Spatial regulation of cell adhesion in the Drosophila wing is mediated by Delilah, a potent activator of βPS integrin expression

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In spite of our conceptual view of how differential gene expression is used to define different cell identities, we still do not understand how different cell identities are translated into actual cell properties. The example discussed here is that of the fly wing, which is composed of two main cell types: vein and intervein cells. These two cell types differ in many features, including their adhesive properties. One of the major differences is that intervein cells express integrins, which are required for the attachment of the two wing layers to each other, whereas vein cells are devoid of integrin expression. The major signaling pathways that divide the wing to vein and intervein domains have been characterized. However, the genetic programs that execute these two alternative differentiation programs are still very roughly drawn. Here we identify the bHLH protein Delilah (Dei) as a mediator between signaling pathways that specify intervein cell-fate and one of the most significant realizers of this fate, βPS integrin. Dei’s expression is restricted to intervein territories where it acts as a potent activator of βPS integrin expression. In the absence of normal Dei activity the level of βPS integrin is reduced, leading to a failure of adhesion between the dorsal and ventral wing layers and a consequent formation of wing blisters. The effect of Dei on βPS expression is not restricted to the wing, suggesting that Dei functions as a general genetic switch, which is turned on wherever a sticky cell-identity is determined and integrin-based adhesion is required.

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Introduction

The differentiation of many cell types and tissues requires remodeling of cells’ adhesive properties. Proper adhesive interactions between neighboring cells, and between cells to the extracellular matrix, are important for tissue morphogenesis and maintenance of tissue integrity. In addition, cell adhesion molecules often function as cell surface receptors important for the communication between cells and their environment. The transduction of extracellular signals via cell-adhesion receptor molecules, such as integrins, can affect cell proliferation, survival, differentiation, migration and many other aspect of cell biology (Bokel and Brown, 2002; Dupuy and Caron, 2008; Harburger and Calderwood, 2009; Hynes, 2002; Perkins et al., 2010; Streuli, 2009; Vicente-Manzanares et al., 2009; Zaidel-Bar et al., 2007). Thus, the genetic program of almost any developmental process should include tight temporal and spatial regulation on the expression of cell adhesion molecules. Very little is known about the regulatory processes that specify adhesive properties of cells.

Through our studies on chordotonal organ development, we became interested in the bHLH protein Delilah (Dei). Dei is expressed during embryonic development almost exclusively in specialized cells that provide anchoring sites to either muscles (tendon cells), or proprioceptors (chordotonal attachment cells) (Armand et al., 1994; Inbal et al., 2004). This unique pattern of expression may indicate that Dei plays an important role in a basic developmental program that is common to different types of attachment cells. It is reasonable to assume that Dei, being a putative bHLH transcription factor, is not involved directly in adhesion processes that characterize such cells. Rather, it may regulate the expression of subordinate genes that are required to confer attachment cells with their unique adhesive properties. The only other cells that express Dei in the embryo are fusion-competent myoblasts, prior to their fusion to founder cells (Artero et al., 2003). It is possible that Dei regulates in these cells the expression of cell surface molecules that are required for their interactions with other cells, similarly to its hypothesized role in chordotonal organs and tendon cells.

The developmental roles of Dei and its possible involvement in the regulation of cell adhesion and attachment have not been investigated so far. To test our hypotheses about the involvement of Dei in the differentiation of ‘sticky’ cell types, we took advantage of a relatively simple system, the fly wing. The wing offers an excellent model system to investigate the molecular mechanisms that regulate the differentiation of cells with different adhesive properties. The wing blade consists of two epithelial sheets that adhere tightly to each other in the intervein regions through integrin-based basal junctions (Fristrom et al., 1993). Five longitudinal and two transverse veins
separate between the sectors of intervein cells. The veins and interveins differ from each other in cell size, shape, cuticle pigmentation, trichome orientation and the repertoire of cell adhesion molecules that they express. For example, βPS integrin is expressed in intervein cells only, whereas laminin A and collagen are expressed only in vein cells (Fristrom et al., 1993).

The *Drosophila* wing starts to develop in the embryo, when 20–30 cells form the embryonic wing disc. Further proliferation and differentiation of the wing disc take place during larval and pupal stages. Patterning of the wing disc is initiated at larval stages when broad proven and intervein regions are established by the activity of the Hedgehog and Dpp pathways (Bier, 2000; Sturtevant et al., 1993) (reviewed in Blair, 2007; De Celis, 2003). Subsequently, the EGFR and Notch pathways are activated within the proven domain, subdividing it into a prospective vein region, where EGFR signaling is active, and two adjacent rows of boundary cells, where the Notch signaling acts to prevent vein differentiation (for reviews see Blair, 2007; De Celis, 2003).

The intervein cells are characterized at the same time by the expression of the *Drosophila* serum response factor—Blistered (Bs) (Fristrom et al., 1994; Montagne et al., 1996; Roch et al., 1998) and the bHLH protein Net (Brentrup et al., 2000). The vein–intervein choice remains plastic for quite some time and it depends on both vein-specific and intervein-specific genes (reviewed in Blair, 2007). For example, antagonistic interactions between Bs and the EGFR pathway are required to maintain the identities of interveins versus veins, respectively (Fristrom et al., 1994; Roch et al., 1998; Sturtevant and Bier, 1995).

