al., 2003), confirming that this site is functional. In fact, introducing this mutation in the context of the *kni-EX* fragment strongly reduces reporter expression in the wing blade (Lunde et al., 2003; see Fig. 5B-C).

### Dpp signalling regulates the expression of the transcription factors positioning the L2 vein

The expression of *salm* and *salr* in the wing blade are regulated by the Dpp pathway (de Celis et al., 1996). As the expression pattern of *optix*, *al* and *kni* are also positioned with respect to the anteroposterior compartment boundary, we checked whether these domains depend on Dpp signalling. We used transient ectopic expression of *brinker* (*brk*) as a way to reduce Dpp signalling in wing discs of *tub-Gal80$^{ts}$; ap-Gal4 UAS-GFP/UAS-brk* wing discs grown at the restrictive temperature (29°C) for 12 h. In these discs, Brk is overexpressed in dorsal cells when the larvae grow at the Gal80$^{ts}$ restrictive temperature. The overexpression of Brk causes a considerable reduction in the size of the dorsal wing compartment compared with the corresponding ventral compartments (Fig. 6B,D). In these dorsal compartments, the expression of Sal is lost, as expected (Fig. 6B-B″, compare with

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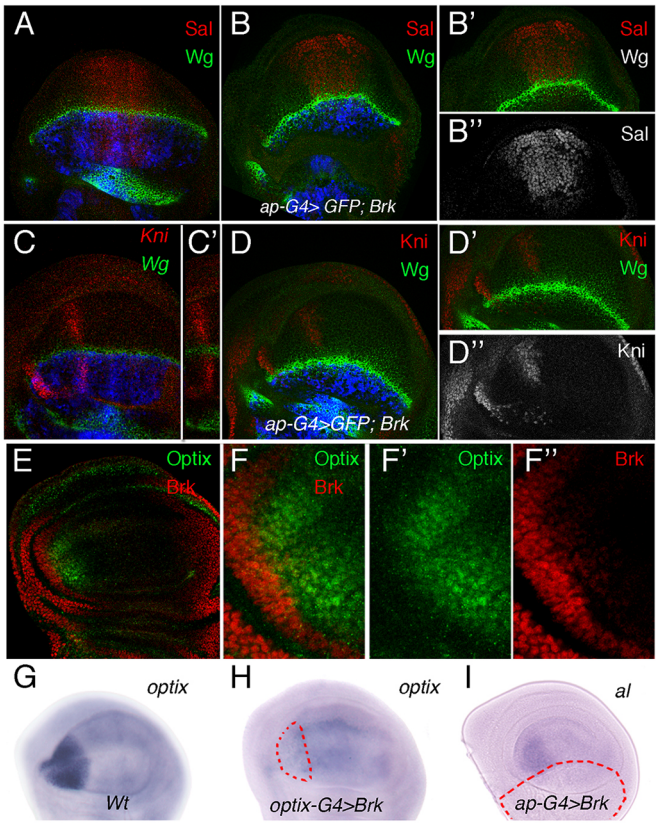


**Fig. 6. Regulatory interactions between *salm*/*salr*, *optix*, *kni* and the Dpp signalling pathway.** (A) Control *ap-Gal4 UAS-GFP*/+ wing disc showing the expression of Salm (red), Wingless (Wg, green) and GFP (blue). (B-B″) Wing disc of *tub-Gal80$^{ts}$*/+; *ap-Gal4 UAS-GFP*/*UAS-brk* after 12 h at 29°C. The domain of Brk overexpression (blue) is reduced in size and the expression of Sal (red in B,B′ and white in B″) is strongly reduced or lost in dorsal cells. (C,C′) Control *ap-Gal4 UAS-GFP*/+ wing disc showing the expression of Kni (red), Wingless (Wg, green) and GFP (blue). The expression of Kni (red) and Wg (green) is also shown in C′. (D-D″) Wing disc of *tub-Gal80$^{ts}$*/+; *ap-Gal4 UAS-GFP*/*UAS-brk* after 12 h at 29°C. The domain of Brk overexpression (blue) is reduced in size and the expression of Kni (red in D-D″ and white in D″) is strongly reduced or lost in dorsal cells. (E) Late third instar *brk-lacZ*/+ wing disc showing the expression of Optix (green) and *brk-lacZ* (red). (F-F″) Higher magnification of the optix domain showing the mostly complementary localization of Optix (green in F-F″) and *brk-lacZ* (red in F,F″). (G,H) *optix in situ* hybridization in wild-type discs (G) and in *optix-Gal4*/*UAS-brk* discs (H). (I) *al in situ* hybridization in *tub-Gal80$^{ts}$*/+; *ap-Gal4 UAS-GFP*/*UAS-brk* after 12 h at 29°C. The expression of both *optix* and *al* is lost when Brk is overexpressed.

