

Dynamic Decapentaplegic signaling regulates patterning and adhesion in the *Drosophila* pupal retina

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The correct organization of cells within an epithelium is essential for proper tissue and organ morphogenesis. The role of Decapentaplegic/Bone morphogenetic protein (Dpp/BMP) signaling in cellular morphogenesis during epithelial development is poorly understood. In this paper, we used the developing *Drosophila* pupal retina – looking specifically at the reorganization of glial-like support cells that lie between the retinal ommatidia – to better understand the role of Dpp signaling during epithelial patterning. Our results indicate that Dpp pathway activity is tightly regulated across time in the pupal retina and that epithelial cells in this tissue require Dpp signaling to achieve their correct shape and position within the ommatidial hexagon. These results point to the Dpp pathway as a third component and functional link between two adhesion systems, Hibris-Roughest and DE-cadherin. A balanced interplay between these three systems is essential for epithelial patterning during morphogenesis of the pupal retina. Importantly, we identify a similar functional connection between Dpp activity and DE-cadherin and Rho1 during cell fate determination in the wing, suggesting a broader link between Dpp function and junctional integrity during epithelial development.

KEY WORDS: Adhesion, BMP, Dpp, Epithelia, Patterning

INTRODUCTION

Guiding cells to their correct positions within a patterned epithelium involves an intricate combination of cellular events. These events are typically coordinated by ‘organizer cells’ within the epithelium that act to set pattern across the tissue. Bone morphogenetic proteins (BMPs) are members of the Transforming growth factor- β (TGF- β) superfamily of proteins, which regulate a wide range of biological processes (Attisano and Wrana, 2002; Hogan, 1996; Ozdamar et al., 2005). Although much is known of the basic signaling pathway, the role of BMPs in cell morphogenesis remains poorly understood. In this paper we use the *Drosophila* pupal retina to explore how temporal and cell type-specific regulation of BMP signaling regulates the positioning of cells within developing epithelia.

TGF- β -family proteins activate signaling by binding Type I and Type II serine-threonine kinase receptors, which in turn recruit and phosphorylate receptor SMADs to regulate transcription of target genes (Shi and Massague, 2003). Dpp is the *Drosophila* ortholog of vertebrate BMP2/4. Its potential receptors include the Type II receptors Punt (Put) and Wishful Thinking (Wit) and the Type I receptors Thickveins (Tkv) and Saxophone (Sax), which activate the downstream target Mad (Letsou et al., 1995; Marques et al., 2002; Newfeld et al., 1997; Penton et al., 1994; Xie et al., 1994). The Dpp signaling pathway regulates multiple developmental processes including dorsoventral patterning of the embryo, gut morphogenesis, growth, patterning and differentiation of the imaginal discs, and epithelial morphogenetic processes such as dorsal closure and imaginal disc spreading (Ferguson and Anderson, 1992; Firth and

Baker, 2005; Greenwood and Struhl, 1999; Neumann and Cohen, 1997; Panganiban et al., 1990; Rogulja and Irvine, 2005; Affolter et al., 1994; Martin-Blanco et al., 2000).

Recently, strong loss of Dpp signaling in the wing has been demonstrated to cause the release of cells from the epithelium and the establishment of a basal cyst (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). This suggests that Dpp pathway activity is required to maintain epithelial integrity. Epithelial integrity and tissue morphogenesis are mediated through dynamic regulation of the apical junctions (Schock and Perrimon, 2002). Dpp signaling is also precisely regulated during development, and one possibility is that it regulates epithelial patterning or maturation through an association with apical junctions.

The *Drosophila* pupal retina has proven a useful system for studying epithelial patterning. Its precise pattern emerges through a series of morphogenetic processes that include changes in cell shape, cell position and programmed cell death (Cagan and Ready, 1989b). Formation of correct cell contacts and selective cell adhesion – collectively known as cell sorting – are also key events during patterning of the pupal retina (Bao and Cagan, 2005; Grzeschik and Knust, 2005; Hayashi and Carthew, 2004; Reiter et al., 1996). The adhesion molecule Roughest (Rst) is the ortholog of vertebrate NEPH1 (also known as KIRREL1) and a member of the immunoglobulin superfamily. Mutations in the *rst* gene result in impaired cell sorting and subsequent blockade in programmed cell death during pupal retinal development (Reiter et al., 1996; Wolff and Ready, 1991). Rst regulates patterning of the pupal retina through selective heterophilic adhesion with Hibris (Hbs) and formation of cell junctions (Bao and Cagan, 2005). Additionally, the adhesion molecule DE-cadherin has been proposed to regulate Rst during stages of maximal cell rearrangements in the pupal retina (Grzeschik and Knust, 2005), although the precise relationship between these two adhesion molecules remains to be elucidated.

In this paper, we demonstrate an essential role for the Dpp pathway in regulating epithelial cell shape and patterning in the pupal retina. We provide evidence that Dpp pathway activity is regulated

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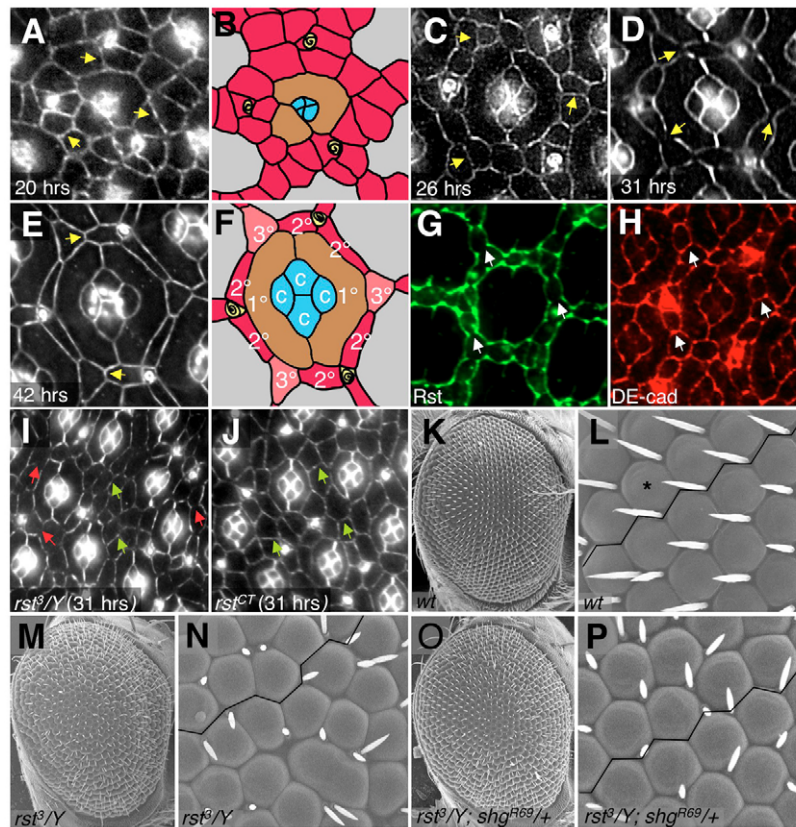