Following larval stages, the wing disc everts and the two wing layers go through two distinct phases of adhesion to form the wing blade. The dorsal and ventral wing epithelia initially appose during the prepupal period, separate at early pupal stages, and then re-approxos 21–36 h after pupariation (AP) (Brabant et al., 1996; Fristrom et al., 1994). Proteins of the Integrin family play cardinal roles in these processes (Brabant et al., 1996; Domínguez-Gimenez et al., 2007; Fristrom et al., 1993). Even though the main pathways that define the initial development of vein versus intervein territories are well established (reviewed in Blair, 2007), very little is known about later events responsible for the realization of the distinct properties of vein versus intervein cells.

In this work we show that *dei* plays an important role in wing development where it provides an important missing link between the major regulatory networks of gene expression that divide the wing to veins and interveins and the actual morphogenesis and terminal differentiation of intervein cells.

*Dei* is expressed in the developing interveins starting at early prepupal stages. Its expression is regulated by the main signaling pathways, which divide the wing to veins versus intervein territories. Phenotypic analyses of *dei* mutant alleles suggest that Dei plays a dual role in wing development. In early prepupal stages it is required to prevent ectopic vein development and promote intervein development. Later on it is required for terminal differentiation and proper adhesion of intervein cells. Finally, we demonstrate that Dei exerts its effects on wing development by means of specifically regulating the expression of βPS integrin. βPS integrin is expressed in both wing layers and is required for the formation of active integrin dimers with both αPS1, in the dorsal wing layer, and αPS2 at the ventral wing layer. Thus, by regulating the expression of βPS alone, Dei affects integrin-mediated signaling and cell adhesion. The effects of Dei on βPS integrin expression are not restricted to the wing, suggesting that Dei acts as a general regulator of βPS expression, thus constituting an important part in the mechanism that generates sticky cells in *Drosophila*.

**Materials and methods**

**Fly strains**

To identify point mutations in the *dei* gene we used the FlyTill service ([http://tilling.fhcrc.org:9366/fly/](http://tilling.fhcrc.org:9366/fly/)) to screen 2500 mutant strains (half of the Zucker collection) for point mutations in a 1500-bp fragment, spanning the entire coding sequence of the gene. Three strains that harbor a point mutation in *dei* and exhibit extra-vein phenotypes when crossed to *Df(3R)ED6232* were identified: *dei*1 (G294E, Zucker stock Z3-PMM-0233), *dei*8 (Z233N, Zucker stock Z3-5654) and *dei*14 (Z243L, Zucker stock Z3-0313). Other mutant strains used in this study were: *dei*01478 and *dei*HBE174 (*Flybase, 1999*) (obtained from the exelixis collection at Harvard and H. Bellen, respectively), *ps* (Collins, 1928), *Ax*5172 (Kelley et al., 1987), *bs*24 and *bs*14 (Fristrom et al., 1994), and *Df(3R)ED6232* (*Flybase, 1999*). The following Gal4 drivers and UAS strains were used: *P[CD4329]v37630* and *P(y+I+t.77) v(+;t.8) = TRIP-J*01995* attP2* (UAS-dei-IR); *P(KK108584)v100606* (UAS-EGS5447-IR); *P(KK107140)v100218* and *P(GD11656)v47331* (UAS-Hex-t2-IR); *P(GD11655)v46573* and *P(GD11655)v46574* (UAS-Hex-t1-IR) (Dieztl et al., 2007; Perkins et al., 2009; *Flybase, 1999*); *UAS-DN-DER* (O’Keefe et al., 1997), UAS-*dei* (a gift of T. Volk), UAS-*dei*-attP2 (this work); UAS-VP16-Dei (this work), UAS-Da (Hinz et al., 1994), 1.65-Gal4 (Sotillos and de Celis, 2006), en-Gal4, MS1096-Gal4, 24B-Gal4 (*Flybase, 1999*). To generate act-Gal4 expressing clones in pupae we crossed *yw, hsFLP, Sp/Cy; act->CD2-Gal4, UAS-GFP Y* flies to either UAS-*dei* or UAS-*dei*-IR and heat-shocked 2nd instar larvae 1 h at 37 °C.

**DNA constructs**

To generate the UAS-VP16-dei construct the full-length *dei* coding sequence was amplified by PCR from *dei* cDNA clone and was cloned into the Xhol–Xhol sites of the pUAST vector, in frame and downstream of the VP16 activation domain (as described in Inbal et al., 2001).

**Immunohistochemistry and in situ hybridization**

Staining of whole-mount embryos was performed using standard techniques. In situ hybridization to embryos was performed as described in Robinow et al. (1997).

Whole-mount in situ hybridization and immunostaining of pupal wings were carried out as described by Sturtevant and Bier ([http://www-bier.ucsd.edu/imagdisc.html](http://www-bier.ucsd.edu/imagdisc.html)). Briefly, pupae were aged at 24° or 29 °C. The pupal case was removed and the head and tip of the abdomen were cut off. Internal tissues were washed away with PBS using a small syringe. Pupae were then fixed in 4% formaldehyde overnight at 4 °C (for in situ) or 1 h at room temperature (for immunostaining). Following several washes in PBT (PBS, 0.1% Tween 20), the wing cuticle was peeled using delicate forceps. Staining was performed as for embryos, except that incubation with antibodies was performed in PBT plus 5% normal goat serum, and the washes were longer (30 min each). Stained embryos and pupal tissues were viewed using bright field and confocal microscopy (Axioskop, Zeiss and LSM510, Zeiss, respectively). Fluorescence intensity was measured from confocal images using ImageJ (NIH, USA). Average values and standard deviations were calculated and Student’s *t*-test analysis was carried out.

