Regulation of *decapentaplegic* expression during *Drosophila* wing veins pupal development

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Abstract

The differentiation of veins in the *Drosophila* wing relies on localised expression of *decapentaplegic* (*dpp*) in pro-vein territories during pupal development. The expression of *dpp* in the pupal veins requires the integrity of the *shortvein* region (*shv*), localised 5' to the coding region. It is likely that this DNA integrates positive and negative regulatory signals directing *dpp* transcription during pupal development. Here we identify a minimal 0.9Kb fragment giving localised expression in the vein L5 and a 0.5Kb fragment giving expression in all longitudinal veins. Using a combination of *in vivo* expression of reporter genes regulated by *shv* sequences, *in vitro* binding assays and sequence comparisons between the *shv* region of different *Drosophila* species, we found binding sites for the vein-specific transciption factors Araucan, Knirps and Ventral veinless, as well as binding sites for the Dpp pathway effectors Mad and Med. We conclude that conserved vein-specific enhancers regulated by transcription factors expressed in individual veins collaborate with general vein and intervein regulators to establish and maintain the expression of *dpp* confined to the veins during pupal development.

1. Introduction

The *Drosophila* wing is formed by the cuticle secreted during metamorphosis by epidermal cells of the wing imaginal disc, the epithelium that gives rise to the wing and thorax of the fly. The veins and interveins are the two major pattern elements of the wing and they differ in cuticle pigmentation and trichome density. The patterning of veins occurs in the wing imaginal disc and involves the activity of several conserved signalling pathways and transcription factors (de Celis, 2003). The first step in vein patterning requires Hedgehog (Hh) and Decapentaplegic (Dpp) signalling to determine the position of each provein and the size of the interveins. Hh and Dpp signalling regulate the expression of both provein- and intervein-specific transcription factors. A second step in vein patterning involves the activity of the Notch and Epidermal Growth Factor Receptor (EGFR) pathways. The expression of several elements of these pathways is activated within each provein. Interactions between Notch and EGFR signalling divide the provein into a central domain, where EGFR is active, and two boundary domains where Notch is active. Finally, during pupal development, the expression of *dpp* is activated *de novo* in the developing veins, where it is required for their differentiation (de Celis, 2003).

Dpp is the ligand of a conserved signalling pathway involved in regulating gene expression and cell behaviour in a variety of developmental processes throughout the animal kingdom (Kingsley, 1994; Spencer et al., 1982; Wrana et al., 1994). In all cases the expression of *dpp* is spatial and temporally regulated and miss-expression of the gene causes profound developmental alterations (Capdevila and Guerrero, 1994; Staehling-Hampton and Hoffmann, 1994). The genetic structure of *dpp* reflects its complex

regulation. Thus, five promoters and many enhancers distributed over 50Kb of DNA contribute to the overall pattern of expression of the single protein-coding sequence (St Johnston et al., 1990). Dpp plays key roles during *Drosophila* embryogenesis in processes such as dorso-ventral patterning, dorsal closure and mesoderm development, to name a few (Frasch, 1995; Staehling-Hampton et al., 1994; Wharton et al., 1993). During dorso-ventral patterning multiple regions within the second intron of *dpp* cooperate to generate the pattern of *dpp* transcription (Huang et al., 1993). This region contains both enhancer elements and ventral-specific repressor elements (Huang et al., 1993).

Dpp also directs growth and patterning of the imaginal discs during larval development and triggers vein differentiation during pupal development (de Celis, 1997; Gelbart, 1989; Posakony et al., 1991). These two independent activities occur at different developmental times and require an exquisite regulation of *dpp* expression. *Dpp* expression in the wing disc is regulated by sequences localised 3' to the coding region (Blackman et al., 1991). These sequences stretch approximately 20Kb of DNA and constitute the dpp^{disc} (dpp^{d}) genetic region of the locus (St. Johnston et al., 1990; Blackman et al., 1991). Mutations in the dpp^{d} region reduce or eliminate the expression of *dpp* along the anterior-posterior compartment boundary and result in small wings with a reduction or elimination of pattern elements. In contrast, mutations in the 15Kb region located 5' to the coding region cause incomplete differentiation of the veins in wings of normal size (Segal and Gelbart, 1985). These alleles, named $dpp^{shortvein}$ (dpp^{s}), reduce the expression of *dpp* in the pupal wings, suggesting they affect the regulatory region responsible for *dpp* expression at this stage (de Celis, 1997). However, this region is

included in a large intron of one *dpp* transcript (St. Johnston et al., 1990) and there is no proof of the existence of regulatory DNA in the dpp^s region responsible for *dpp* expression during pupal development.

We have studied the dpp^s region to identify and characterise the regulatory sequences driving *dpp* expression in the veins during pupal development. We found cis-regulatory regions that activate the expression of *dpp* in individual veins. These regions contain binding sites for vein-specific transcription factors such as Iroquois (Iro; veins L3 and L5) and Knirps (Kni; vein L2) as well as binding sites for transcription factors expressed in all veins such as phosphorylated Mothers against Dpp (P-Mad) and Ventral veinless (Vvl). The fragments we identified in reporter constructs show very high sequence conservation among several *Drosophila* species and they are able to bind *in vitro* the Iro, Vvl and Mad transcriptional regulators. Our results demonstrate the existence of modular vein-specific regulatory sequences in the dpp^s region. This observation implies that each vein has individual characteristics susceptible to independent variation during evolution.

2. Results and Discussion

2.1. Expression and cis-regulation of decapentaplegic in pupal wings

The expression of *dpp* in the wing disc is restricted to a narrow stripe of anterior cells localised at the anterior/posterior compartment boundary (Sanicola et al., 1995; Fig. 1C). This expression is regulated by sequences localised 3' to the *dpp* coding region, and the

function of the gene at this stage is required for the growth and patterning of the wing (Fig.1A-D). The expression of *dpp* is still detected at the A/P boundary during the first 8 hours of pupal development. Later, at 14h APF novel domains of *dpp* expression appear corresponding to the developing wing veins (de Celis, 1997; Ralston and Blair, 2005; Yu et al., 1996; Fig. 1E). The function of *dpp* during pupal development requires the integrity of the *shv* region, which is localised 5´to the *dpp* coding region (de Celis, 1997; Segal and Gelbart, 1985; Fig. 1F). There are two different transcripts expressed during pupal development, transcripts *dpp-RA* and *dpp-RC*, whose promoters (P5 and P3, respectively) are separated by approximately 20Kb of DNA (St Johnston et al., 1990) Figure 1A). This DNA includes the first exon of transcript *dpp-RC* (Figure 1A) and corresponds to the place where all dpp^s alleles map (St Johnston et al., 1990). Because the strength of dpp^s alleles correlates with their distance to the P3 promoter, it is likely that *dpp* function in pupal development is mediated mainly by transcript *dpp-RC*. This suggests that dpp^s mutations affect regulatory sequences necessary to drive *dpp* expression in presumptive vein territories during pupal development. We confirmed this possibility by analysing the expression of a 8.5Kb construct containing most of the *shv* region fused to the reporter gene *lacZ* ($shv^{8.5}$-*lacZ*) (Capovilla et al., 1994). The expression of βGal in $shv^{8.5}$-*lacZ* is detected exclusively in the pupal veins, indicating that this region includes all *dpp* wing veins regulatory regions (Figure 1E). We made several constructs using different sub-fragments from the original 8.5Kb dpp^s DNA to identify with more precision the sequences that regulate *dpp* expression during pupal development (Figure 2A). These fragments were cloned in front of a dicistronic *lacZ-Gal4* reporter gene and the activity of these constructs was analysed by looking at the expression of βGal in pupal wings from transgenic flies (Figure 2). In addition, to