Fig. 1. Morphogenesis of the *Drosophila* pupal retina and the role of cell adhesion. (A-F) Time course of retinal development. Apical cell profiles were visualized with anti-Armadillo to highlight adherens junctions. Anterior is to the right; times refers to hours after puparium formation (APF). Unpatterned arrays of IPCs (A,B) sort to single file (C); IPC number continues to decrease as patterning tightens (D) until the final pattern is achieved (E,F). B and F are schematics of the central ommatidium from A and E, respectively; cone cells (c, blue shading), primary pigment cells (1°, brown), IPCs (red), 2° (red), 3° (pink) and bristles (yellow) are indicated. Arrows in A,C,D,E point to IPC:IPC adherens junctions. (G,H) 26 hours APF retina stained with anti-Rst (G) and anti-DE-cadherin (H). Arrows point to the IPC:IPC junctions, where DE-cadherin is expressed and Rst is absent. (I,J) 31 hours APF *rst* mutant retinas; magnification is reduced to show additional ommatidia. Green arrows point to IPC:IPC junctions that failed to clear (compare with wild type, D). This defect correlated with the failure in mutant IPCs to sort out into single-cell rows, as observed in mutants for the hypomorphic allele *rst³*, which is subject to position effect variegation (I). Red arrows in I point to *rst³* regions where IPCs have sorted out into single layers and have also cleared out their junctions; these are likely to contain normal levels of Rst protein. (K-P) Scanning electron micrographs of adult eyes (genotypes as listed) taken at 180× (K,M,O) and 800× (L,N,P). K and L show a wild-type adult eye; a single ommatidium is indicated with an asterisk. Note the straight ommatidial rows, highlighted by a line drawn between ommatidia. The aberrant ommatidial packing observed in an *rst³* eye (M,N) is rescued by removing a single functional copy of *shg* (O,P).

dynamically across time and that it acts as a new component and functional link between two adhesion systems, Hbs-Rst and DE-cadherin. Our data support a novel role for temporal and cell-type specific Dpp/BMP signaling to direct shape and positioning of individual cells into an emerging epithelial pattern.

MATERIALS AND METHODS

Fly lines

All crosses and staging were conducted at 25°C unless otherwise noted. Wild-type (Canton-S), *GMR-gal4*, *dpp-lacZ*¹⁰⁶³⁸, *tkv*^{k16713}, *Rho1*^{72F} and *put*^P flies were kindly provided by the Bloomington *Drosophila* Stock Center, *dpp*^{e90} by Kristi Wharton (Brown University, Providence, RI), *UAS-tkv*^{Q253D} (constitutively active) by Michael O'Connor (University of Minnesota, Minneapolis, MN), *tkv*⁴ by Nick Baker (Albert Einstein College of Medicine, Bronx, NY), *tkv*⁸ and *Mad*¹² by Laurel Raftery (Massachusetts General Hospital, Charlestown, MA), *shg*^{R69} by Richard Carthew (Northwestern University, Evanston, IL), *UAS-CD4:GFP*, *hs-FLP*; *Gal80*, *FRT40* by Andreas Bergmann (UT M.D. Anderson Cancer Center, Houston, TX), *scalloped-gal4* by Sarah Bray (University of Cambridge, Cambridge, UK)

and *rst*^{CT} by Karl Fischbach (Albert-Ludwigs-Universitaet, Freiburg, Germany). The *rst*³ allele was described previously (Tanenbaum et al., 2000). We constructed two independent *UAS-tkv-IR* (*tkv-IR*) lines by subcloning inverted repeats into pGEM-WIZ (Bao and Cagan, 2006): *tkv-IR1*, which targets part of the 5' half; and *tkv-IR2*, directed towards the 3' half of the *tkv* mRNA.

RNA extraction from pupal retinas and RT-PCR

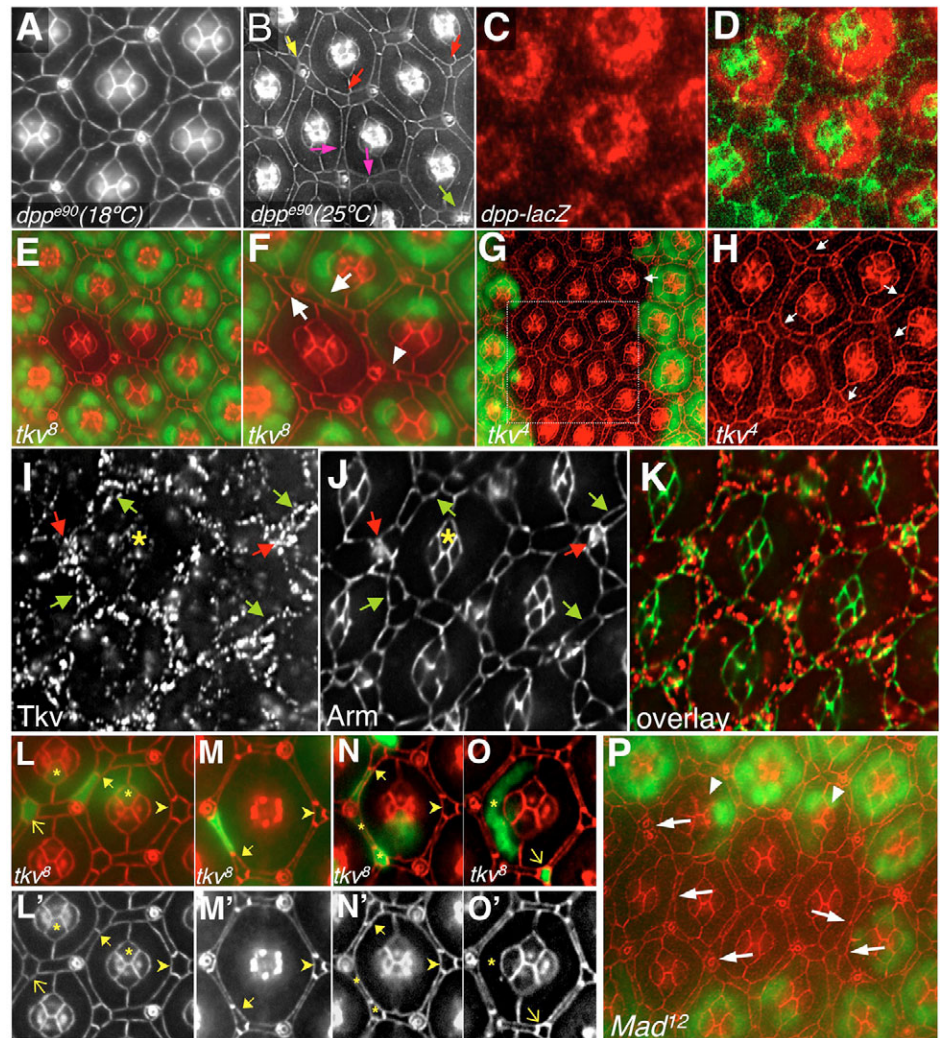
Retinal-brain complexes from 10-15 pupae per genotype were dissected under RNase-free conditions. Retinas were separated from the brains using a sterile surgical razor blade and subject to RNA extraction using TRIzol (Invitrogen). The RNA was then used to detect *tkv* transcript levels by RT-PCR.