**Probes and antibodies**

A *dei*-specific probe was made by amplifying a 400-bp fragment from a cDNA template, using the following primers: 5′ GACAAA-GAGGGGCTTCA A and 5′ GCTCCCAGTCGACTTC A. A *mys*-specific probe was made by amplifying a 545-bp fragment from exon 6, using the following primers: 5′ TGGAGAGAGAGATCAC AT 3′ and 5′ GTACCATGAGGGGAGATG 3′. A new-specific probe was prepared by amplifying a 563-bp fragment, which is included in both the A and B transcripts of the gene, using the following primers: 5′ TACAT- CAGTGGCATTACCA 3′ and 5′ TCCACCCGAGGATACAC 3′. An *if*-specific probe, which recognizes four transcript variants of the
Dei is expressed in the intervein domains of prepupal and pupal wings

The expression pattern of Dei in the developing wing has not been described previously. We therefore characterized the expression pattern of dei in larval and pupal wing discs by in situ hybridization and immunostaining. dei’s expression was not detected in wing discs of third instar larvae or immediately after pupariation (AP) (Fig. 1A and data not shown). In wing discs dissected approximately 2 h AP dei-specific mRNA was observed in the forming wing blade in a spatially restricted pattern, which probably corresponds to certain intervein territories (Fig. 1B). No expression was detected in the dorso-ventral wing margin or in the hinge region. At 18 h AP the expression of dei expanded to the entire prospective intervein domain, in both the dorsal and ventral wing layers (data not shown). dei’s expression remained restricted to the intervein domain and was excluded from the forming veins in pupal wings dissected 30–35 h AP (Fig. 1C–D). The Dei mRNA and protein accumulated at somewhat higher levels in the vein/intervein border cells that are defined by the activity of Notch (Fig. 1D–E). The Dei protein also accumulated at high levels in cells of sensory organs of the anterior wing margin (Fig. 1F).

The wing disc is divided to provein and intervein zones in the 3rd larval wing disc, prior to the onset of dei’s expression. The main pathways responsible for specifying provein domains are EGFR and Notch. The EGFR pathway is active in the prospective vein region, promoting vein differentiation, whereas Notch acts in the vein–intervein boundaries, delimiting vein differentiation (Blair, 2007; De Celis, 2003; de Celis et al., 1997; Sturtevant et al., 1993). We hypothesized that dei’s expression is normally turned on in cells that have been already determined to differentiate as intervein cells, and that its expression in these cells is essential for their proper differentiation. If this is correct, then dei’s expression should be

Cuticle preparations

Anesthetized flies were boiled in 10% NaOH solution for 10 min, squeezed against the tube wall and washed in water. Wings were dissected, and mounted in Hoyer’s medium. Alternatively, flies were stored in isopropanol at 4 °C and wings were mounted in Canada balsam (SIGMA-ALDRICH, St. Louis, MO, USA). To compare the severity of ectopic vein phenotypes of different genotypes, wing cuticles were prepared and photographed. The length of ectopic veins was measured using Adobe Photoshop.

Results

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affected by vein-determining signals, such as EGFR signaling, as well as vein-repressing signals such as Notch and Bsh.

To establish whether dei’s expression is affected by the EGFR and Notch pathways, we examined the distribution of dei’s mRNA in pupal wings with abnormal activity of these pathways. The gain-of-function allele of Notch, Axl6172, suppresses the competence to form veins in several regions of the wing, where intervein domains are established instead (Fig. 2A) (de Celis et al., 1997; Kelley et al., 1987). In accordance with our hypothesis, dei’s expression expanded into the vein-to-intervein transformed regions of Axl6172 pupal wings (Fig. 2B), suggesting that dei is positively regulated by Notch activity.

To test whether the EGFR pathway normally suppresses dei’s expression in the vein territory, we expressed a dominant negative form of the EGF receptor (DERDN) throughout the developing wing under the regulation of MS1096-Gal4. This expression leads to a partial loss of the L3, L4 and L5 veins in adult wings. In UAS-DERDN/+; MS1096-Gal4/+ pupal wings dei’s expression expanded into the ectopic intervein domains (Fig. 2C–D), suggesting that Dei’s