amplify the signal of the dicistronic *lacZ-Gal4* gene, we also monitored the expression of a reporter gene regulated by UAS sequences (Figure 2). This expression should reveal all places where the Gal4 protein is present. Constructs including the 5´ end of the 8.5Kb *shv* fragment (*2.7Kb EcoRI/SalI* and *1.85Kb SalI/SalI* do not direct reporter gene expression in pupal wings (not shown). In contrast, a *3Kb KpnI/KpnI* fragment from the 3´end of *shv* reproduces most aspects of *dpp* expression in pupal wings (Figure 2A,C). The expression of βGal in *3Kb KpnI/KpnI* pupal wings includes the longitudinal veins L2-L5 (Figure 2C). This expression is detected in all longitudinal veins at least from 14 until 40 hours after puparium formation (APF), a time window similar to the establishment of *dpp* transcription in the pupal wing (Sotillos and de Celis, 2005; Ralfston and Blair, 2005). The *3Kb KpnI/KpnI* fragment was further subdivided into a *1.65Kb KpnI/XhoI* and *1.35Kb XhoI/KpnI* fragments. Only the *1.65Kb KpnI/XhoI* fragment drives expression of *lacZ-Gal4* in the *3Kb KpnI/KpnI* pattern (Figure 2A, D). Most aspects of this expression were also retained in the *1.1Kb KpnI/SacII* and *0.5Kb SacII/Xho* fragments (Figure 2A, F, G). An additional enhancer able to drive reporter expression exclusively in L5 was also identified in a large *3.7Kb XhoI/KpnI* fragment located 5' to the *3Kb KpnI/KpnI* region (Figure 2A, B). This enhancer most likely resides in the *0.9Kb SalI/KpnI* fragment, because these constructs were also able to direct reporter gene expression in the L5 (Figure 2A, E). In every fragment that drives expression in the pupal wing, we detected βGal protein only in the developing veins (Figure 2B'-D'). In addition the expression of a reporter under UAS control also revealed expression in intervein territories in most constructs included in the *3Kb KpnI/KpnI* fragment (Figure 2C-G), suggesting that the expression of *lacZ* in these regions was under the level of detection. These observations are compatible with *dpp*

being expressed at very low levels in the interveins. Alternatively, the absence in these constructs of sequences mediating repression of *dpp* in the interveins might result in low level of reporter expression in these territories.

In summary we have identified several regulatory regions that control *dpp* expression in the veins during pupal development. One regulatory sequence is localised in a *1.1Kb* fragment localised 6.5Kb from P3, and drives high levels of expression in most pupal veins and low levels of expression in some interveins. Additional regulatory sequences that drive efficiently expression in most veins are localised in an adjacent *0.5Kb* fragment, and further vein-specific regulatory sequences for L5 are localised in the *0.9Kb SalI/KpnI* fragment.

2.2. Embryonic expression of the dpp^{s} constructs

The expression of *dpp* during embryogenesis is highly dynamic and several independent regulatory regions controlling embryonic *dpp* expression have already been identified (Hursh et al., 1993; Masucci and Hoffmann, 1993; Capovilla et al., 1994). The *shv* constructs included in the 8.5Kb *EcoRI* fragment drive reporter expression during embryonic development from stage 12/13 mainly in three regions of the mesoderm: the oesophagus, gastric caeca and midgut (Figure 3). Regulatory regions controlling *dpp* expression in the oesophagus appear to be duplicated, because they are localised in the 2.7Kb *EcoRI/SalI* fragment and also in the 3Kb *KpnI/KpnI* fragment (Figure 3 D, E, H, I). Similarly, regions controlling *dpp* expression in the gastric caeca are also present in the two adjacent fragments 0.9Kb *SalI/KpnI* and 3Kb *KpnI/KpnI* (Figure 3 J-K, H-I). As

previously described (Hursh et al., 1993; Capovilla et al., 1994), the regions driving reporter expression in the gut are localised in the 3' end of the *shv* region (Figure 3 H-I, L-M).

2.3. In vitro binding of vein-development regulatory proteins to the shv enhancer

To better understand the regulation of *dpp* expression during vein development, we analyzed the interactions between a 2.5Kb region including wing veins pupal enhancers and several proteins involved in the regulatory network controlling the formation of veins (Bier, 2000; de Celis, 2003). For this purpose, the 2.5Kb region was subdivided into overlapping fragments of 250-300 bp (Figure 4A) used as probes to detect the binding of different transcription factors by electrophoretic mobility-shift assays (EMSAs). We tested both prepattern specific genes that control vein development, such as *Ventral veinless* (*Vvl*) and the Araucan protein (Ara), and transcription factors belonging to the Dpp pathway (Mad and Medea).

The POU-Homeodomain protein Vvl is expressed in all vein territories throughout pupal development and is required for vein differentiation (de Celis et al., 1995). We found that Vvl was able to bind all the probes included in the *2.5Kb SalI/SacII* region (Figure 4B). The Vvl binding was competed by cold DNA as well as by specific oligonucleotides previously described to compete Vvl binding to the *vestigial* quadrant enhancer (Certel et al., 2000). The *shv*-Vvl binding was not competed with primers specific for Medea (Xu et al., 1998) Figures 4C,D and data not shown), confirming the specificity of Vvl binding to the *shv* enhancer. To further characterise the

binding of Vvl to the *shv* enhancer we focussed in the 0.5Kb SacII fragment, which drives expression in all longitudinal veins. This fragment was subdivided into two overlapping probes (S9 and S10) and both of them bind Vvl specifically (Figures 4C, D). We competed these bindings using oligonucleotides covering these probes and found Vvl-binding regions in S9 and in S10 (Figures 4C,D). Interestingly these sequences contain previously identified consensus sequences for Vvl binding (Certel et al., 2000). These data suggest that Vvl participates in vein formation during pupal development through the regulation of *dpp* expression via the *shv* enhancers.

The Iroquois complex includes three genes, *araucan (ara)*, *caupolican* (*caup*) and *mirror*, encoding highly related homeodomain-containing proteins (Gómez-Skarmeta et al., 1996). The genes *ara* and *caup* are expressed in the presumptive veins L3 and L5 and their activity is required for the formation of the distal region of these two veins (Gómez-Skarmeta et al., 1996). The activity of these genes is required during imaginal development to regulate the expression of *rhomboid* in the L3 and L5 veins, but it is not known whether they are also required during pupal development (Gómez-Skarmeta et al., 1996). We used the Ara homeodomain and the different probes included in the *2.5Kb SalI/SacII* region, finding strong binding using S1, S5, S9 and S10 as probes. Only high amounts of Ara protein caused bandshift of the S2-4 and S6-8 probes (data not shown). We were able to displace these bindings using cold DNA, suggesting specific interactions between Ara and these DNA fragments (Figure 5 B, C and data not shown). When we competed the binding with oligonucleotides included in the S9 and S10 probes, we found two binding regions in S9 and three in S10. The sequence a/t ACAnnTGT t/a has been recently defined as a binding site for the Iro-C component Mirror in random oligo-

selection experiments (Bilioni et al., 2005). This sequence is specific for Mirror, although other members of the Iro-C, Ara or its homolog Irx4, also bind this sequence with a weaker affinity (Bilioni et al., 2005). We found three sites with similar consensus sequence in the enhancer, located in the S1, S9 and S10 probes. However in our case the palindrome is in opposite direction. Interestingly, the consensus we identified included in S9 and S10 are in a highly conserved regions between *D. melanogaster* and *D. pseudoscura* (see below) and is present in oligonucleotides that compete efficiently in the binding assays, pointing to the relevance of these sequences to mediate Ara binding to the *shv* enhancer (Figure 5B, C and see below). The binding of Ara to the *shv* enhancer suggests that the Iro-C proteins participate in the development of the L3 and L5 veins acting as transcriptional activators to regulate positively the expression of *dpp* during pupal development.