Temperature-sensitive experiments and clonal analysis

To examine the role of *dpp* in pupal patterning, *dpp*^{e90} homozygous flies were kept at 18°C (permissive temperature), pupae were selected at 42 hours [equivalent to approximately 20 hours after puparium formation (APF)] at 25°C, and then switched to 25°C (restrictive temperature) for 22 hours and dissected. As a control, *dpp*^{e90} pupae were held at the permissive temperature until dissection.

Fig. 2. Dpp signaling regulates IPC patterning in the *Drosophila* pupal retina.

All retinas are 42 hours APF and apical membrane profiles are highlighted with anti-Armadillo, except where noted. (A,B) Retinas from animals carrying the temperature-sensitive allele *dpp^{ts90}* raised at the permissive (A) or non-permissive (B) temperature. (B) Mutant retinas show: abnormal IPC:IPC contacts (pink arrows); 3°s, which failed to establish a correct position as a vertex of the hexagon (red arrows); 2°/3°s abnormally arranged around sensory bristles (yellow arrow); and aberrant bristle-bristle contacts (green arrow). (C,D) *dpp* was expressed in primary pigment cells at 26 hours APF (red). (E,F) *tkv⁸* clone marked by the absence of nuclear GFP (green). (F) Magnification of the clonal tissue in E. Arrows in F point to typical 2°/3°s patterning defects; arrowhead points to ectopic 2°/3°. (G,H) *tkv⁴* clone (cells marked as in E,F). H is a magnified view of the boxed region in G. Arrows in H indicate examples of typical 2°/3° defects. Arrow in G indicates a rare cone cell defect (five versus four). (I-K) Tkv localization at 26 hours APF. (I) Tkv protein was found primarily at the surface of IPCs (green arrows), sensory bristles (red arrows) and at lower levels in cone cells (asterisk). (L-O') *tkv⁸* single-cell clones were marked by the presence of GFP (green). Full arrows point to cases where *tkv* mutant 2°s failed to fully expand into their proper niche, as evidenced by their shortened apical profile, while wild-type neighboring 3°s elongated to compensate. Arrowheads point to examples of the apical profile characteristic of wild-type 3°s. Asterisks in N and N' indicate how neighboring mutant cells typically show normal apical profiles. Thin arrows and asterisks in L,L',O,O' point to 3°s, cone cells and primary pigment cells, whose shape was not affected by the absence of Tkv activity. (P) A *Mad¹²* clone marked by the absence of nuclear GFP (green). The arrows point to a subset of the 2°/3°s patterning defects and aberrant bristle-bristle contacts within the clone. Arrowheads indicate rare, abnormal cone cell clusters.



Whole eye mutants for *punt^P* were generated using the EGUF system (Stowers and Schwarz, 1999) using pupae with the following genotypes: (1) control: *ey-Gal4, UAS-FLP; FRT82B, cl, GMR-hid/FRT82B lacZ*; (2) experimental: *ey-Gal4, UAS-FLP; FRT82B, cl, GMR-hid/FRT82B punt^P*.

Discrete *tkv* and *Mad* mutant clones were generated by FRT-mediated recombination (Golic and Lindquist, 1989; Xu and Rubin, 1993). Recombinant clones were induced by heat shocking larvae 72 hours after egg laying for 1 hour at 37°C. For MARCM *tkv⁸* clones (Lee and Luo, 1999), third instar larvae were heat shocked for 30 minutes at 37°C. Clonal analysis was performed in pupae with the following genotypes: (1) *tkv⁸* clones: *hs-FLP; tkv⁸ FRT40/ub-nGFP FRT40*; (2) *tkv⁴* clones: *hs-FLP; tkv⁴ FRT40/ub-nGFP FRT40*; (3) *Mad¹²* clones: *hs-FLP; Mad¹² FRT40/ub-nGFP FRT40*; (4) *tkv⁸* single-cell clones: *UAS-CD4:GFP, hs-FLP; Gal80, FRT40/tkv⁸ FRT40; tubulin-Gal4/+*.

Immunostaining and imaging

Pupal retinas and wings were processed as described previously (Bao and Cagan, 2005; Blair and Ralston, 1997). Antibodies used were: mouse anti-Armadillo and rat anti-DE-cadherin (1:3 and 1:10, respectively, from the Developmental Studies Hybridoma Bank at the University of Iowa); mouse anti-Rst (1:50, from Karl Fischbach); rabbit anti-β-galactosidase (1:2000,

Cappel); rabbit anti-luminal Tkv (1:10, from Marcos Gonzalez-Gaitán, Max Planck Institute, Dresden, Germany); rabbit anti-GFP (1:2000, from Pam Silver, Harvard Medical School, Boston, MA); mouse anti-Rho1 and mouse anti-Tubulin (E7) (1:10, from the Developmental Studies Hybridoma Bank at the University of Iowa); mouse anti-Srf (1:50, from M. Gilman, Cold Spring Harbor Laboratory, NY); and rabbit anti-p-Mad (1:5000, from Tetsuya Tabata, University of Tokyo, Tokyo, Japan). Alexa488- and Alexa568-conjugated secondary antibodies were used (1:1000, Molecular Probes).

Whole-mount in situ hybridization was carried out as previously described (Bao and Cagan, 2005). Cell surface-associated Tkv was visualized with an antibody directed against the extracellular domain of Tkv (Kruse et al., 2004); dissected tissue was incubated with the antibody at 4°C prior to fixation (Strigini and Cohen, 2000). The antibody did not work when added after fixation and permeabilization.

Images were captured with a Zeiss Axiophot microscope equipped with a Quantix CCD camera (Photometrics) and Image Pro Plus software. Images were processed with Photoshop (Adobe). Confocal xzy projections were taken on a Leica confocal microscope using the Leica confocal software. For scanning electron microscopy, flies were prepared by ethanol fixation followed by critical-point drying. Images were captured using a Hitachi S-2600H scanning electron microscope.