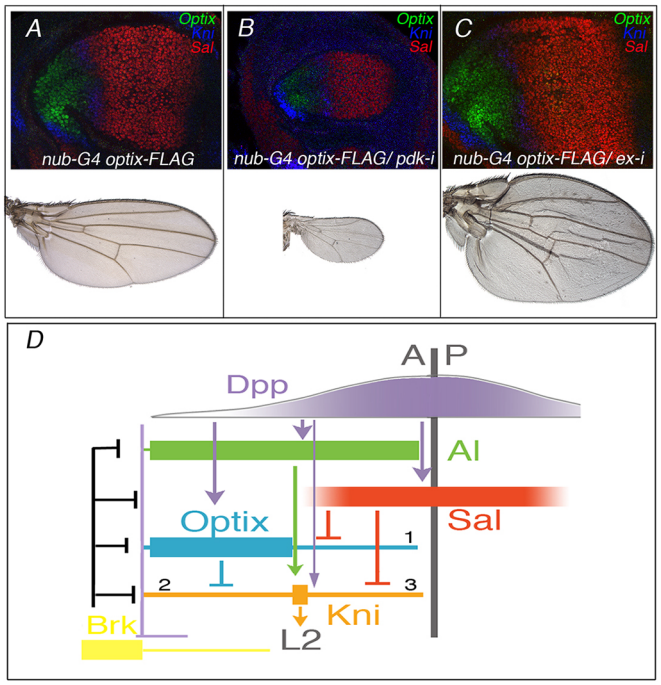


**Fig. 7. The regulatory interactions participating in the positioning of the L2 vein.** (A) Wing disc (top) and adult wings (bottom) of *nub-Gal4 fTRG optix-GFP-Flag*/+ controls. (B,C) Wing disc (top) and adult wing (bottom) *of nub-Gal4 fTRG optix-GFP-Flag*/*UAS-pdk-RNAi* (B) and *nub-Gal4 fTRG optix-GFP-Flag*/*UAS-ex-RNAi*. All discs show the expression of *fTRG optix-GFP-Flag* (Optix; green), Kni (blue) and Sal (red). (D) Schematic model of the regulatory interactions taking place downstream of Dpp. The Dpp gradient (purple) activates *al* (green), *kni* (orange), *salm*/*salr* (red) and *optix* (blue). The restriction of *kni* expression to its domain requires a positive input from Al, and negative inputs from Sal and Optix. In this manner, *kni* is expressed only within the Al domain in the region where there is enough Sal to repress *optix*, but not enough Sal to repress *kni* (indicated by light red). Horizontal coloured lines represent the extension of the expression domains of *optix* (blue) and *kni* (orange) in *salm*/*salr* knockdown (1), in *optix* knockdown (2) and in *optix*/*salm*/*salr* triple knockdown (2+3). The expression of *al*, *salm*/*salr*, *optix* and *kni* is abolished upon increased expression of *brk* (yellow), indicating that all these expression domains are repressed by Brk (black lines). The expression of at least *optix* and *kni* is repressed in the posterior compartment, most likely by the En protein (not shown).