The DNA-binding activity of *Drosophila* Smad proteins, Mad and Medea, is crucial for the expression of Dpp target genes (Kim et al., 1997; Newfeld et al., 1996; Newfeld et al., 1997; Sekelsky et al., 1995). The expression of phosphorylated Mad (p-Mad), the activated form of the Mad protein, is restricted to the developing veins during pupal development (Sotillos and de Celis, 2005; Ralfston and Blair, 2005). We found that the efficiency of ectopic *dpp* expression to direct vein differentiation depends on the integrity of the *shv* region, suggesting that Dpp signalling is sufficient, directly or indirectly, for driving the expression of additional *dpp* transcription via the *shv* enhancer (Figure 6C-E). Therefore, we studied whether the Smads proteins can bind to the dpp^{s} enhancer. We obtained specific binding using all probes (S1-S10, Figure 6 and data not shown) as shown by competition both with cold DNA and with specific oligonucleotides containing

consensus binding sequences for Medea (D3up) and for Mad and Medea (D3) (Xu et al., 1998). Other oligonucleotides with consensus for the transcription factor Nubbin (Neumann and Cohen, 1998) did not compete the binding (Figure 6). The three main regions of competition with Mad and Medea binding included in the S9 and S10 probes correspond to GC rich sequences characteristic of Smad-response elements (Figures 6 and 7). However, only a single consensus sequence for Mad/Med (GCGGCTGT) in S10 is localised in a highly conserved region of different Drosophilids (see below).

2.4. Sequence comparison between Drosophila melanogaster and other Drosophilids

The pattern of four longitudinal veins is very similar in all Drosophilids despite the differences in wing size and pigmentation existent between species (de Celis and Diaz-Benjumea, 2003). This conservation suggests that the mechanisms underlying vein pattern formation are conserved. The availability of the genomic sequence for different *Drosophila* species allows a direct comparison between their dpp^s regions. We compared two *Drosophila* species from the *melanogaster* group (*D. melanogaster* and *D. ananassae*), one *Drosophila* from the *obscura* group (*D. pseudoobscura*) and *D. virilis* from the *virilis* group. It is expected that sequence similarity in non-coding regions correspond to functional regulatory DNA. We found in the 2.5Kb region analysed several clusters of sequence conservation including most of the binding sites identified by *in vitro* analysis, (Figure 7A). Thus, there are four highly conserved regions corresponding to the S1, S4-5, S7-8 and S9-S10 probes containing conserved binding sequences for Vvl, Mad, Med and Ara. This conservation reinforces the importance of these regions to regulate the expression of *dpp* in the pupal veins. In the case of Vvl we

have shown specific DNA binding to all probes. However, the putative Vvl binding sequences localised in the conserved regions are only in S1, S3, S7, S8 and S10. In the case of the Dpp pathway transcription factors Mad and Med, putative binding sites are present throughout the enhancer, and accordingly we have shown binding of them to all probes. However, only the S5 and S10 probes contain putative binding sites in regions of high sequence conservation. Interestingly, these conserved Mad/Med binding regions contain overlapping binding consensus for the Brinker repressor (Figure 7A; (Rushlow et al., 2001). This suggests a competition mechanism between Mad/Med and Brinker for binding to the *shv* enhancer. Competition mechanisms between activator and repressor also occur in several Dpp-downstream genes such as *zen* and *omb* (Rushlow et al., 2001). We also found four consensus binding sequences for the transcription factors of the Knirps-complex. The *kni* genes are expressed in the L2 vein, where they are required for its formation (Lunde et al., 1998). We found three Kni-binding sites in the *1.1Kb KpnI/SacII* enhancer and one in the *0.5 SacII* regions. Only two of the sequences located in the *1.1Kb KpnI/SacII* enhancer present some conservation between Drosophilids. Interestingly, the *0.9Kb SalI/KpnI* enhancer responsible of *dpp* expression in the L5 veins does not contain any putative Knirps binding sequence. Although we have not analyse whether Kni binds directly to the *shv* enhancer, the presence of Kni-binding sites in conserved regions of the enhancer suggests that, in addition to its role during imaginal development (Lunde, et al., 1998), Kni might also activate *dpp* transcription during pupal development.

3. Concluding Remarks

The enhancers are key elements in the regulation of gene expression, as they integrate activator and repressor signals from different transcription factors. Thus, the analysis of regulatory regions helps us understand the molecular networks behind developmental decisions. One example of such network operates during vein pattern formation, where specific transcription factors and signalling pathways interact and contribute to establish and maintain vein territories (reviewed in de Celis, 2003; de Celis and Diaz-Benjumea, 2003 and Bier, 2000). We have identified regulatory sequences driving *dpp* expression in the pupal veins in 2.5Kb of the dpp^{s} region. This regulatory DNA can be subdivided into three fragments, a 1.1Kb fragment that recapitulates almost completely the pupal expression of *dpp*, a 0.9Kb upstream fragment, which drives expression in the proximal part of L5, and a 0.5Kb fragment that directs expression in all veins. We found binding sites in these fragments for general transcription factors involved in the development of all veins (Vvl) and for the downstream activators of the dpp pathway, Mad and Medea. The regulatory region we identify also contains binding sites for transcription factors expressed and required only in specific veins, such as Ara (L3 and L5) and Kni (L2). Most of these sequences are located in highly conserved regions of the *dpp* gene in different *Drosophila* species, indicating a general conservation of *dpp* regulation in the Drosophilids.

4. Experimental Procedures

4.1. Genetic strains

We have used the *dpp* alleles dpp^{d5}, dpp^{s4} and dpp^{s22} (Segal and Gelbart, 1985) and the Gal4 line *Gal4-1348* (de Celis, 1997). We also used the following UAS lines: *UAS-GFP* (Ito et al., 1997), *UAS-tauGFP*, *UAS-taulacZ* (Hidalgo et al., 1995) and *UAS-dpp* (Staehling-Hampton and Hoffmann, 1994). Unless otherwise stated, crosses were done at 25º C. Wings were mounted in lactic acid-ethanol (1:1) and photographed with a Spot digital camera and a Zeiss Axioplan microscope.

4.2 Generation of shortvein-Gal4 fusion constructs

DNA fragments from 8.5Kb of the *shv* region (Capovilla et al., 1994) were cloned in pBS and the *KpnI/XbaI* fragments subcloned in the pZGL vector (Hart and Bienz, 1996). This vector contains a dicistronic Gal4/lacZ gene separated by an internal ribosomal entry site. P-element-mediated transformation (Rubin and Spradling, 1982) was performed with 0.3 µg/µl of DNA of the transforming plasmid and 0.15 µg/µl of pUChsπΔ2-3 as Transposase source. The recipient stock was $y\ w^{1118}$.

4.3. DNA binding assays

Recombinant proteins expressed in bacteria were used in Electrophoretic Mobility Shift Assays (EMSA). M. Frasch provided *mad* and *med* MHI DNA binding fragments and bandshifts were performed as described (Xu et al., 1998). The ARA homeodomain was provided by J.L. Gomez-Skarmeta and bandshifts performed as described in (Gomez-Skarmeta et al., 2001). W.A. Johnson provided Vvl protein, and the bandshifts were

performed as described in (Certel et al., 2000). Probes were PCR-labelled radioactively using α dCTP and purified using QIAquick PCR Purification Kit (Qiagen). The oligonucleotide sequences for each PCR will be provided on request and the oligonucleotides used for competition assays are shown in Figure 7. The D3 sequence is described in (Xu et al., 1998), vgQ WT in (Certel et al., 2000) and Nub in (Neumann and Cohen, 1998). Oligonucleotides for competition were used at 10 μM.