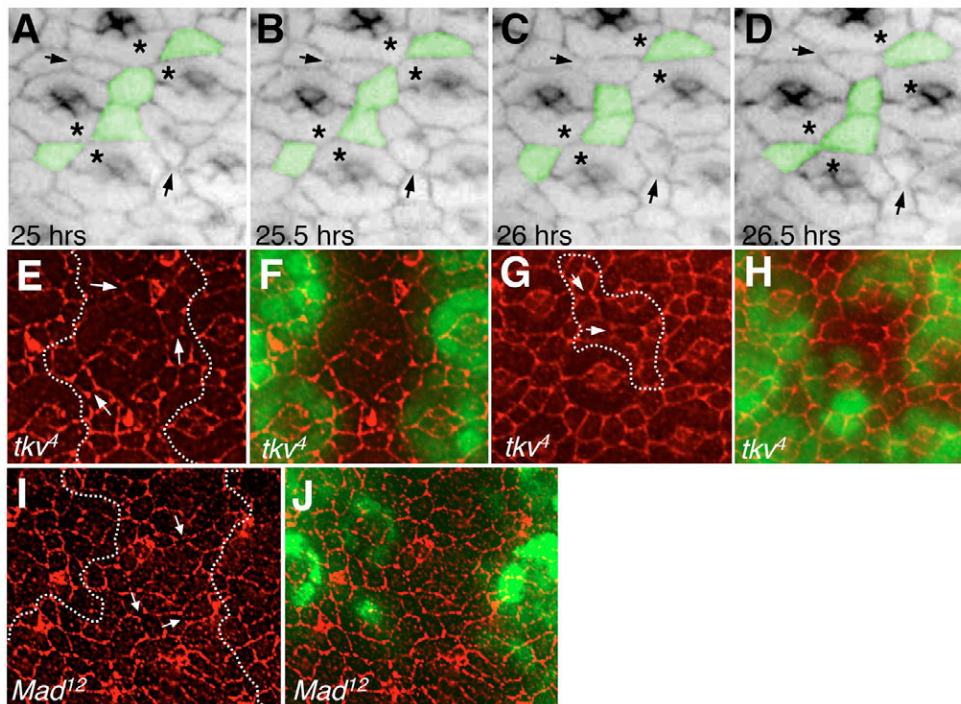


Fig. 3. Aberrant IPC morphogenesis and unstable IPC-IPC junctions in retinas with reduced Dpp signaling. (A-D) In vivo imaging of a *Drosophila* retina with reduced Tkv activity [*GMR-gal4/+; UAS- α Catenin-GFP/tkv⁸; UAS-tkv-IR1(2X)/UAS-tkv-IR1(2X)*] (see also Movie 2 in the supplementary material). Hours APF are indicated. Pseudo colored in green are examples of IPCs that transiently lose their apical contact and leave primary pigment cells from adjacent ommatidia (asterisks) in direct, aberrant contact. Arrows point to examples of adherens junctions, which disappear as the IPC-IPC surface contact decreases. (E-J) Clones of *tkv^A* (E-H) and *Mad¹²* (I,J) dissected at 25 hours APF and stained with anti-DE-cadherin (red; E-J). Clonal tissue is marked by the absence of GFP (green; F,H,I) or outlined by dotted lines (E,G,I). Arrows point to IPC-IPC junctions with abnormally low-to-undetectable DE-cadherin staining.

In vivo visualization

In vivo imaging was performed in pupae with the following genotypes: (1) experimental: *GMR-gal4/+; UAS- α Catenin-GFP/tkv⁸; UAS-tkv-IR1(2X)/UAS-tkv-IR1(2X)* (Fig. 3; see Movie 2 in the supplementary material); (2) control: *GMR-gal4/+; UAS- α Catenin-GFP/+* (see Movie 1 in the supplementary material). Larvae were allowed to pupate at 29°C (0 hours APF). Pupae were then collected and staged at 25°C until 25 hours APF. The pupal case was removed in the head area and the animal was placed with the eye region pressed against a coverslip. Temperature and humidity were controlled and images were captured every 15 minutes.

RESULTS

The *Drosophila* retina develops from a monolayer epithelium composed of approximately 750 unit eyes, or ‘ommatidia’ (Fig. 1E,F,K,L). Each ommatidium has a core composed of four cone cells (Fig. 1F), two primary pigment cells (1°; Fig. 1F) and eight underlying photoreceptors. Secondary and tertiary pigment cells (2° and 3°, respectively; Fig. 1F) interweave between the ommatidial cores to form a precise honeycomb pattern (Cagan and Ready, 1989b) with three sensory bristles at alternating vertices. We will collectively refer to mature interommatidial cells as ‘2°/3°s’. This precise interommatidial pattern emerges 18–31 hours after puparium formation (APF) (Fig. 1A,C,D). ‘Interommatidial precursor cells’ (IPCs) are precursors to the 2°/3°s (Fig. 1A,B). They undergo dynamic cell rearrangements that are necessary to direct them into a precise 2°/3° hexagonal array (Fig. 1E,F). In an early-stage pupal retina, IPCs are initially arranged in double and triple rows between ommatidia (Fig. 1A,B). These cells then rearrange into single-cell rows (Fig. 1C–E). Throughout this patterning process, excess IPCs are eliminated by programmed cell death (Rusconi et al., 2000).

At least two adhesion systems are involved in directing IPC patterning: Hbs-Rst and DE-cadherin. Reducing the activity of Rst or Hbs led to a failure of IPC cell movement (Bao and Cagan, 2005; Reiter et al., 1996). Rst is found primarily at the junction between IPCs and 1°s and is excluded from IPC:IPC junctions (Reiter et al., 1996; Bao and Cagan, 2005) (Fig. 1G,H). Rst regulates patterning

of the pupal retina through selective heterophilic adhesion with Hbs and formation of DE-cadherin-rich adherens junctions (Bao and Cagan, 2005).

The relationship between these adhesion systems is complex. Experiments that altered the activity or expression of DE-cadherin suggested that DE-cadherin is required to drive Rst to the adherens junctions (Grzeschik and Knust, 2005). Conversely, overexpression of Rst led to an increase in DE-cadherin (Bao and Cagan, 2005). Additionally, we observed that mutations in *rst* disrupted the dynamic localization of IPC:IPC adherens junctions (Fig. 1I,J). Normally, the adherens junctions between IPCs are strongly reduced by 31 hours APF (Bao and Cagan, 2005; Grzeschik and Knust, 2005) (Fig. 1, compare D with A,C,E). Retinas from *rst* mutants failed to clear these junctions (Fig. 1I,J). Consistent with the previous result, taking away one functional copy of *shotgun* (*shg*), the gene encoding DE-cadherin, significantly suppressed the rough eye phenotype of *rst* mutants (Fig. 1K–P). Together, these data emphasize the complexity of the relationship and epistatic order between Hbs-Rst and components of the adherens junctions. To better understand this relationship, we examined other potential regulators of IPC patterning.