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**Fig. 3.** dei mutant alleles. (A) Schematic representation of the dei locus and the identified mutant alleles. The dei gene is comprised of two exons (black boxes) separated by a 6-kb intron (thin black line). The ORF (green box) is contained within the second exon. The light grey box at the top panel marks the extension of the dei33–2 deletion. The lower panel shows the various domains of the Dei protein and the position of the point mutations. (B–C) Wings (35-h APF) stained with anti-Dei (B, C) and anti-DSRF (B’, C’); merge of the two channels are shown in (B”, C”). (B–B”) A normal wing. (C–C”) A dx homozygous wing. Note the loss of Dei’s expression from intervein cells. The arrowhead points to sensory organs at the anterior wing margin that continue to express Dei in the dx mutant. (D–E) Lateral view of three abdominal segments of stage 16 embryos stained with anti-Dei (green) anti-Sr(red) and MAb21A6 (blue, marks the scolopale cells of chordotonal organs). The cap (c), ligament (l), ligament attachment (la) and tendon cells (t) express Dei in wildtype embryos (D–D”). In dei01478 homozygous embryos, Dei’s expression is lost from tendon cells and the chordotonal attachment cells and is elevated in cap and ligament cells (E–E”). (F–G) In situ hybridization with a dei-specific probe to 30–35-h APF wildtype (F–F”) and homozygous dei01478 wings (G–G”). The wings were processed in parallel.
Fig. 4. dei is required for normal wing patterning and adhesion of wing layers. (A) A wildtype wing displaying normal venation. Veins L2, L3, L4 and L5 are indicated. (B) A dei\textsuperscript{1478} wing displaying the typical phenotype of creased lengthwise (arrow) pointed wing. (C) A dei\textsuperscript{1478} by displaying a typical wing blister (arrow) and held out wings. (D) A tx\textsuperscript{1} mutant wing displaying the typical crease (arrow) and extra bristle phenotype (arrowheads and inset); (E) A homozygous dei\textsuperscript{1478} fly with typical wing blisters and held out wings; (F) A dei\textsuperscript{1478}/Df(3R)ED6232 wing displaying ectopic cross-vein (arrow) and ectopic bristles (arrowheads and inset). (H) An en-Gal4\textsuperscript{+}; UAS-dei-IR/+ (PGD4229)v37630 fly with blisters in the posterior region of its wings. (I) A 24B-Gal4/P(GD4229)v37630 fly exhibiting held out, pointed and blistered wings. (J) A wing of 1.65-Gal4/UAS-dei fly. Loss of vein properties is evident in L4 and posterior cross-vein (arrowheads).

expression is normally negatively regulated by the activity of the EGFR pathway.

To establish whether dei's expression is regulated by the intervein-determining gene hs, the distribution of dei's mRNA was examined in wings homozygous for the viable allele hs\textsuperscript{12}. The intervein territory of these mutants was shown to harbor partial vein tissue identity (Fristrom et al., 1994) and, in agreement with that, a reduction in the level of dei's expression was observed in hs\textsuperscript{12} homozygous wings as compared to control wings processed in parallel (Fig. 2E–F and data not shown).

Altogether, these results suggest that the pattern of dei expression in the wing is an accurate readout of the signaling pathways that divide the wing to zones of vein and intervein identities, where Dei's expression is on in cells defined as intervein cells and off in vein cells.

Generation and characterization of dei LOF alleles

As a first step towards functional analysis of the dei gene we have isolated and characterized a series of loss-of-function (LOF) dei alleles, which included point mutations, insertions and deletion alleles. The different alleles used for this work are described shortly below.

The dei\textsuperscript{1478} allele (FlyBase, 1999) harbors a piggyBac intronic insertion 1006 bp upstream from the initiation codon (Fig. 3A); the dei\textsuperscript{MB3174} allele (Bellen et al., 2004) harbors a Minos insertion 267 bp upstream to the coding exon (Fig. 3A); three EMS-induced hypomorphic alleles dei\textsuperscript{1}, dei\textsuperscript{2} and dei\textsuperscript{14} were identified by TILLING (see Materials and methods) and they all harbor point mutations that lead to amino acid substitutions (Fig. 3A). A null allele of dei, dei\textsuperscript{1722}, was generated by imprecise excision of the MB3174 Minos element as described in Metaxakis et al. (2005). This allele carries a 2.7-kb fragment of the Minos element (inserted 267 bp upstream to the coding exon) combined with a 6.86-kb deletion that removes the entire coding sequence of dei and two neighboring genes, hex-t1, hex-t2 as well as part of cg5447 (Fig. 3A).

The very prominent wing phenotypes of some of the dei alleles (described below), combined with the fact that the dei locus can be readily mutated, made it improbable that dei mutants have not been already isolated and described during more than hundred years of Drosophila research. A thorough browse through Lindsley and Zimm's Red Book (Lindsley and Zimm, 1992) identified a prime suspect, called taxi wings (tx), which was firstly identified in 1924 by J.L. Collins (Collins, 1928). The beautiful drawing, the accompanying phenotypic description of tx mutants, and the fact that tx maps to cytological location 97 strongly suggested that tx and dei are allelic. A series of complementation tests between the tx\textsuperscript{1} allele and several alleles of dei suggested that dei is indeed allelic to tx. Molecular analysis of the tx\textsuperscript{1} allele did not reveal any alterations in the coding sequence of dei. However, immunostaining of tx\textsuperscript{1} pupal wings with anti-Dei antibodies revealed a loss of Dei's expression from intervein cells (Fig. 3B–C), suggesting that tx\textsuperscript{1} is a regulatory allele that disrupts the normal pattern of dei's expression.

Dei\textsuperscript{1478} is also a regulatory allele that disturbs the normal expression pattern of the dei gene. In dei\textsuperscript{1478} homozygous embryos dei's expression was lost from all tendon cells and chordotonal attachment cells, but was maintained (or even elevated) in the cap and ligament cells of the chordotonal organs (Fig. 3D–E). In situ hybridization to dei\textsuperscript{1478} homozygous pupal wings revealed a significant reduction in the level of dei's mRNA (Fig. 3F–G).