4.4. DNA comparison and sequence analysis

For DNA analysis we used the GCG package (Wisconsin University) and Blastn (NCBI). For transcription factors binding sites prediction we used MatInspector (Genomatix). Comparison between different Drosophilids was done using VISTA Browser (NCBI).

4.5. Immunocytochemistry

We used rabbit anti-βGalactosidase (Cappel) and secondary antibodies from Jackson Immunological Laboratories (used at 1/200 dilution). Pupal wings were dissected, fixed and stained as described in de Celis (1997). Confocal images were captured using a BioRad confocal microscopy.

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Figure Legends

Figure 1

Genomic organisation of the *dpp* locus

(A) Above: Genetic structure of the *dpp* locus, showing the relative positions and extent of the *shortvein* (*shv*), *Haploinsufficient* (*Hin*) and *disc* (*disc*) regions. Below: Representation of the different *dpp* mRNAs and coding region (red rectangles). The coloured squares represent 5' non-coding RNA belonging to the transcripts *dpp-RA* (red), *dpp-RB* (pink), *dpp-RC* and *dpp-RB* (blue) and *dpp-RE* (green). These transcripts use the promoters P5, P4, P3, P2 and P1, respectively. The position of two dpp^{shv} alleles (*s22* and *s4*) is indicated on the bottom line representing the size of the gen in Kb. The position of the 8.5Kb fragment used as the starting point of this analysis is also indicated as a red line. (B) Wild type wing indicating the position of the four longitudinal veins (L2-L5). (C) Third instar wing disc showing *dpp* expression in dpp^{disc}*-Gal4/UAS-GFP* (green). (D) dpp^{d5} adult wing. (E) βGal expression (green) driven by the *shv8.5Kb EcoRI-lacZ* construct in the pupal veins at 24-28 h APF. (F) dpp^{s22}/dpp^{s4} adult wing showing the absence of the longitudinal veins L2, L4 and L5.

Figure 2

Pupal expression of dpp^{s} reporter constructs

(A) Above: Genomic dpp^{s} DNA in the 8.5Kb *EcoRI* fragment. The restriction sites used in the generation of the different constructs are indicated below the line. Constructs giving expression in the pupal wing are coloured in blue, and constructs not expressed in this tissue are coloured in red. (B-D) Pupal expression of GFP (green) and βGal (red) in

dpp^{s}*-lacZ/Gal4* constructs combined with *UAS-GFP*. The expression of βGal is present only in the L5 vein (3.7*XhoI*/*KpnI*; 3.7KX, B'), in the longitudinal veins L2, L3, L4 and L5 (3*KpnI*/*KpnI*; C' and 1.65*KnpI*/*XhoI*; D'). In addition, expression of GFP is also detected in many intervein cells in the combinations 3*KpnI*/*KpnI*/*UAS-GFP* (3KK, C) and 1.65*KnpI*/*XhoI*/UAS-GFP (1.65 KX, D). (E-G) Expression of GFP in pupal wings of the genotypes *0.9Kb SalI*/*KpnI*/*UAS-GFP* (0.9SK, E), *1.1Kb KpnI*/*SacII*/*UAS-GFP* (1.1KS, F) and *0.5Kb SacII*/*XhoI*/*UAS-GFP* (0.5SK, G). The age of all pupal wings is 24-28 hours APF.

Figure 3

Embryonic expression of *dpp*^{s} reporter constructs

(A) Map of the 8.5Kb *shv* region showing the constructs driving reporter expression in embryos in blue and those not expressed in red. (B-M) Localization of GFP directed by the following *shv* constructs: 8Kb *EcoRI* (8R1; B, C), 2.7Kb *EcoRI*/*SalI* (2.7RS; D, E), 3.7Kb *XhoI*/*KpnI* (3.7XK; F, G), 3Kb *KpnI*/*KpnI* (3KK; H, I), 0.9Kb *SalI*/*KpnI* (0.9SK; J, K) and 1.35Kb *XhoI*/*KpnI* (1.35XK; L, M). The 8RI fragment directs reporter expression in ps4, ps7 and in some cells of the oesophagus at stage 13 (st13; B). (C) The expression is observed at stage 17 (st17) in the oesophagus (1), gastric caecae (2) and in the second midgut constriction (3). The 2.7RS fragment directs GFP expression only in some cells at stage 13 (1; D), which give rise to the oesophagus (E, stage 17). The expression of the reporter in the 3.7XK (F, G) and 0.9SK (J, K) fragments is restricted to the gastric caecae. The 3KK fragment directs reporter expression in the ps4 and ps7 at stage 13 (H) and later in the oesophagus and midgut (stage 17, I). The only subfragment included in

the 3K region able to direct reporter expression is the most proximal (1.35 XK), in which GFP is expressed in ps4 and ps7 at stage 13 (L) and in the midgut at stage 17 (M).

Figure 4

Vvl binding to the dpp^{s} enhancer

(A) Above: schematic representation of the 2.5Kb genomic region of the *dpp^{s}* enhancer including the 0.9Kb *SalI*/*KpnI* (0.9S/K; red), 1.1Kb *KpnI*/*SacII* (1.1K/S; purple) and 0.5Kb *SacII*/*SacII* (0.5S/S; blue) fragments. The position of the probes used for the bandshift experiments is indicated below the map (S1-S10). The overlapping probes S1-4 correspond to the 0.9S/K fragment (red), which drives LacZ expression in L5. Probes S5-S8 correspond to the 1.1K/S fragment (purple), which directs reporter expression in all wing veins except proximal L5. Probes S9-S10 are included in the 0.5S/S fragment (blue), which drives expression of the *lacZ* reporter in all veins. Below: Schematic representation of the wing showing the expression pattern of Ventral veinless (Vvl; grey), 0.9S/K (red), 1.1K/S (purple) and 0.5S/S (blue). (B) *In vitro* eletrophoretic mobility shift assay (EMSA) of Vvl with S1-S8 PCR-radiolabeled probes. (C) *In vitro* DNA binding of Vvl to the S9 probe. The binding is competed by increasing quantities of unlabeled wild-type probe and by a specific oligonucleotide previously described to compete Vvl binding to the *vestigial* quadrant enhancer (VgQ). Oligonucleotides bound by Med (D3up) do not compite the binding. Only the S9 specific oligonucleotides p1-2, p4-5 and p7-8 compete Vvl binding to S9. (D) *In vitro* DNA binding of Vvl to the S10 probe. Increased quantities of unlabeled wild-type probe competed Vvl binding to S10 as well as the oligonucleotide VgQ and the S10 oligonucleotides p12-15 and p17. The oligonucleotide D3up and the specific oligonucleotides p9-11, p16 and p18-19 are unable to compete the

binding to Vvl. Arrows point to specific binding whereas asterisks indicate unspecific ones.

Figure 5

Analysis of Ara binding to the *dpp*^{s} enhancer

(A) Above: schematic representation of the 2.5Kb genomic region of the dpp^{s} enhancer included in the 0.9Kb *Sal*/*KpnI* (0.9SK; red), 1.1Kb *KpnI*/*SacII* (1.1KS; purple) and 0.5Kb *SacII*/*SacII* (0.5 SS; blue) fragments. The position of the probes used for the bandshift experiments is indicated below the map (S1-S10). Below: Schematic representation of the wing showing the expression pattern of Ara and Caup (Iro-C; grey), 0.9S/K (red), 1.1K/S (purple) and 0.5S/S (blue). (B) Competition of Ara homeodomain binding to S9 with specific oligonucleotides included in this probe. Only the oligos p1 and p4-5 compete Ara-homeodomain binding to the S9 PCR-radiolabeled probe. (C) Binding of Ara homeodomain to the S10 probe and competition with specific oligonucleotides. Only p11, p14-15 and p19 are able to displace the binding of the Ara-homeodomain to the S10 probe. Arrowheads point to DNA-protein complexes.