Fig. 6A). The same result is observed with Kni, the expression of which is eliminated from dorsal cells (Fig. 6D-D″, compare with Fig. 6C,C′). The domain of Optix accumulation is mostly complementary to the region of maximal expression of Brk (Fig. 6E-F″), suggesting that Brk repression might define the most anterior boundary of Optix in the wing blade. In agreement, ectopic or increased expression of *brk* in the optix domain (*optix-Gal4/UAS-brk*) eliminates optix transcription (Fig. 6G,H). The expression of *al* is regulated in the wing disc by Wingless signalling (Campbell et al., 1993). Here, we have found that transient and ectopic expression of *brk* in dorsal cells causes the loss of *al* expression in the dorsal compartment of the wing disc (*ap-Gal4/tub-Gal80$^{ts}$; UAS-brk/+*; Fig. 6I), suggesting that Dpp signalling also regulates *al* expression in anterior wing cells. The position of the L2 domain (*kni* expression) is set by a combination of transcription factors regulated by the Dpp gradient through the complementary Brk gradient (Schwank et al., 2008). In addition, the Dpp/Brk system adjusts to wing disc size, allowing for the coordination of patterning to the growth of the disc (Ben-Zvi et al., 2011; Hamaratoglu et al., 2011). We find that wings of very different sizes retain a similar distribution of Optix-Kni-Sal expression domains (Fig. 7A-C′), further confirming that the Dpp/Brk system coordinates pattern in a growing epithelium by its ability to scale and through the regulation at different thresholds of Brk expression of genes encoding transcription factors that engage in cross-regulatory interactions to set and reinforce gene expression boundaries.

## DISCUSSION

In this work we have identified *optix* as a novel component of the regulatory machinery that sets the position of the *Drosophila* longitudinal veins. We show that *optix* is expressed in an anterior domain of the developing wing blade under the regulation of Dpp signalling. In turn, Optix participates in the regulation of *kni* expression in the primordium of the L2 vein, setting the anterior limit of this domain. Making a comparison with the genetic hierarchy regulating blastoderm segmentation, *optix* occupies a position analogous to a gap gene, because it is regulated in a broad domain

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by the gradient of Dpp signalling, and participates in the regulation of one downstream component expressed in a single stripe (*kni*).

### Expression, regulation and function of *optix*

The expression of *optix* is first detected in mid third instar wing discs as a broad domain located in the most anterior region of the wing blade. In all wing discs where we visualised the localisation of Optix and Salm, both domains appeared complementary to each other. Furthermore, the domain of Optix extends posteriorly in discs with reduced *salm* and *salr* expression, indicating that Sal activity sets the posterior border of *optix* expression (Fig. 7D). Cells expressing *optix* in the wing blade correspond to anterior cells with low levels or no expression of Brk. In addition, Dpp downregulation of *brk* is a prerequisite for *optix* expression, suggesting that the *optix* domain corresponds to a territory of low Dpp signalling where two repressors regulated by the pathway, Brk and Salm/Salr, are either expressed at low levels (Brk) or not expressed at all (Salm/Salr). Because the domain of *salm/salr* is present before the start of *optix* expression, we suggest that *optix* initiates its expression when the wing disc has expanded in size to an extent that allows the generation of an anterior territory containing low enough levels of Brk and no Salm/Salr. We do not know whether *optix* transcription in the wing blade requires an additional direct positive input mediated by other components of the Dpp pathway, as it is the case of *salm* (Barrio and de Celis, 2004).

The main morphological characteristic of *optix* knockdown wings is the reduction in size of the region between the L2 vein and the anterior wing margin. This reduction is in part caused by lower cell division in the corresponding imaginal disc, suggesting that Optix might promote cell proliferation in its domain of expression. We have not explored the mechanistic bases of this effect. One possibility is that Optix contributes to define the particular characteristics of the anterior-most wing pouch, in a similar manner to the way Salm/Salr proteins determine the characteristics of the wing central region (Organista and de Celis, 2013). Alternatively, the defects observed in cell proliferation might be the consequence of the inappropriate expression of Optix target genes in the anterior wing pouch. Thus, knockdown of *optix* causes ectopic expression of *kni*, and the presence of Kni could interfere with the development of this territory (Lunde et al., 1998). In fact, the anterior displacement of the L2 vein is one of the several phenotypic consequences described for *kni* over-expression in anterior cells (Lunde et al., 1998).