Dei is required for proper differentiation and adhesion of intervein cells

To establish whether dei is required for terminal differentiation of intervein cells and proper adhesion of the wing layers we characterized the wing phenotypes of multiple allelic combinations of dei. In addition, we used RNA interference (RNAi) to abrogate dei's activity. The dei mutant alleles and the RNAi caused abnormal wing phenotypes that implicate Dei in the restriction of vein territories and in intervein differentiation and adhesion processes. The dei alleles fall into two phenotypic classes: mild alleles (dei\textsuperscript{1}, dei\textsuperscript{2}, dei\textsuperscript{14}, dei\textsuperscript{MB3174}) that cause mild extra vein phenotypes, and stronger alleles (dei\textsuperscript{1478}, dei\textsuperscript{1722}, tx\textsuperscript{1}) characterized by held out, arched, creased lengthwise, smaller and pointed wings that often harbor very large blisters filled with liquid (Fig. 4B–D). The dei\textsuperscript{14} (hemizygous over Df
(3R)ED6232 and tx1 alleles, or dei^{14}/tx1 transheterozygotes, exhibit also ectopic sensory bristles on the wing (Fig. 4D, G).

The dei locus resides in a gene-rich genomic region and we know that at least the dei^{33-2} allele affects three additional genes (Fig. 3A). To verify that the observed phenotypes were caused by the reduction in dei’s activity, we used RNAi to down-regulate dei’s expression in the developing wing and tested in parallel RNAi transgenes directed against the three neighboring genes. When double stranded RNA (dsRNA) of dei (Dietzl et al., 2007) was produced in the posterior compartment of the wing under the regulation of the en-Gal4 driver, a significant reduction of dei’s mRNA and protein was observed (Fig. 5J–K and data not shown). The wing layers of such flies failed to adhere properly and blisters were detected in the posterior wing region in 100% of the flies (n = 50, Fig. 4H). When expressed under the regulation of the 24B-Gal4 driver, which drives expression in the proximal region of the wing blade and in flight muscles (Fig. s1A), the dsRNA of dei caused most of the observed LOF phenotypes, including held out, pointed, arched and blistered wings as well as semilethality at the pupal or pharate adult stage (Fig. 4I). RNAi directed against the other three genes deleted in the dei^{33-2} chromosome did not cause any of these phenotypes (Fig. s1B–E). These observations strongly suggest that the loss of dei’s activity is the cause of the observed phenotypes. Curiously, in the RNAi crosses of all these genes we saw occasionally a short extra-vein located in parallel, either anteriorly or posteriorly, to L2 (Fig. s1B–E). This observation suggests that this region in the wing is prone to develop ectopic vein tissue upon various perturbations, complicating the analysis of dei’s ectopic vein phenotype.

The blisters and extra vein phenotypes suggest that Dei is required to promote intervein differentiation and adhesion and to prevent vein development. To further test this hypothesis we over-expressed dei in the wing under the regulation of the 1.65 Gal4 driver, which drives expression mainly in the developing veins (Sotillos and de Celis, 2006). Ectopic expression of dei in the developing veins often led to loss of vein properties and intervein-like differentiation in the L4 and posterior cross-vein territories (Fig. 4J). This observation corroborates the idea that Dei plays an instructive role in intervein differentiation.

Dei is a potent activator of βPS, but not αPS1 or αPS2, integrin in intervein cells

During prepupal and pupal stages, the wing layers go through two phases of adhesion (Brabant et al., 1996; Fristrom et al., 1994). The βPS integrins are restricted to basal plaques in the intervein region during the second pupal apposition (Brabant et al., 1996; Fristrom et al., 1993). Homozygous mutant clones of myospheroid (mys, the gene encoding for βPS integrin) lead to a failure in re-apposition of the wing layers and the consequent formation of wing blisters (Brabant et al., 1996), similar to the blisters seen in dei mutant flies, or in flies expressing dsRNA of dei in the developing wing. In addition, weak mys alleles, such as mys^{nm2}, display venation defects that are very similar to the venation defects of weak dei alleles, such as dei^{14} and dei^{8} (DAvino and Thummel, 2000; Wilcox et al., 1989). The similar expression pattern (Fig. 5A–D) and mutant phenotypes of dei and βPS integrin in the wing led us to test for possible interaction between Dei’s activity and βPS expression.