Dpp is required for patterning the pupal retina

To explore the role of Dpp specifically in the pupa, we utilized the temperature-sensitive allele *dpp^{e90}* (Fig. 2A,B). Genotypically *dpp^{e90}* animals were kept either at 18°C (Fig. 2A) or switched to the non-permissive temperature at 20 hours APF just prior to the stage of cell rearrangements in the pupal retina (Fig. 2B). Downregulation of *dpp* resulted in an abnormal hexagonal pattern due to disruption in the shape and patterning of 2°/3°s as assessed with the junctional marker Armadillo (Fig. 2B). The main 2°/3° defects we observed included: (1) abnormal IPC:IPC contacts; (2) a failure of 3°s to establish a correct position within the vertex of the hexagon; (3) 2°/3°s that were abnormally arranged around sensory bristles; and (4) misplaced bristle organs. As previously reported (Wharton et al., 1996), there was some

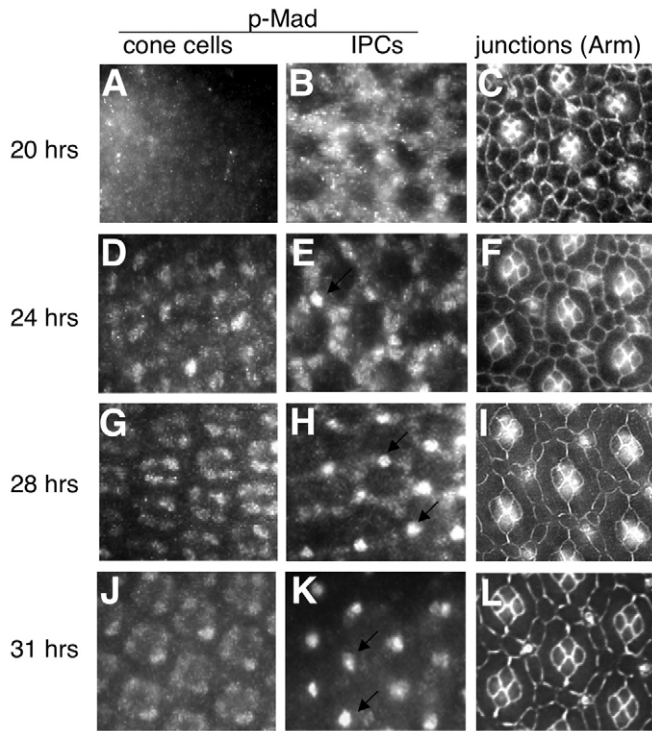


Fig. 4. Dynamic Dpp signaling activity in the *Drosophila* pupal retina. (A-L) Dpp pathway activity is visualized in pupal retinas at different developmental stages using anti-p-Mad antibody. (A,D,G,J) p-Mad staining at the level of the cone cell nuclei; (B,E,H,K) p-Mad staining at the level of the IPC nuclei. (C,F,I,L) The maturing IPC pattern from age-matched retinas as development proceeded; cell membranes were stained with anti-Armadillo antibody. Time refers to hours APF. Arrows in E,H,K point to nuclear p-Mad in the sensory bristles.

variability in the penetrance and expressivity of the phenotype (for quantification of defects, see Table S1 in the supplementary material).

Our previous cell ablation experiments indicated that primary pigment cells act locally to direct patterning of neighboring IPCs (Miller and Cagan, 1998). A *dpp-lacZ* reporter line indicated that Dpp was expressed exclusively in all primary pigment cells at the time of active IPC morphogenesis (Fig. 2C,D). Together, these results suggest a model in which Dpp is provided by primaries to regulate patterning of neighboring IPCs.

Type I and Type II receptors are required for patterning the pupal retina

We next utilized the *hs-FLP/FRT* system (Golic and Lindquist, 1989; Xu and Rubin, 1993) to generate clonal patches bearing reduced activity of the Type I receptor *Tkv*, or the Type II receptor *Punt*, in the pupal retina. Clones of the null allele *tkv⁸* (Nellen et al., 1994) (Fig. 2E,F) or of the hypomorphic allele *tkv⁴* (Penton et al., 1994) (Fig. 2G,H), led to defects in the shape and patterning of $2^\circ/3^\circ$ s similar to those observed when Dpp activity was reduced (Fig. 2B). Additionally, we observed cases of ectopic $2^\circ/3^\circ$ s. Whole eye clones of genotypically *punt^P* tissue showed patterning defects that were similar to, but milder than, those seen in *dpp* and *tkv* mutants, and discrete *punt^P* clones showed infrequent defects (data not shown). Weaker phenotypes of *punt* versus *tkv* clones have

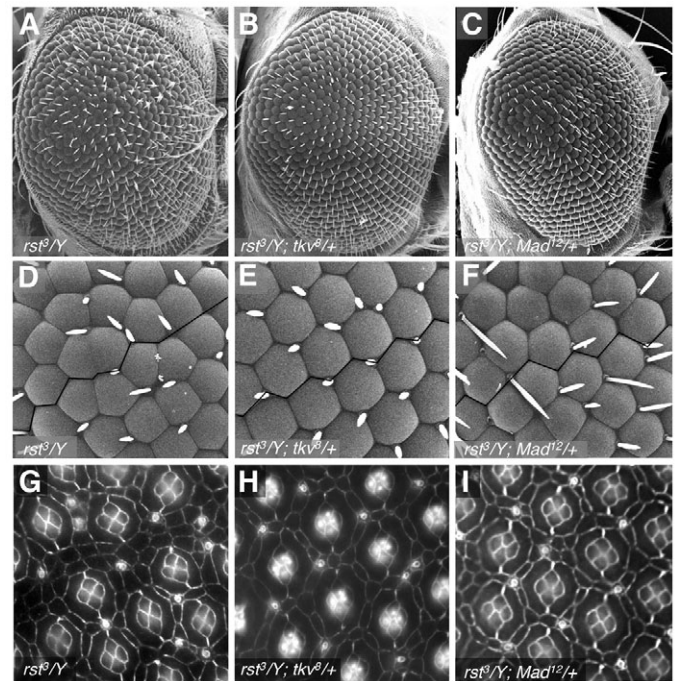


Fig. 5. Dpp signaling and *Rst* work in opposition in the *Drosophila* pupal retina. Genetic interactions between *dpp* pathway components and *rst*. (A-F) Scanning electron micrographs of adult eyes taken at (A-C) 180 \times and (D-F) 800 \times . (G-I) 42 hours APF retinas stained with anti-Armadillo antibody. Removing a single functional genomic copy of *tkv* (B,E,H) or *Mad* (C,F,I) suppressed the rough eye phenotype of *rst³* mutants (A,D,G).

previously been documented in the wing disc (Burke and Basler, 1996), perhaps reflecting the hypomorphic nature of available *punt* alleles. Clones of null alleles of *wit* or *sax*, which encode alternative Type II and Type I receptors, respectively, gave no mutant phenotype (data not shown).

In situ hybridization experiments and a *tkv-lacZ* reporter indicated that *tkv* and *punt* transcripts were expressed in IPCs and cone cells during stages of IPC patterning (data not shown). Antibody staining (Kruse et al., 2004) detected surface-exposed *Tkv* in puncta along the surface of IPCs, cone cells and sensory bristles (Fig. 2I-K). Therefore, Dpp and its receptors *Tkv* and *Punt* are mainly expressed in complementary cell types, supporting a model in which Dpp from primary pigment cells binds to *Tkv* and *Punt* in the IPCs to regulate their shape and positioning.