### Regulation of *kni* by a combination of two repressors and one activator

The position of the L2 vein in the wing pouch corresponds to the stripe of cells expressing the Kni genes (Lunde et al., 1998). In this manner, understanding the elements and mechanisms that position this vein requires the unravelling of the regulation of this gene complex during imaginal development. It has previously been shown that the regulatory region of Kni contains both repressor and activator regions (Lunde et al., 1998). Furthermore, these authors identified consensus sequences for the Scalloped (Sd), Engrailed (En) and Brk transcription factors, which might mediate the repression of *kni* in the posterior compartment (En) and in the anterior-most cells of the anterior compartment (Brk), as well as its activation in the wing blade (Sd). We find here that Optix and Sal have all the characteristics required to set the anterior and posterior limits of *kni* expression. Thus, the anterior border of *kni* coincides with the posterior border of Optix localisation, and manipulating the expression of *optix* displaces this boundary. The situation is slightly more complex in the case of the posterior limit of *kni*, because in this case the complementarity with the territory of Sal-expressing cells is not perfect (Fig. 7D). Thus, Kni and Sal proteins colocalise in the most anterior region of the *salm/salr* domain of expression, a territory that is characterised by consistently lower levels of Sal accumulation (Fig. 7D). This situation is compatible with a lower efficiency of Sal compared with Optix to repress *kni*, leading to an effective repression of this gene only above a certain threshold of Sal protein concentration. In contrast, Sal repression of *optix* must be more effective that its repression of *kni*, occurring even at low levels of Sal accumulation (Fig. 7D). The sequences mediating Sal repression are still unknown, but might include A/T rich regions (de Celis and Barrio, 2009), as those present in the activator domain of the *kni* enhancer that are present in the reporter *kni-EC-Δ3-GFP* but absent in *kni-EC-Δ4-GFP*.

The cross-interactions between Optix and Sal, and between Optix/Sal and *kni* described here ensure that given a generalised activation of *kni* in the anterior compartment of the wing pouch, its expression would become restricted to anterior cells expressing low levels of *salm/salr* and no *optix* at all (Fig. 7D). The position of this population of cells would therefore be determined by the different efficiencies of Dpp regulation of *salm/salr* and *optix*, by the repression of *optix* by Salm/Salr, and by the repression of *kni* by Optix and Salm/Salr (Fig. 7D). The regulatory region of *kni* contains all the necessary information to translate Sal and Optix repression, in this last case through a group of five consecutive Optix-binding sites present in the repressor part of the *kni-EX* enhancer. For the activation of *kni*, we identified the homeobox transcription factor Al as an additional candidate. Thus, *kni* expression is nested within the domain of Al expression, and Al activity is necessary and sufficient to activate *kni* in the anterior wing pouch. Furthermore, the activator region of the *kni* enhancer contains a canonical Al binding site (Al1) that is necessary for the complete expression of a reporter in this territory.

### The implementation of Dpp signalling in patterning of the anterior wing blade compartment

The expression of *al* in the wing blade, as for *salm/salr*, *kni* and *optix*, also depends on Dpp signalling. This group of genes appears as the set of downstream components of the pathway implementing pattern information in the anterior compartment of the wing pouch. The cross-regulatory interactions between then lead to the formation of one vein, the L2, and one intervein, the L2/anterior margin territory. Interestingly, the interactions between Al, Optix, Salm/Salr and Kni bear a strong similarity to the mechanisms regulating the expression of the pair-rule gene *even-skipped* (*eve*) during embryonic segmentation. In this case, the combination of broadly expressed activators (provided by Jak-Stat signalling and the maternal zinc-finger protein Zelda) and two repressors (the gap genes Hunchback and Kni) regulate the expression of the *eve* enhancer operating in stripes 3 and 7 (Small et al., 1996; Struffi et al., 2011). Furthermore, a similar mechanism works in the regulation of *eve* in the stripe 2, in this case using (as repressors) Giant in anterior regions and Kruppel in central regions, and (functioning as activators) Bicoid and zygotic Hunchback (Stanojevic et al., 1991). In this manner, the territorial subdivision of the wing disc and blastoderm segmentation share a similar patterning mechanism in which 'cardinal' genes initiate transcriptional cascades along the length of the cellular space that self-generate smaller domains of transcription factor expression culminating in a periodic pattern of signalling domains (parasegments and vein territories). The two key differences between these two developmental processes are that early blastoderm segmentation occurs in a pre-cellular territory and that the formation of the wing implies extensive cell proliferation, which results in the growth of the epithelium. The increase in the size