Figure 6

Mad and Med binding to the 2.5Kb *shv* region

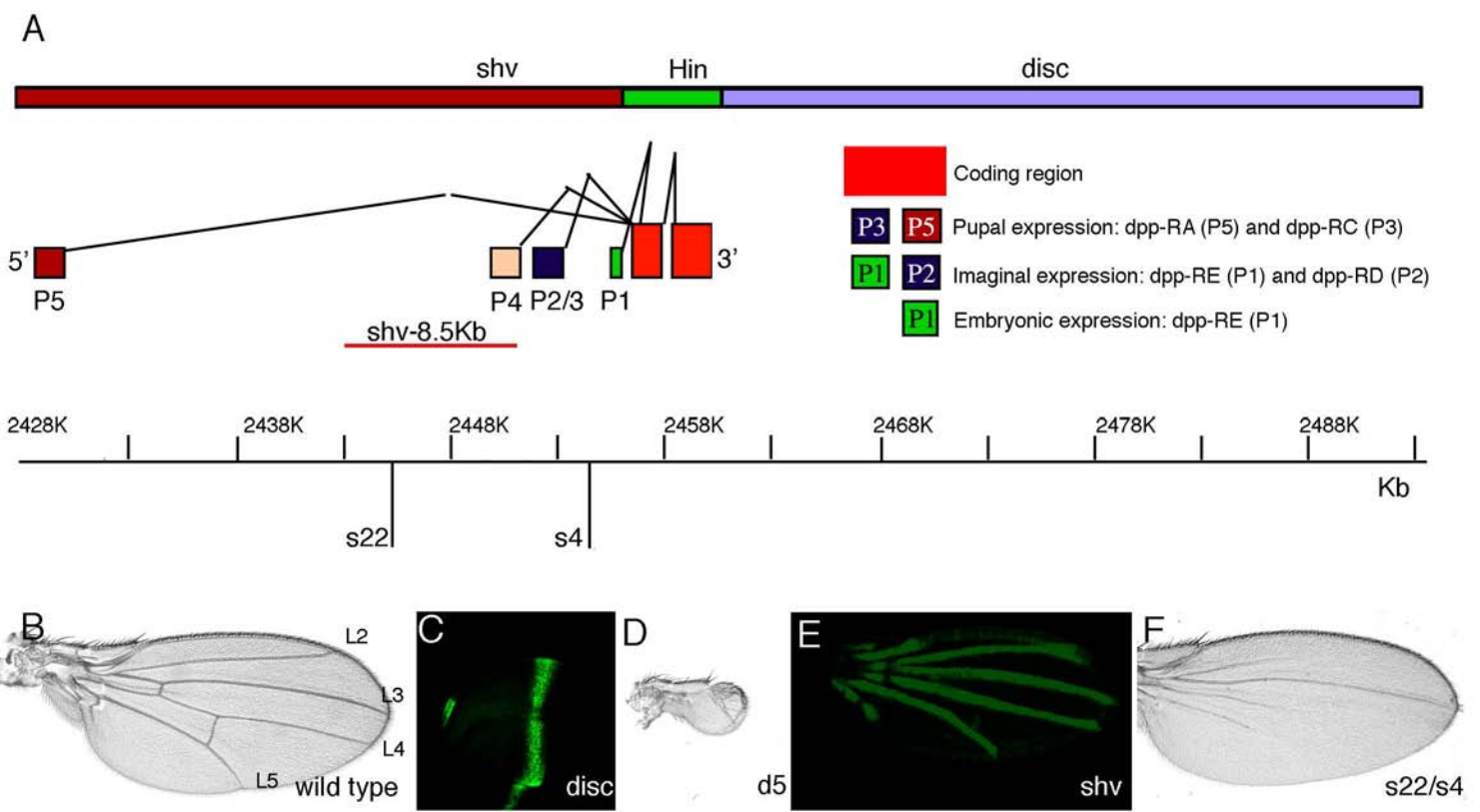
(A) Mad binding to the S9 probe (left panel) and S10 probe (right panel) is competed by increasing amounts of cold DNA as well as by specific oligonucleotides (D3), whereas competition with oligonucleotides specific for Nubbin (Nub) or Med (D3up) do not interfere with the binding. Cold oligonucleotides were used to reveal that p1 and p6 (S9 probe) and p10-p16 and p19 (S10 probe) specifically competed Mad binding. (B) Med

binding to S9 (left panel) and S10 (right panel) is competed by increasing quantities of cold DNA and with specific oligonucleotides (D3 and D3up), but not with oligonucleotides containing the Nubbin binding site (Nub). Oligonucleotides p1-2 and p6-p10 in S9 and p13-16 in S10 specifically competed Med binding. Arrows point to DNA-protein complexes. (C-E) Adult wings showing the requirement of the *shv* region for the excess of vein phenotype caused by *dpp* over-expression in the pupal wings. (C) shv^{s12}/shv^{s4} wing (shv). (D) shv^{s22}/shv^{s4}*; shv-3KpnGal4/UAS-dpp* wing (shv;UAS-dpp). (E) *shv-3KpnGal4/UAS-dpp* (UAS-dpp).

Figure 7

Sequence comparison among *Drosophila* species.