Fig. 5. Dei is a positive regulator of βPS integrin expression in the wing. Wings (30–35-h APF) immunostained with anti-βPS antibodies (A, E, F, J) or co-stained for anti-Dei (blue) and anti-βPS (red) (B–D, G–I, K–M). (A–D) Wildtype wings. (E–I) en-Gal4/UAS-dei wings show a parallel upregulation of Dei and βPS in the posterior compartment of the wing. Note the unusual expression of βPS in vein cells (arrow in F). (J–M) en-Gal4/++; UAS-dei-IR/+ (P{GD4329}v37630) wings show a parallel reduction in the level of Dei and βPS in the posterior compartment of the wing (39.3% and 39.2%, respectively).
Fig. 6. Dei regulates the expression of βPS, but not αPS1 or αPS2 integrin. Pupal wings, 30–35-h APF, harboring act-Gal4-expressing clones (labeled by the expression of GFP) in which the expression of either a UAS-dei or a UAS-dei-IR (P GD4329 v37630) transgene is induced. (A–D) A wing expressing UAS-dei-IR in GFP-labeled clones (A) stained for Dei (B, blue channel in D) and βPS (C, red channel in D). Note the parallel reduction in the level of Dei and βPS in the clones. (E) A graph summarizing the quantification of average fluorescence intensity in GFP positive (dei-IR expressing) versus negative clones (wt tissue). An average reduction of 26% was measured for Dei, and a parallel reduction of 22.6% in the level of βPS integrin, was measured in dei-IR expressing clones as compared to wt clones (n = 17 and 19, P < 0.004 and P < 0.04, respectively). (F–I) A wing expressing UAS-dei in GFP-labeled clones (F) stained for Dei (G, blue channel in I) and βPS (H, red channel in I). A parallel up-regulation of Dei and βPS is evident in the clones (arrow). Quantification of the average fluorescence intensity in GFP positive versus negative clones is shown in J. An average increase of 181% was measured for Dei, and a parallel, though lower, increase of 29.8% in the level of βPS integrin, was measured in dei overexpressing clones as compared to wt clones (n = 8 for each class; P < 0.0001 and P = 0.031 respectively). (K–N) A wing expressing UAS-dei in GFP-labeled clones (K) stained for Dei (L, blue channel in N) and αPS1 (M, red channel in N). Up-regulation of Dei did not lead to parallel up-regulation of αPS1 in clones (arrow). (O–R) A wing expressing UAS-dei-IR in GFP-labeled clones (O) stained for Dei (P, blue channel in R) and αPS2 (Q, red channel in R). Down-regulation of Dei did not lead to parallel down-regulation of αPS2 in clones. (S–V) Wings stained for αPS1 (S–T) and αPS2 (U–V). (S) and (U) show normal wings, whereas (T) and (V) show en-Gal4/UAS-dei and en-Gal4/UAS-Vp16-dei wings, respectively.
To establish whether dei is normally required to activate \( \beta \)PS expression in intervein cells, we examined \( \beta \)PS expression in \( dei^{01478} \) mutant pupal wings. A significant reduction of \( \beta \)PS expression was evident (data not shown). To verify that the observed reduction in \( \beta \)PS staining is not a mere reflection of the abnormal morphology (lack of adhesion between wing layers) and the resulting difficulty to handle \( dei^{01478} \) homzygous wings, we used the Gal4 system to alter the level of \( dei \) expression in restricted regions of the wing and examined the effects on \( \beta \)PS expression. When \( dei \) was over expressed in the posterior compartment of the wing, dramatic enhancement of \( \beta \)PS expression was evident (Fig. 5E–I). Most significantly, \( \beta \)PS expression was detected in vein cells (in L4 and L5), which normally never express this integrin (Fig. 5F). When, in contrast, dsRNA of \( dei \) was expressed in the posterior compartments of the wing, a significant reduction of \( \beta \)PS expression was evident (Fig. 5J–M). Similar reduction was evident when the level of myRNA was examined by in situ hybridization in wings expressing dsRNA of \( dei \) in their posterior compartments (Fig. S2). These results demonstrate that \( dei \) is required for activation of \( \beta \)PS expression in intervein cells and is sufficient to induce \( \beta \)PS expression when expressed ectopically in vein cells. The effect of \( dei \) on \( \beta \)PS expression is cell-autonomous as shown by clonal analysis (see below, Fig. 6).

\( \beta \)PS integrin is expressed in both the dorsal and ventral wing layers, whereas the \( \alpha \)PS subunits are expressed differentially, \( \alpha \)PS1 in the dorsal layer and \( \alpha \)PS2 in the ventral layer (Brower et al., 1984; Wilcox et al., 1981). Terminal differentiation of intervein cells relies on accurate levels of expression of all these integrin subunits. To test whether \( dei \) affects the expression of \( \alpha \)PS subunits, similarly to its effect on \( \beta \)PS, we generated clones that over-expressed \( dei \) and examined them for \( \beta \)PS, \( \alpha \)PS1 and \( \alpha \)PS2 expression. All the clones that over expressed \( dei \) exhibited elevated levels of \( \beta \)PS, but such clones did not show consistent changes in \( \alpha \)PS1 or \( \alpha \)PS2 expression (Fig. 6F–N and data not shown). Similarly, unlike its effects on \( \beta \)PS expression, overexpression of \( dei \) in the posterior compartment of the wing did not lead to upregulation of \( \alpha \)PS1 or \( \alpha \)PS2 levels (Fig. 6S–V). In addition, clones expressing dsRNA of \( dei \) consistently exhibited a reduction in \( \beta \)PS expression, but no consistent changes in \( \alpha \)PS1 or \( \alpha \)PS2 levels (Fig. 6A–E, O–R and data not shown). These results suggest that \( dei \) regulates specifically the \( \beta \)PS gene, but does not regulate the multiple edematous wings and \( \beta \)PS expression.

\( Dei \) functions downstream to \( Bs \) in regulating integrin expression

It was previously suggested that \( Bs \), a major determinant of intervein identity, acts upstream to the integrin genes in intervein cells (Fristrom et al., 1994; Walsh and Brown, 1998). \( Bs \) mutants display wing phenotypes that resemble very much those of \( Bs \) mutants. In addition, \( dei \) interacts genetically with \( Bs \). Wings transheterozygous for \( bs^{14} \) and \( dei^{23-2} \) exhibited more pronounced extra-vein phenotypes than wings of flies carrying \( bs^{14} /+ \) or \( dei^{23-2} /+ \) alone (Fig. 7A–C). This result suggests that \( bs \) and \( dei \) function in the same genetic pathway. Based on its expression pattern, \( Dei \) is likely to act downstream to \( Bs \) in intervein domains. To test whether \( dei \) functions downstream to \( Bs \) in regulating \( \beta \)PS expression, we tested whether \( dei \) can rescue the reduced expression of \( \beta \)PS in \( Bs \) mutants. Over expression of \( dei \) under the regulation of \( en-Gal4 \) in homzygous \( bs^{12} \) wings resulted in elevated expression of \( \beta \)PS in the posterior compartment (Fig. 7D–F). This result indicates that \( Dei \) can activate \( \beta \)PS expression in the absence of normal \( Bs \) activity and suggests that \( dei \) normally acts downstream to \( Bs \) in the \( \beta \)PS regulatory pathway.