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of the wing primordium imposes a dynamic spatial and temporal component to the regulation of gene expression to ensure a certain degree of size invariance in the pattern, as observed in wings discs that have been genetically modified to reach very different final sizes but retain the same pattern. The set of transcription factors engaged in cross-regulatory interactions and regulating *kni* act downstream of Dpp signalling, and likely this ensures that the L2 primordium (*kni* expression domain) is permanently and dynamically established with reference to a gradient of Dpp signalling/*brk* expression that continuously matches pattern to size in the growing wing pouch (Ben-Zvi et al., 2011; Hamaratoglu et al., 2011).

## MATERIALS AND METHODS

### Genetic strains

We used the following lines: the *Gal4*lines, *sal$^{EPv}$-Gal4* (Cruz et al., 2009), *ap-Gal4*, *nub-Gal4, C5-Gal4* and *optix-Gal4* (Jory et al., 2012); the Gal80 lines, *tub-Gal80$^{ts}$* and *sal$^{EPv}$-Gal80*; the *UAS* lines, *UAS-GFP*, *UAS-salm-i* (ID 3029 VDRC), *UAS-salr-i* (ID 28386 VDRC), *UAS-dicer2* [31], *UAS-optix-i* (ID 18455R-2), *UAS-pdk*-i (109812-KK), *UAS-ex-i* (109281/KK), *UAS-salm*, *UAS-optix*, *UAS-Flag-optix* (see below) and *UAS-aristalles* (*UAS-al*; a gift from Dr Rosa Barrio, CIC-Biogune, Spain). The expression of *sal$^{EPv}$-Gal4* is restricted to the central region of the wing imaginal disc between the vein L2 and intervein L4-L5; the expression of *nub-Gal4* occurs in the entire wing pouch and hinge; the expression of *C5-Gal4* occurs at low levels in the wing blade; and the expression of *ap-Gal4* is restricted to the dorsal wing compartment. The *UAS* lines used to express RNA interference were obtained from Bloomington Stock Center, Vienna Drosophila RNAi Center (VDCR) and NIG-FLY RNAi. Unless otherwise stated, crosses were made at 25°C. Detailed information about the lines used can be found in FlyBase.

### Immunocytochemistry

We used rabbit anti-Salm and rat anti-Salm (de Celis and Barrio, 2000; 1:200); mouse anti-Wg and mouse anti-FasIII (Hybridoma Bank at Iowa University; 1:100); guinea pig anti-Dll (Estella et al., 2008; 1:100) and anti-Kni (Kosman et al., 1998; 1:200); rabbit anti-PH3 and anti-Dcp1 (Cell Signalling Technology, 9701 and 9578, respectively; 1:200); and anti-βGal (Promega, Z3781; 1:200). Secondary antibodies were from Jackson Immunological Laboratories (used at 1/200 dilution). Imaginal wing discs were dissected in PBS, fixed with 4% paraformaldehyde in PBT (0.1% Triton X-100 in PBS), washed and blocked in PBT-BSA (Triton plus 1% BSA) (PBT-BSA). The discs were incubated overnight with primary antibodies at 4°C in PBT-BSA. After four washes in PBT the discs were incubated with secondary antibodies diluted 1:200 in PBT-BSA. Confocal images were captured using a LSM510 confocal microscope. All images were processed with the program ImageJ 1.45s (NIH) and Adobe Photoshop CS6.