(A) Sequence comparison between *D. melanogaster* and the Drosophilis *D. pseudoscura* (1), *D. virilis* (2) and *D. ananassae* (3) shown regions of high homology inside the *shv* enhancer (red shadow in the upper panel). The scheme shown below corresponds to the three fragments analysed and the probes used for EMSA assays 0.9Kb *SalI/KpnI* (S1-S4, red), 1.1Kb *KpnI/SacII* (S5-S8, purple) and 0.5Kb *SacII/SacII* (S9-S10, blue). Coloured boxes distributed along the enhancer represent consensus binding sequences for Vvl (purple, upper row), Ara (green, second row), Mad/Med (blue, third row), Brk (red, four row) and Knirps (yellow, lower row). (B) DNA sequence of the 0.5Kb *SacII/SacII* enhancer showing the alignment between *D. melanogaster* (Dm) and *D. pseudoscura* (Dp). The two probes, S9 and S10, are indicated in blue and purple boxes, respectively. The oligonucleotides used for competition (p1-19) are indicated by alternating black and red bars. The conserved consensus sequences for Ara, Vvl and Mad/Med are boxed in green, purple and blue, respectively.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shv
Hin
disc
5'
3'
P5
P4 P2/3 P1
shv-8.5Kb
Coding region
P3 P5 Pupal expression: dpp-RA (P5) and dpp-RC (P3)
P1 P2 Imaginal expression: dpp-RE (P1) and dpp-RD (P2)
P1 Embryonic expression: dpp-RE (P1)
2428K
2438K
2448K
2458K
2468K
2478K
2488K
Kb
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D
d5
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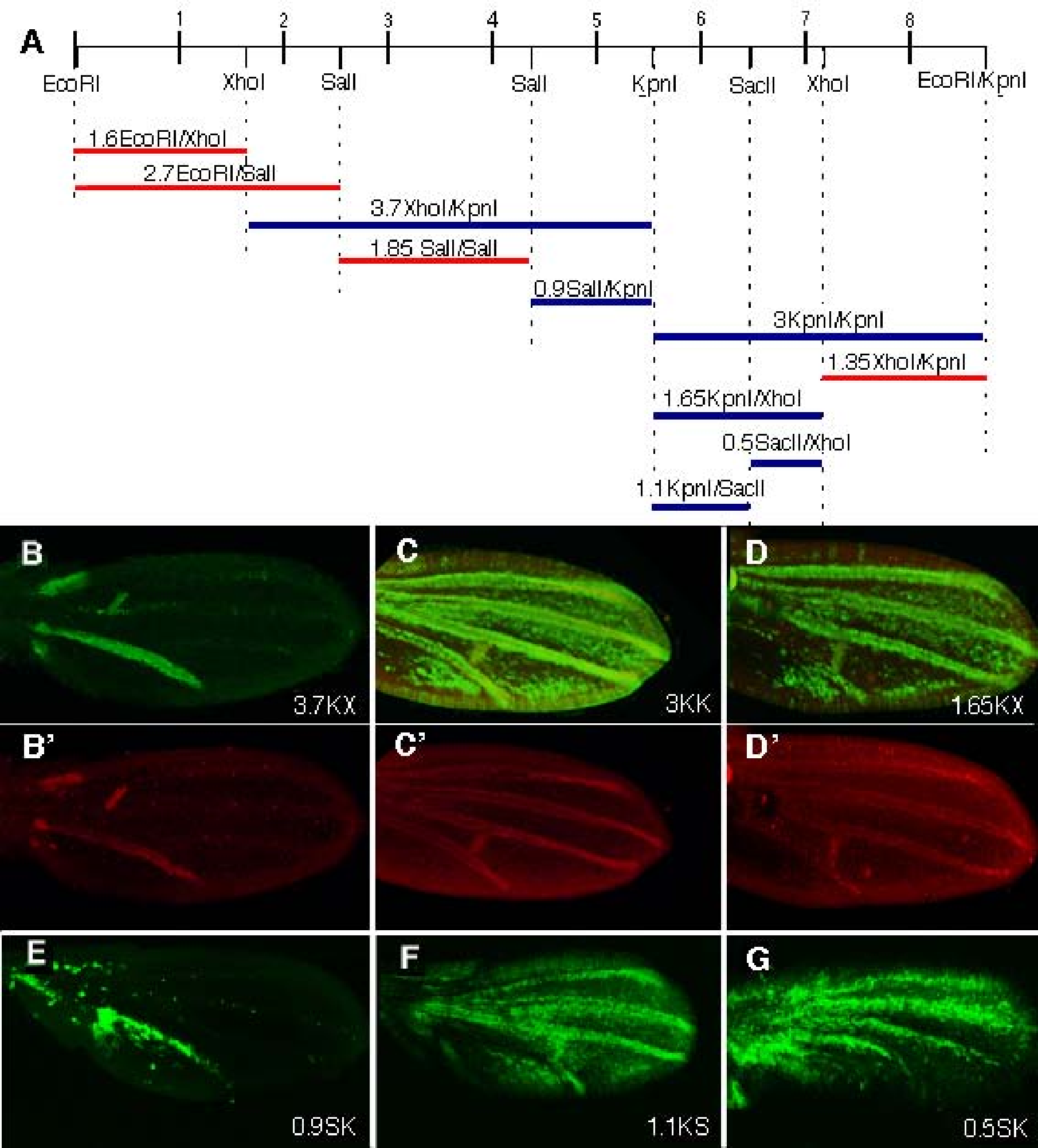
s22/s4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6
7
8
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XhoI
SalI
SalI
KpnI
SacII
XhoI
EcoRI/KpnI
1.6EcoRI/XhoI
2.7EcoRI/SalI
3.7XhoI/KpnI
1.85 SalI/SalI
0.9SalI/KpnI
3KpnI/KpnI
1.35XhoI/KpnI
1.65KpnI/XhoI
0.5SacII/XhoI
1.1KpnI/SacII
B
3.7KX
C
3KK
D
1.65KX
B'
C'
D'
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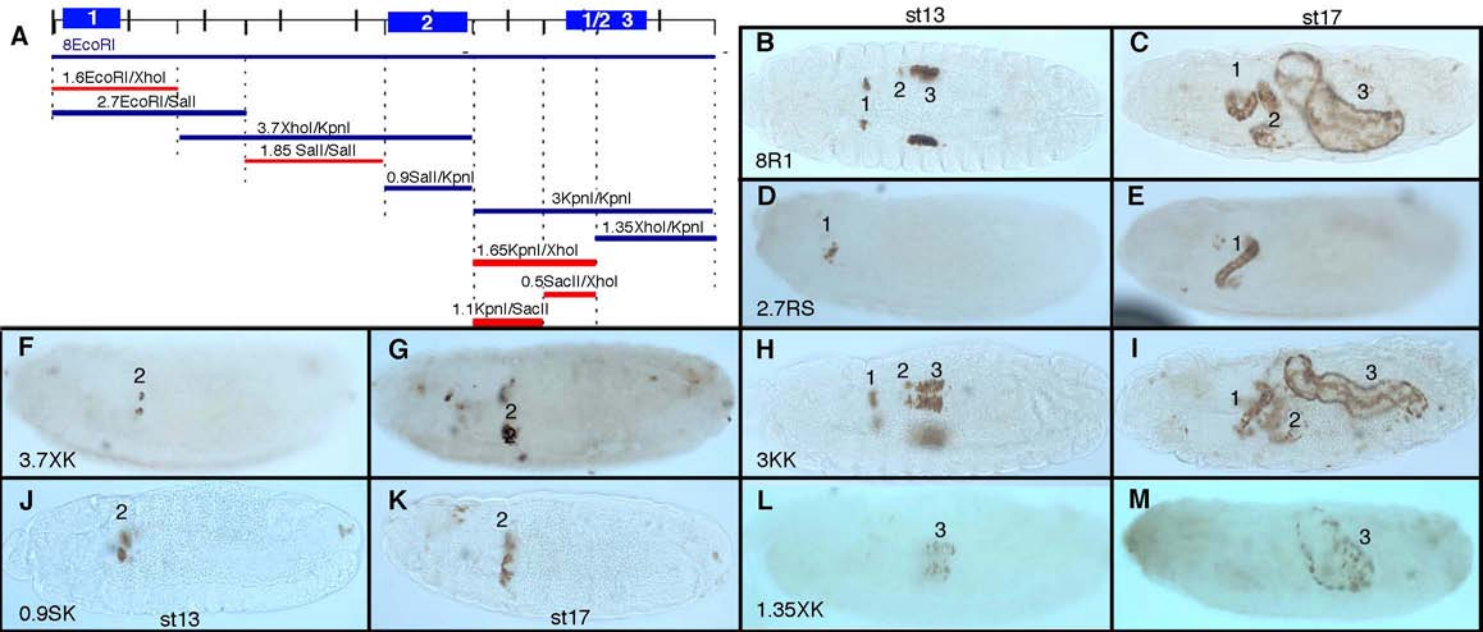
0.9SK
F
1.1KS
G
0.5SK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1/2 3
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1.6EcoRI/XhoI
2.7EcoRI/SalI
3.7XhoI/KpnI
1.85 SalI/SalI
0.9SalI/KpnI
3KpnI/KpnI
1.35XhoI/KpnI
1.65KpnI/XhoI
0.5SacII/XhoI