\( Dei \) is a general regulator of \( \beta \)PS expression in Drosophila

The overlap between \( Dei \)’s expression and \( \beta \)PS expression is not restricted to the developing wing. In the embryo such co-expression is evident in the accessory and attachment cells of the chordotonal organs and in tendon cells (Fig. 8F–H and (Bogaert et al., 1987; Inbal et al., 2004; Leptin et al., 1989). In the pupa, other cell types that express high levels of \( Dei \) express also high levels of \( \beta \)PS integrin, for instance the cone cells in the eye (Fig. 8A–E), where \( \beta \)PS is required for anchoring rhabdomeres to the cone cell plate (Longley and Ready, 1995). We therefore tested whether the effect of \( Dei \) on \( \beta \)PS expression is specific to intervein cells, or is a more general phenomenon.

To establish whether \( Dei \) is involved in regulating \( \beta \)PS expression in other pupal tissues we examined mitotic eye clones that expressed double strand RNA of \( dei \) under the regulation of \( act-Gal4 \). As shown in Fig. 8, cone cells with diminished \( Dei \) expression exhibited consistent reduction in the level of \( \beta \)PS expression (Fig. 8A–E), indicating that \( Dei \) is involved in the regulation of integrin expression in these cells.

The ability of \( Dei \) to induce the expression of \( \beta \)PS in the embryo was tested by ectopically expressing \( dei \) under the regulation of \( en-GAL4 \). In \( en-GAL4/UAS-dei \) embryos a low, but significant, level of
βPS expression was evident in the epidermal stripe of Dei-expressing cells (Fig. 8I–K). When an activating form of the gene, Vp16-Dei, was expressed under the regulation of en-Gal4, a dramatic enhancement of βPS expression was observed both at the mRNA and protein levels (Figs. 8L–N and s2), indicating that Dei can activate βPS expression in embryonic tissues. We hypothesized that the expression of Dei alone could not strongly activate βPS expression due to a lack of its bHLH dimerization partner. Indeed, co-expression of Dei and its putative

![Image](https://Example.com/image.jpg)

Fig. 8. Dei affects βPS expression in multiple tissues. (A–D) A pupal eye (35-h APF) expressing UAS-dei-IR in GFP-labeled clones under the regulation of act-Gal4 (B), stained for Dei (C, blue channel in A) and βPS (D, red channel in A). Dei is expressed in the cone cells, but not in the surrounding pigment cells. Therefore the reduction in the level of Dei leads to parallel reduction in the level of βPS only in cone cells within the clones (arrows in D). (E) Quantification of the average fluorescence intensity in GFP positive versus negative clones. An average decrease of 61.3% in the intensity of anti-Dei staining, and a parallel though lower decrease of 23.3% in the level of βPS integrin, was measured in individual facets contained within dei-IR expressing clones as compared to wt clones (n = 8 for each class; P = 0.0001 and P = 0.016, respectively). (F–Q) Lateral view of stage 16 embryos stained with anti-Dei (red) and anti-βPS (cyan) antibodies. (F–H) A wildtype embryo. Co-localization of Dei and βPS is evident in the cap (C) and ligament (L) cells of the lateral chordotonal organs, as well as in tendon cells (T). (I–K) An en-Gal4/UAS-dei embryo. Ectopic expression of Dei in epidermal stripes within the en domain (arrows in J) leads to parallel up-regulation of βPS expression (arrows in I). (L–N) An en-Gal4/UAS-Vp16-dei embryo. Ectopic expression of Vp16-Dei (arrows in N) leads to strong up-regulation of βPS expression in the Vp16-dei-expressing cells (arrow in M). (O–Q) An en-Gal4/UAS-dei, UAS-da embryo. Concomitant ectopic expression of Dei and Da leads to strong up-regulation of βPS expression in the expressing cells (arrows in P).
dimerization partner Daughterless (Da) (Armand et al., 1994) induced much higher levels of βPS expression (Fig. 80–Q). Such induction of βPS expression was not seen upon ectopic expression of Da alone (data not shown). These observations indicate that Dei can function as an activator of βPS expression in the embryo and that this activity depends on its binding to Da. Homozygous dei01478 or dei132 embryos did not display consistently an obvious reduction in the levels of the βPS protein (data not shown). This observation may indicate that Dei is not a major regulator of integrin expression during embryogenesis. However, due to the high level of maternal contribution of βPS we cannot rule out the involvement of Dei in regulating mys expression in late stages of embryogenesis or in larval stages. In this respect, it is worth noting that despite the overall normal morphology of muscles, tendons and chordotonal organs in dei01478 homozygous embryos, homozygous 3rd instar larvae are sluggish and less coordinated than heterozygous siblings, suggesting that dei mutant animals have impaired contractile and/or proprioceptive functions.