### *In situ* hybridisation

Imaginal discs were dissected and fixed in 4% formaldehyde for 20 min at room temperature, washed in PBS-0.1% Tween (PBT) and re-fixed for 20 min at room temperature with 4% formaldehyde and 0.1% Tween. After three washes in PBT, discs were stored at −20°C in hybridisation solution (HS; 50% formamide, 5× SSC, 100 µg/ml salmon sperm DNA, 50 µg/ml heparin and 0.1% Tween). Disc were pre-hybridised for 2 h at 55°C in HS and hybridised with digoxigenin-labelled RNA probes at 55°C. The probes were previously denaturalised at 80°C for 10 min. After hybridisation, discs were washed in HS and PBT, and incubated for 2 h at room temperature in a 1:4000 dilution of anti-DIG antibody (Roche). After incubation, the discs were washed in PBT and the detection of probes was carried out using NBT and BCIP solution (Roche). The discs were mounted in 70% glycerol. Pictures were taken using a Spot digital camera coupled to a Zeiss Axioplan microscope using 20× objective lenses. All images were processed using Adobe Photoshop CS6. The probes were generated the cDNAs LD05472 (*optix*) and RE68460 (*al*) from the Expression Sequence Tags (EST) collection of the Berkeley Drosophila Genome Project.

### Generation of *kni* reporter lines

The *kni-EX* and *kni-EC* regulatory regions (Lunde et al., 2003) and *kni-EX-Δ1* and *kni-EC-Δ1* to *Δ4* fragments (see Fig. 5) were amplified by PCR from genomic DNA (oligonucleotides are described in Table S1). In the case of *kni-EX-Δ1*, we added an *Avr*II restriction site at the 5′-end of the inner oligonucleotides, and the PCR products were digested and their sticky ends bound by T4 ligase. We used the Gateway system (Invitrogen) to clone into pENTR/D-TOPO vectors and performed LR recombination (Invitrogen) into pHPDest-eGFP (Boy et al., 2010). Positive clones were sequenced using the oligo CTTCGGGCATGGCGGACTTG (GFPreverse), and the selected plasmids were injected and integrated into the *attP* site of the 3R chromosome-arm of *y$^{1}$ M[vas-int.Dm]ZH-2A w; M[3xP3-RFP.attP]ZH-86Fb* genotyped flies using the *ϕC31* integration system (Bischof et al., 2007).

Substitution of the single cytosine present in the *kni* alelle *kni$^{ri53j}$* was performed over the pENTR-*kni-EX* vector (*kni-EX* C596A) and mutagenesis of the chosen presumptive Aristaless binding sites (Al1 and Al2) over the pENTR-*kni-EC-Δ2* vector (substitutions $^{587}$TAATTAA>GCCGGCC for Al1 and $^{416}$GTAATTAT>TGCCGGCG for Al2). In all cases, we used QuickChange II Site-Directed Mutagenesis Kit (Aligen Technologies; oligos described in Table S1). LR recombination into pHPDest-eGFP and fly transformation was performed as described below.

### Generation of the *UAS-optix* y *UAS-Flag-optix* lines

We cloned into pENTR-D-TOPO (Invitrogen), a PCR fragment containing the coding sequence of *optix*, using the pENTR-D-TOPO Cloning Kit (Invitrogen) and made the recombination reactions with the destination vectors pTFW (*UAS-Flag-optix*) and pTW (*UAS-optix*) from the DRGC using LR-Clonasa II (invitrogen). Positive clones were sequenced using the oligo GGCATTCCACCACTGCTCCC and injected into *w$^{1112}$ Drosophila* embryos.

### Optix-binding sites and sequence comparison of the EX DNA

Optix-binding sites were obtained from the JASPAR data base (JASPAR CORE insecta; http://jaspar.binf.ku.dk) (Fig. S1). We considered those sites in which sequence is conserved in at least 7 *Drosophila* species to be Optix-binding sites. To compare the EX sequence in different *Drosophila* species, we obtained the corresponding sequences from the UCSC genome browser (https://genome.ucsc.edu) for the following species: *D. melanogaster*, *D. simulans*, *D. yacuba*, *D. erecta*, *D. ananasse*, *D. pseudooscura* and *D. mojavensis*. We aligned these fragments using the Pro-coffee tool from the T-coffee website (http://tcoffee.crg.cat/; see Fig. S1).

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Validation: M.M.; Investigation: M.M., C.M.O., J.F.d.C.; Writing - original draft: M.M., J.F.d.C.; Writing - review & editing: J.F.d.C.; Supervision: J.F.d.C.; Project administration: J.F.d.C.; Funding acquisition: J.F.d.C.

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#### Supplementary information

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