1.1KpnI/SacII
st13
st17
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H
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st17
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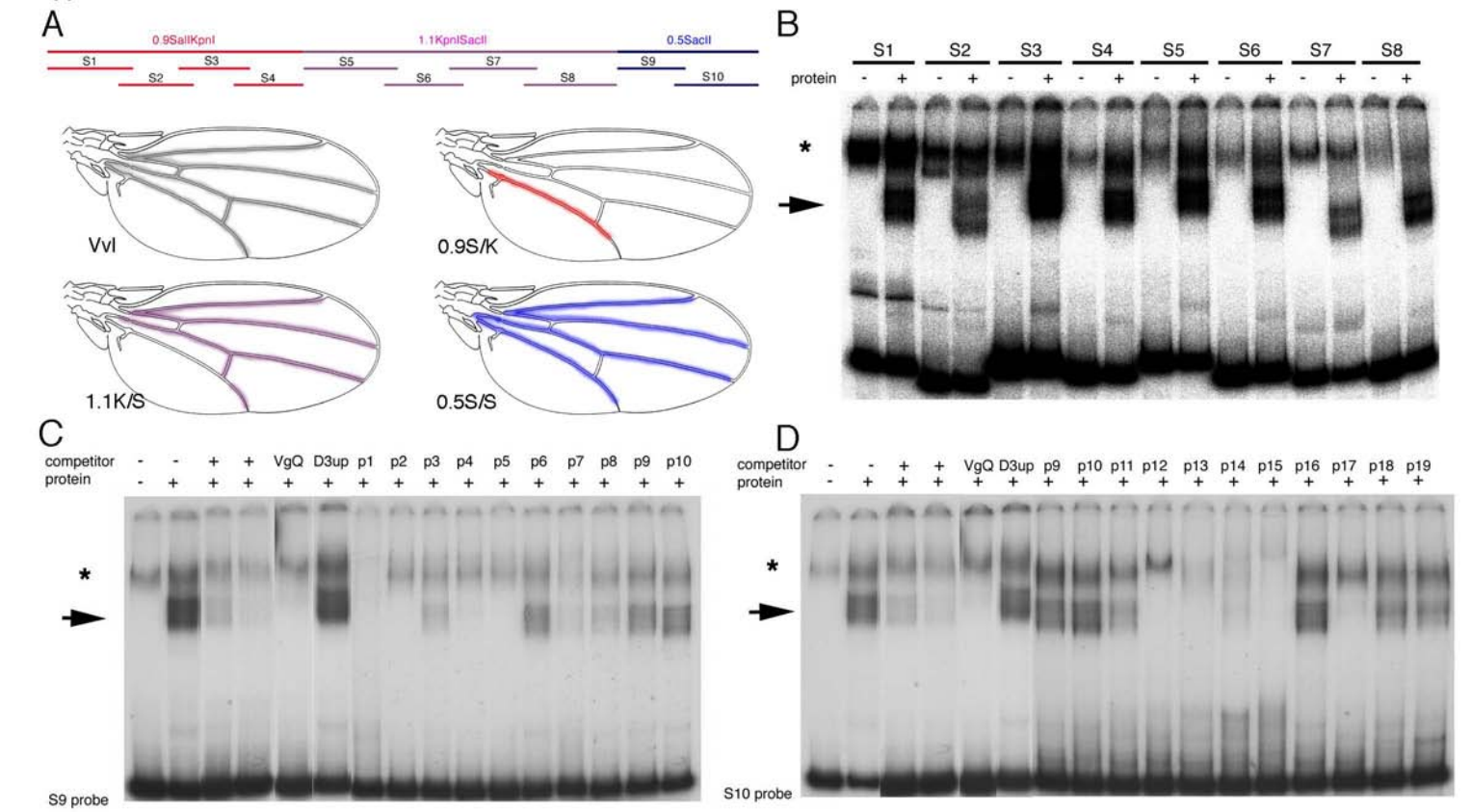
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1.1KpnISacII
0.5SacII
S1
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S8
S9
S10
Vvl
0.9S/K
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0.5S/S
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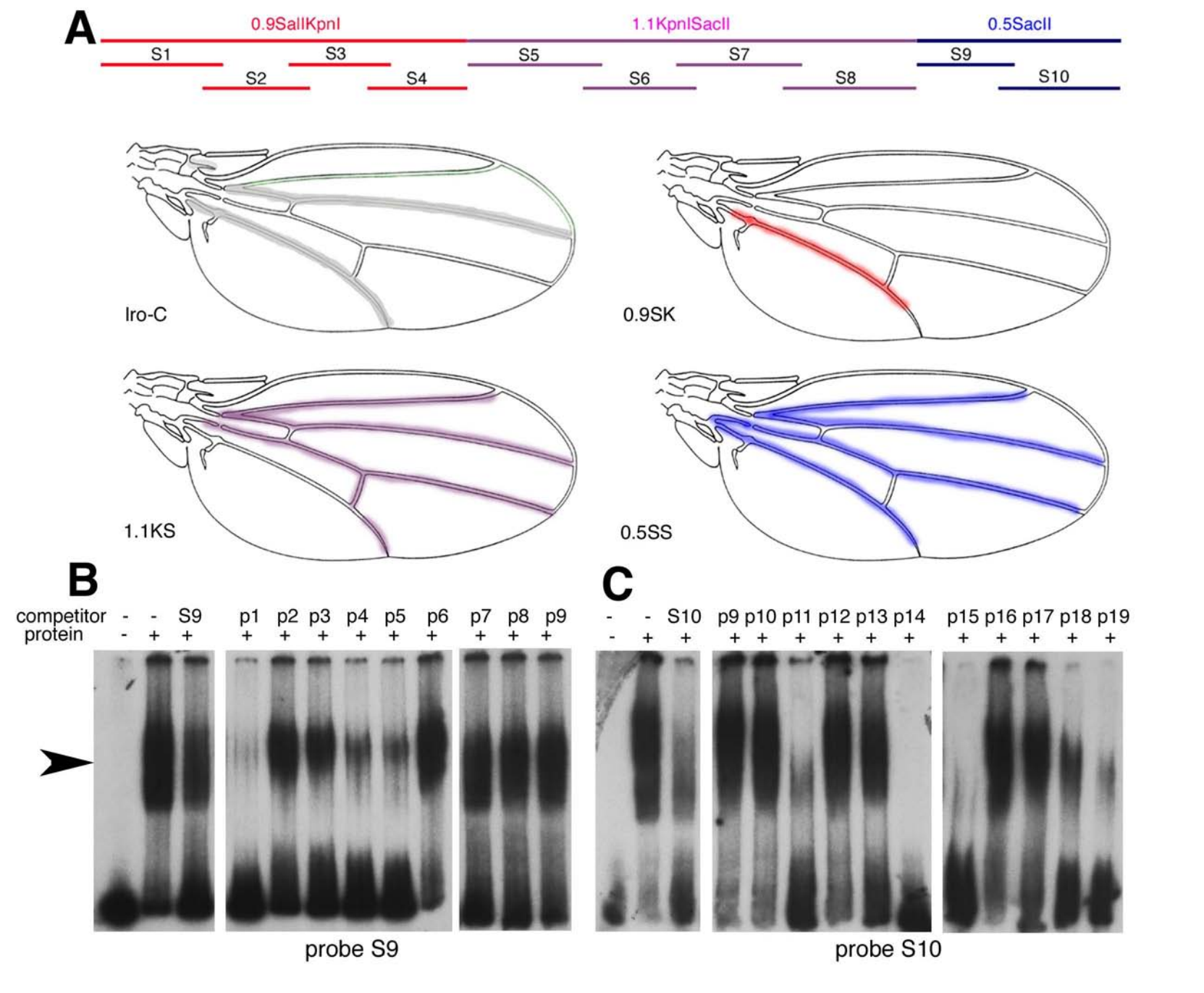
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competitor
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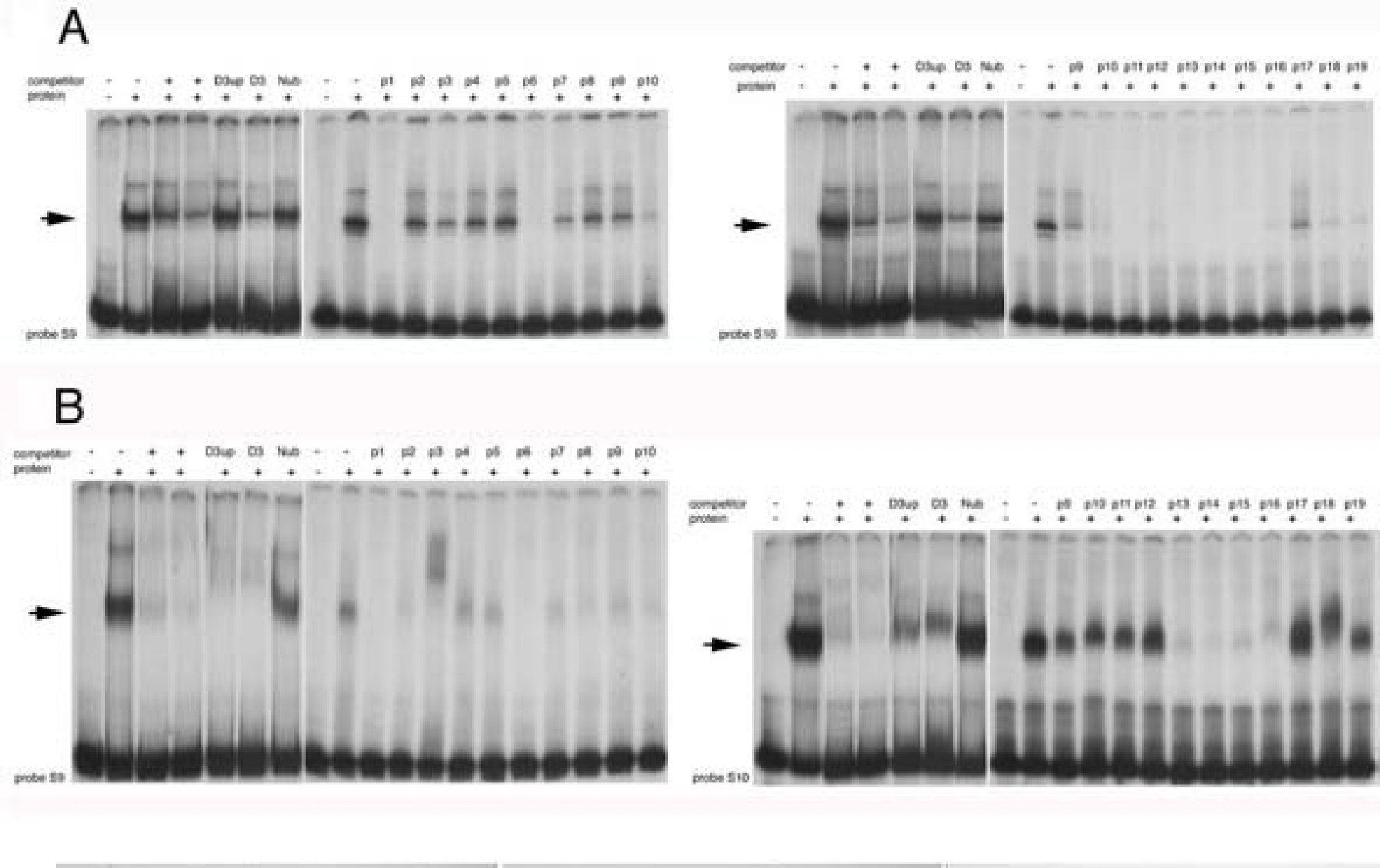
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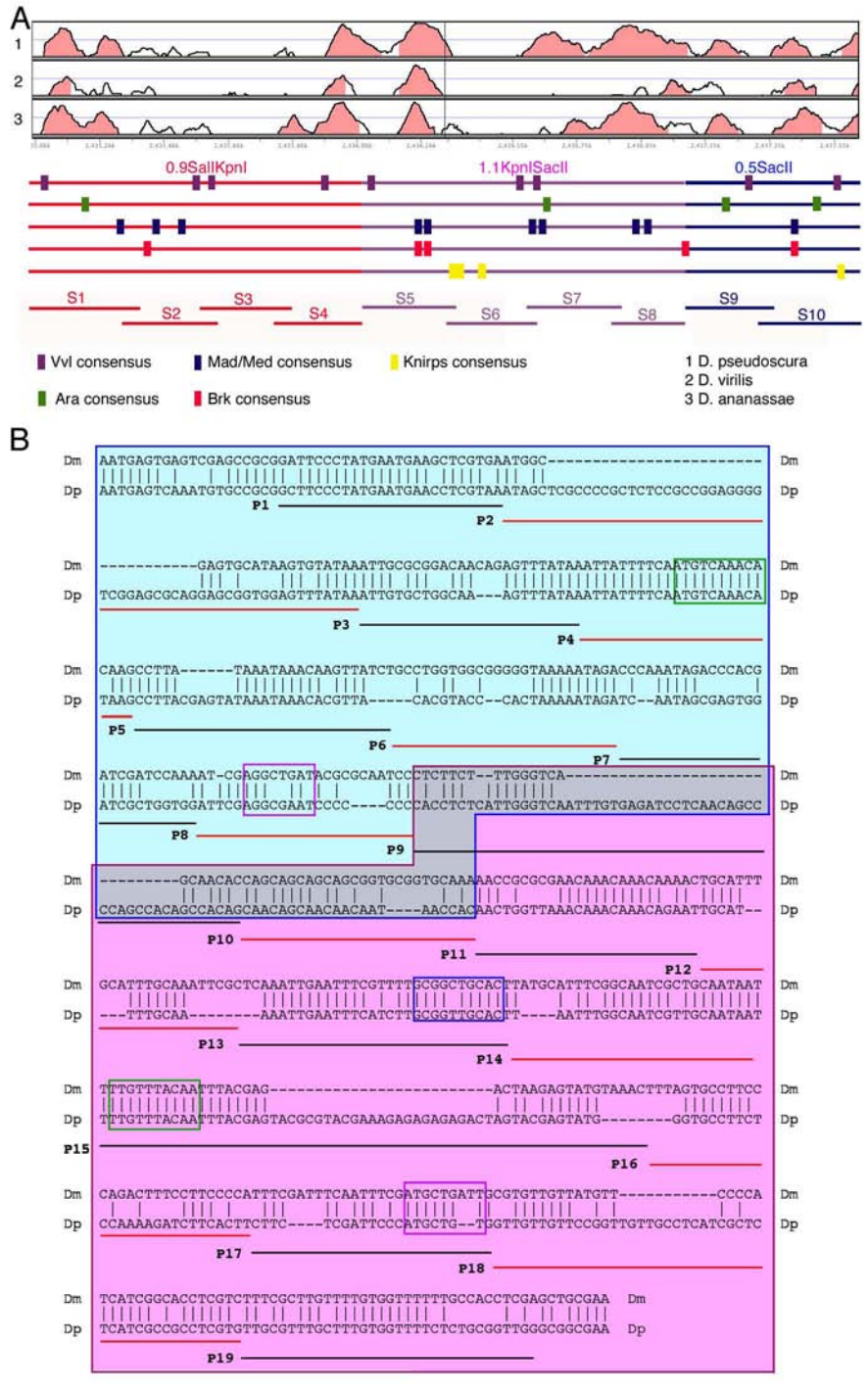
UAS-dpp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S7
S8
S9
S10
Vvl consensus
Mad/Med consensus
Knirps consensus
Ara consensus
Brk consensus
1 D. pseudoscura
2 D. virilis
3 D. ananassae
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Dm
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