Tkv regulates cell shape autonomously in the pupal retina

To more closely explore Dpp pathway activity, we generated single-cell clones of *tkv⁸* using the MARCM system (Lee and Luo, 1999). Each cell within the pupal retina has a stereotyped apical profile (Fig. 1E,F), and deviations are readily observed. The apical profiles of isolated, genotypically *tkv⁸* cells failed to stretch out and fill their proper niche within the hexagon (65%, $n=30$; Fig. 2L-N). Instead, their shortened profile was typically compensated for by a wild-type neighbor, which expanded to fill the unoccupied space (Fig. 2L-N).

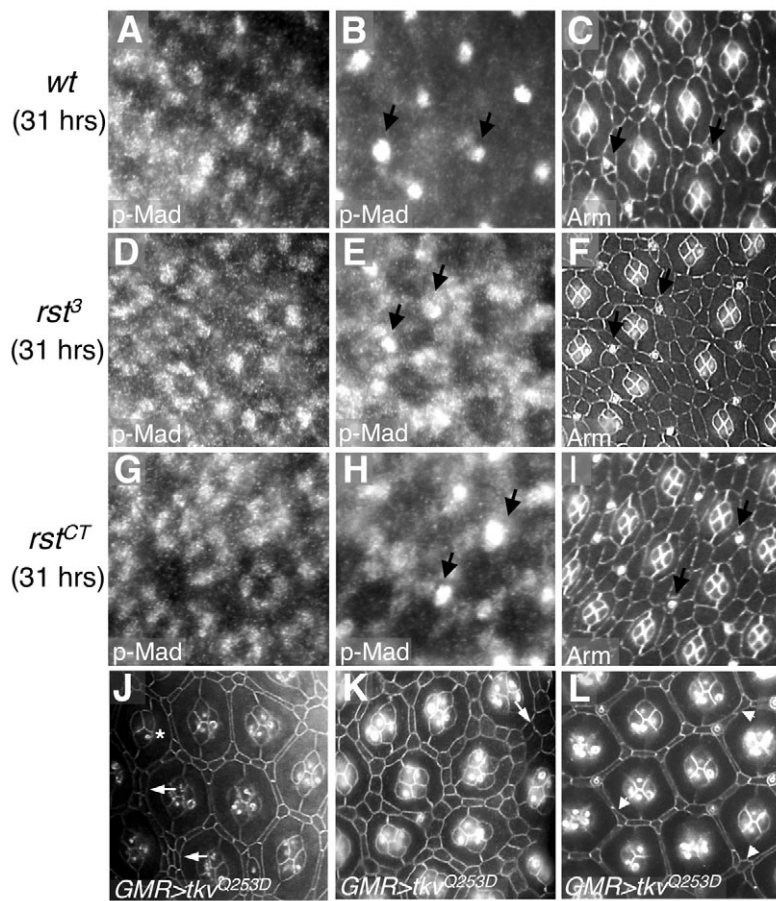


Fig. 6. Rst function is required to achieve proper Dpp signaling activity in the *Drosophila* pupal retina. (A,B,D,E,G,H) p-Mad staining in 31 hours APF retinas from wild-type (A,B) and *rst* mutant animals (D,E,G,H). (C,F,I) Anti-Armadillo staining of the retinas to visualize the cells. Abnormally high levels of p-Mad were observed in genotypically *rst* IPCs (E,H; compare with B). Arrows point to sensory bristles. (J-L) 42 hours APF retinas overexpressing activated TkV (*GMR>tkv^{Q253D}*). The predominant phenotype observed (J,K, arrows point to examples of multi-layered IPCs), except for the most-anterior part of the retina which showed only minor IPC patterning defects (L, arrowheads).

Interestingly, when two *tkv⁸* cells were juxtaposed they typically exhibited normal apical profiles (88%, $n=40$; Fig. 2N), indicating that relative levels of Dpp signaling between neighboring cells determine cell shape.

Strikingly, the effects of losing *tkv* activity was specific to 2°s. No cell profile defects were observed when *tkv* activity was reduced in cone cells, primaries or 3°s (Fig. 2L,O). These data suggest that the defects in 3° positioning frequently observed in larger *dpp* and *tkv* clones is an indirect consequence of the patterning defects in the neighboring 2°s, and that Dpp activity is required autonomously in 2°s to direct proper overall hexagonal patterning.

IPC patterning defects are due to a failure in proper cell movement and morphogenesis

To better assess the role of Dpp signaling we used *in vivo* imaging analysis (Monserrate and Baker Brachmann, 2007; Vidal et al., 2006) to observe morphogenesis progression between 25 and 30 hours APF (Fig. 3A-D; see Movies 1, 2 in the supplementary material). To facilitate these studies, we generated two transgenic lines that reduced *tkv* activity through RNA interference: *tkv-IR1* targets within the 5' region and *tkv-IR2* within the 3' region of the *tkv* mRNA. The phenotypes of the two lines were identical, except that the phenotypes observed in flies with *tkv-IR1* were consistently stronger than in those with *tkv-IR2* (data not shown). The following observations further validated the specificity of our *tkv-IR* constructs: (1) expressing either *tkv-IR* line with the wing pouch driver *scalloped-gal4* (*sd>tkv-IR*) or the eye driver (*GMR>tkv-IR*) phenocopied *dpp* and *tkv* loss-of-function phenotypes (Terracol and Lengyel, 1994) (Fig. 2); (2) removing a functional genomic copy of

tkv significantly enhanced the *sd>tkv-IR2* phenotype; and (3) wing imaginal discs from *sd>tkv-IR* larvae showed significant downregulation of the levels of the phosphorylated form of Mad (p-Mad) in the wing pouch region (data not shown).

To visualize *tkv* development in living tissues, multiple copies of the transgene were targeted specifically to the eye (*GMR>tkv-IR*). The most common and striking phenotype observed within developing *GMR>tkv-IR* eyes was a failure to maintain stable IPC:IPC contacts (see Movie 2 in the supplementary material). Neighbors established contact but then broke apart leading to direct contact between primary pigment cells from adjacent ommatidia (Fig. 3A-D; see Movie 2 in the supplementary material) in a manner that was not seen in control retinas (see Movie 1 in the supplementary material). This failure to maintain contact was briefly preceded by dissolution of the visible IPC:IPC adherens junction (Fig. 3A-D; see Movie 2 in the supplementary material). Further, these abnormal IPC:IPC interactions were accompanied by aberrant changes in cell shape that included abnormal expansions and/or reductions of their apical profiles. IPC:IPC contacts were often later reformed, reducing the severity of the final phenotype (Fig. 3D). These aberrant phenotypes repeated themselves across the retina over the time of visualization (see Movie 2 in the supplementary material). They were consistent with the abnormal IPC:IPC contacts observed in *dpp*, *tkv* and *Mad* mutants (Fig. 2), and in 25 hours APF *tkv* and *Mad* mutant clones (Fig. 3E-J), which frequently exhibited premature clearing of the IPC-IPC DE-cadherin junctions suggestive of junction dissolution. RT-PCR results indicated that *shg* expression levels were not detectably