Discussion

Integrins, which are conserved between all metazoans, are heterodimer receptors composed of α and β subunits. Among other functions, integrins mediate stable adhesion between cells to the extracellular matrix. These strong attachments are crucial for tissue integrity and performance. In flies, in the absence of members of the integrin family, muscles detach from their tendons and blisters form between the two wing layers (reviewed in Brown et al., 2000). Similarly, in mammals, mutations in integrin subunits result in different pathological phenotypes. For example, in the autosomal recessive disorder epidermolysis bullosa, genetic defects in α6 and β4 integrin subunits cause disruption of the mechanical link between the basement membrane and the basal keratinocyte layers, leading to epidermal blistering of the skin (reviewed in Wehrle-Haller and Imhof, 2003). This disease can be also caused by reduction in integrin concentration caused by the generation of anti-integrin autoantibodies. Abnormal levels of integrin expression are also associated with tumor progression and metastasis (Guo and Giancotti, 2004).

The role of integrins as regulators of cell adhesion in Drosophila has been extensively studied, however, we still lack a full understanding of how integrin expression is regulated and by which transcription factors. Here we identify the bHLH transcription factor Dei as an important positive regulator of the expression of βPS, the major β subunit in Drosophila.

During embryonic development Dei’s expression is confined mainly to cells that adhere strongly to other cells and are able to withstand mechanical strain, for instance, tendon cells that attach body wall muscle to the cuticle. Moreover, when different types of cells arise from within a uniform cell population, or through asymmetric cell division, Dei’s expression is restricted to the ‘stickier’ types of cells. For example, in the chordotonal organ lineage, Dei is expressed in the four types of support cells (cap, ligament, cap-attachment and ligament-attachment), but is excluded from the neuron and glia. Similar phenomenon is seen in the developing wing where Dei is expressed only in intervein territories, where the ventral and dorsal layers adhere to each other, and is not expressed in vein cells that do not adhere to cells of the opposite layer. In all these systems, Dei does not function as a selector of cell identity, but it is required to realize the selected fate by activating a developmental program that specifies adhesive properties of cells.

Although we have not characterized dei’s expression in all developmental stages and tissues, published data of various microarray analyses suggest that dei is expressed in other developmental and physiological contexts where up-regulation of βPS integrin is required. For example, dei was up-regulated when larvae were exposed to immune challenge, or when mutant larvae exhibited an increase in lamellocyte cell population (Irving et al., 2005; Kwon et al., 2008). Lamellocytes represent a subset of hemocytes in Drosophila, which differentiate in response to specific immune challenge. The lamellocytes aggregate around large pathogens to form a rigid laminated capsule that confines the pathogen and enables its elimination (for a review see Crozatier and Meister, 2007). This encapsulation process requires members of the integrin family that presumably mediate the lamellocyte’s attachment (Irving et al., 2005).

In this work we focused mainly on the role of dei in intervein cells and showed that dei provides a missing link between the genetic specification of these epithelial cells and their differentiation. Our data place dei downstream to the major signaling pathways that divide the wing to regions of veins and interveins and downstream to B5, which works as a selector of intervein identity. It remains to be determined whether dei is a direct target of B5, and whether it is a direct regulator of βPS, however the results of the rescue experiment suggest that the effect of B5 on integrin expression is mediated, at least in part, by the activity of Dei.

The venation phenotypes caused by weak dei alleles could be also attributed to the effects of Dei on βPS expression. Even though vein and intervein territories are established during early stages of wing development, the decision remains plastic for at least 24-h APF (reviewed in Blair, 2007). Maintenance of the right fates depends on both vein-specific and intervein-specific genes. Appropriate levels of integrin expression are required for the maintenance of intervein fate, as suggested by the ectopic vein phenotype of certain mys alleles, which is very similar to the venation phenotype of weak dei alleles (Wilcox et al., 1989).

It is reasonable to assume that Dei regulates multiple target genes in different cells and tissues. However, as for integrins, Dei regulates specifically βPS integrin. We did not find any evidence for regulation of αPS1 or αPS2, which are expressed differentially in the two wing layers, by Dei. Since βPS is the dimerization partner of both αPS1 and αPS2, by regulating its expression Dei practically affects all integrin-based adhesion processes at both the dorsal and ventral wing layers. Our data also suggest that the effects of Dei on integrin-dependent adhesion are not restricted to the wing. Ectopic expression of Dei led to up-regulation of βPS expression in embryonic tissues, whereas loss of Dei’s activity caused a reduction in the level of βPS expression in the cone cells of the eye.

In summary, we think of Dei as a general switch that turns on βPS integrin expression wherever a sticky cell has to develop. Since such a switch needs to be turned on in different tissues and different developmental and physiological contexts, it is predicted that the dei gene can respond to various signaling pathways and transcription factors. Indeed, analysis of the regulatory region of the dei locus demonstrated that it harbors multiple regulatory modules that respond to different transcription factors working in different developmental contexts (Nachman et al., unpublished data).

How transcription factors and signaling networks specify cell fates is a central question in developmental biology. Although we have a conceptual picture of how differential gene expression is used to generate different types of cells, we still lack a full understanding on how any cell is specified and how it acquires its unique properties. The identification of Dei as a mediator between signaling pathways that specify intervein cell-fate and one of the most significant realizers of this fate, βPS integrin, adds to our understanding and fits in a new component into a genetic network that is, so far, roughly drawn.

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