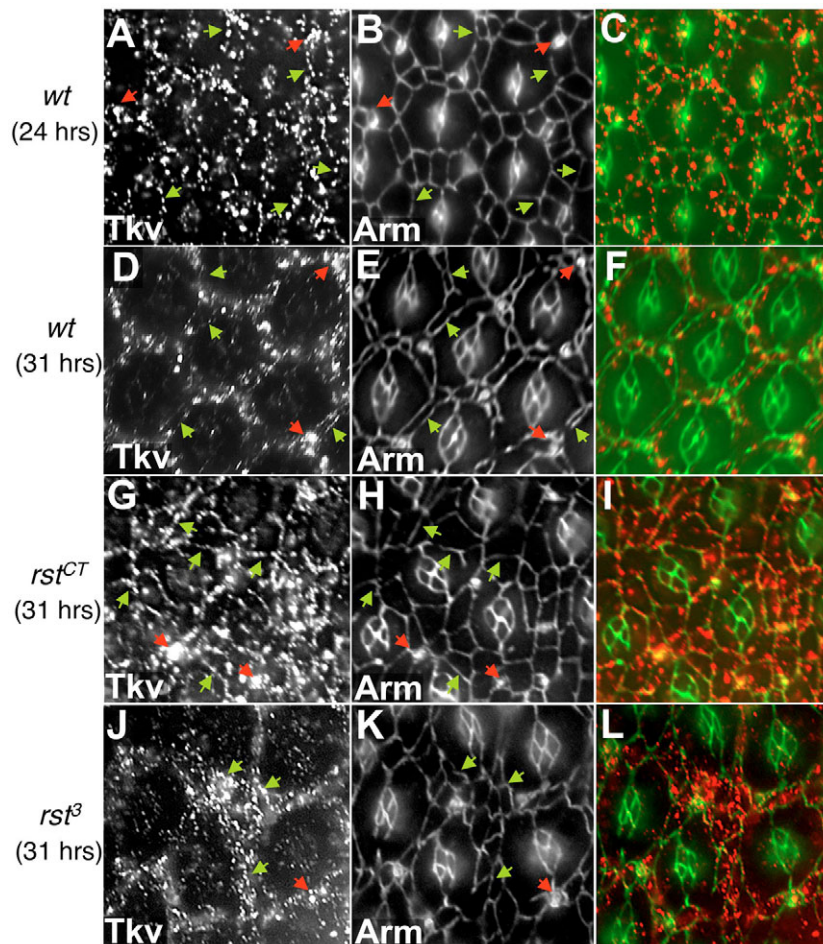


Fig. 7. *Rst* function is required to regulate cell surface levels of Tkv in the *Drosophila* pupal retina. (A,D,G,J) Levels of cell-surface Tkv in pupal retinas. (B,E,H,K) Matched anti-Armadillo staining to visualize cells. (C,F,I,L) Overlay. Genotype and developmental stages (hours APF) are indicated at the left; wt, wild type. Green arrows indicate cell membranes where Tkv is localized. Red arrows indicate sensory bristles, which accumulated high levels of Tkv. Mutants in *rst* failed to downregulate surface Tkv protein in IPCs (G,J; compare with D).

altered (data not shown). Together, these results indicate that Dpp signaling is required to maintain normal IPC:IPC contacts, junction stability and cell shape during morphogenesis of the pupal retina.

Dpp signaling activity is tightly regulated in IPCs

Activation of Tkv leads to phosphorylation of the conserved transcription factor Mad, promoting multimerization and transcriptional activity (Newfeld et al., 1997; Sekelsky et al., 1995). Clones of the null allele *Mad*¹² led to 2°/3° patterning defects that mimicked those observed in *dpp* and *tkv* mutants (Fig. 2P). These results indicate that Dpp-dependent IPC patterning in the pupal retina requires classical pathway signaling that includes Mad activity.

Nuclear levels of p-Mad serve as a readout of Dpp signal transduction activity (Tanimoto et al., 2000). Our data suggest that, in the context of the pupal retina, primary pigment cells might act as a source of Dpp that is then provided to surrounding cells to influence their patterning. To further test this hypothesis, we utilized an antibody specific for p-Mad (see Fig. S1 in the supplementary material) to identify the cells that exhibit active Dpp signaling. Fig. 4 presents a time course of Dpp pathway activity in the pupal retina. Consistent with our ligand/receptor expression pattern and phenotypic results, p-Mad was detected in cone cells, IPCs and sensory bristles but not in primary pigment cells. IPCs contained high levels of p-Mad during the period of maximal IPC patterning (20-26 hours APF; Fig. 4B,E; data not shown). Subsequently, IPC levels decreased at 28 hours APF and were undetectable by 31 hours

APF (Fig. 4H,K). Mad activity in cone cells and bristles was evident after 20 hours APF and remained unchanged through all stages examined (Fig. 4A,D,G,J); we did not observe consistent defects when *dpp* activity was reduced in these cells and the functional relevance of Mad activation in either cell type is unclear.

Consistent with our phenotypic analysis, therefore, IPCs exhibited a dynamic pattern of Dpp activity that was highest at the time of active cell rearrangement and was then rapidly downregulated.

Dpp signaling works in opposition to *Rst* during IPC patterning

No significant genetic modifier interactions were observed (data not shown) between components of the Dpp pathway and *Notch*^{fa-g}, *Egfr*^{El} or *wingless*^{cx4} which were previously implicated in IPC patterning (Cagan and Ready, 1989a; Cordero et al., 2004; Freeman, 1996; Miller and Cagan, 1998).

The results from our phenotypic analysis and in situ visualization indicated that mutations in the Dpp pathway affected cell shape, cell movements and cell-cell contacts, making *Rst* an attractive candidate to mediate Dpp function during IPC patterning. Consistent with this view, removing one genomic copy of *tkv* strongly suppressed the rough eye phenotype of *rst*³ mutants; removing one copy of *Mad* also produced a milder but significant suppression (Fig. 5). Independent *tkv* and *Mad* alleles gave similar results (data not shown). These results suggest that *Rst* and Dpp are functionally linked and that they act in opposition during patterning of the pupal retina.

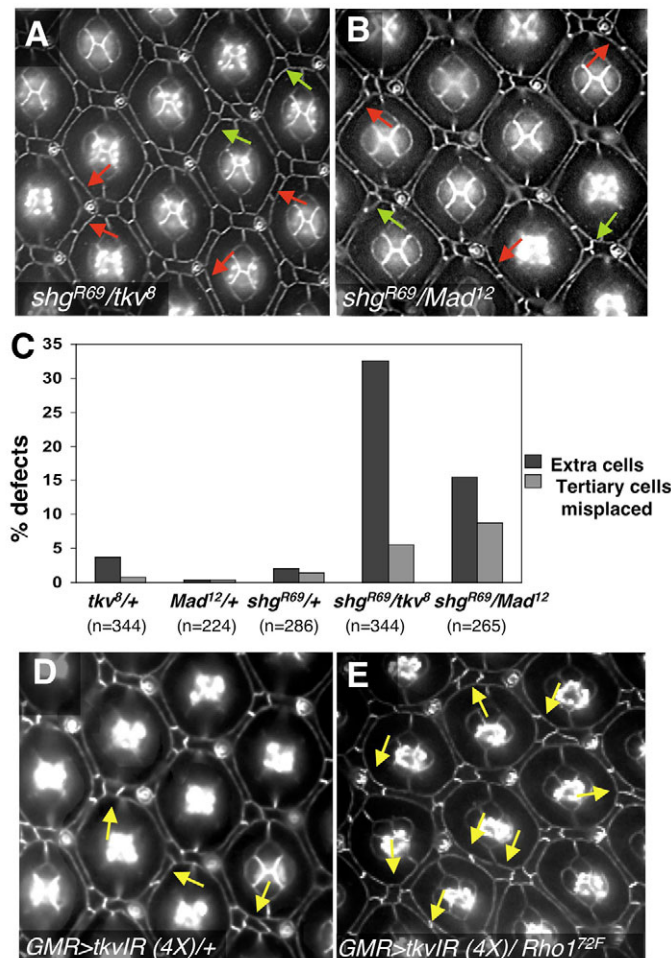


Fig. 8. Dpp signaling works together with DE-cadherin and Rho1 to pattern the *Drosophila* pupal retina.

(A, B) Removing one genomic copy of *shg* and either *tkv* (A) or *Mad* (B) resulted in a significant increase in the incidence of ectopic 2°/3°s (red arrows) and misplaced 3°s (green arrows). Most misplaced 3°s were accompanied by an extra cell; conversely, we observed many cases of extra cells without a misplaced tertiary (red arrow). (C) Quantification of 2°/3°s defects. The data are expressed as the percentage of ommatidia with defects out of the total number of ommatidia counted. *n*=number of ommatidia counted from at least four different animals for each genotype. (D, E) Removing one genomic copy of *Rho1* in retinas expressing four copies of *tkv-IR* enhanced the frequency and severity of IPC patterning defects (arrows, compare D with E). The full genotypes were: (D) *GMR-gal4/+; UAS-tkv-IR2(2X)/+; UAS-tkv-IR1(2X)/+*; (E) *GMR-gal4/+; UAS-tkv-IR2(2X)/Rho1^{72F}; UAS-tkv-IR1(2X)/+*.

Next, we assessed the epistatic relationship between Rst and Dpp signaling. We found no changes in Rst protein levels or localization when Dpp pathway activity was reduced (see Fig. S2A, B in the supplementary material). By contrast, we observed a striking failure of genotypically *rst³* and *rst^{CT}* pupal eyes to properly downregulate Dpp pathway activity: the normal reduction in p-Mad within IPCs at 28 hours APF did not occur (*n*=7 retinas for each genotype; Fig. 6, compare E, H with B). We could not unambiguously compare early-stage p-Mad in IPCs owing to its normally high levels. Levels of p-Mad in cone cells and bristle organs were not affected, providing a further, internal control. Together, these data indicate that Rst acts as a negative

regulator of Dpp signaling activity as IPC patterning in the pupal retina progresses. In this scheme, loss of Rst activity leads to heightened Dpp pathway activity that in turn leads to defects in IPC patterning. Further supporting this model, expression of activated Tkv (*GMR>tkv^{Q253D}*) in the developing eye led to a robust and fairly specific phenotype in the IPCs that at least partially phenocopied the defects observed in *rst* mutant retinas (Fig. 6J-L).

We next sought to determine at what level Rst acts to downregulate Dpp signaling. Pathway activation has been shown to directly correlate with the levels of surface-associated Tkv (Jekely and Rorth, 2003). Indeed, we observed that Tkv surface presence correlated with p-Mad levels: the levels of IPC surface-associated Tkv were highest during the period of maximal IPC patterning and decreased subsequently (Fig. 7A-F). In *rst* mutant retinas, surface Tkv protein failed to decrease as patterning progressed (Fig. 7G-L). Mutant retinas for the null allele *rst^{CT}* showed uniform IPC patterning defects accompanied by general upregulation of cell surface Tkv (Fig. 7G-I). Mutants for the hypomorphic allele *rst³*, which is subject to position effect variegation (PEV) (Tanenbaum et al., 2000), showed highest levels of cell surface Tkv in the areas with strongest IPC patterning defects (Fig. 7J-L). Co-immunoprecipitation experiments from tissue failed to detect physical interaction between Rst and Tkv (not shown), suggesting that the regulation of Tkv protein by Rst might require additional intermediaries. Furthermore, we saw no change in the levels of *tkv* transcript in control versus *rst* mutant retinas (see Fig. S3 in the supplementary material). Together, these data suggest that Rst opposes Dpp signaling by regulating the levels of cell surface-associated Tkv protein.

Dpp signaling works in conjunction with DE-cadherin and Rho1 during IPC patterning

DE-cadherin, along with α -catenin and Armadillo (also known as β -catenin), constitute the core components of the adherens junctions and can help mediate cell-cell adhesion and cell rearrangement (Peifer and Wieschaus, 1990; Tepass et al., 1996; Uemura et al., 1996). Notably, we identified defects in adherens junction coherence and function when Dpp pathway activity was reduced (Fig. 3; see Movie 2 in the supplementary material). To further explore the relationship between Dpp and DE-cadherin, we tested null alleles of components of the Dpp pathway in trans to a null allele of *shotgun* (*shg^{R69}*). Control retinas heterozygous for *tkv⁸*, *Mad¹²* or *shg^{R69}* were essentially wild type except for infrequent 2°/3° defects (Fig. 8C). The trans heterozygous combinations *tkv⁸/shg^{R69}* and *Mad¹²/shg^{R69}* led to a significant increase in the percentage of 2°/3° defects (Fig. 8A-C). This genetic enhancement is consistent with our observation that DE-cadherin and Rst act in opposition (Fig. 11-P) and, together with the junction phenotype observed in *tkv* and *Mad* mutant retinas (Fig. 3; see Movie 2 in the supplementary material), further supports a model in which Dpp signaling regulates IPC patterning at least in part by regulating DE-cadherin-mediated cell adhesion in the retina.

Members of the Rho family of small GTPases – Rac, Rho and Cdc42 – have been linked to regulation of the actin cytoskeleton in diverse organisms (Van Aelst and D'Souza-Schorey, 1997). Rho1 interacts with DE-cadherin-associated proteins and regulates cadherin-based cell junctions (Magie et al., 2002). Using a lower copy number of *tkv-IR* (*GMR>tkv-IR*) to direct a mild IPC patterning phenotype (Fig. 8D), we found that removing one copy of *Rho1* (*Rho1^{72F}*) led to significant enhancement of IPC patterning defects (Fig. 8E; 60% of at least 15 retinas scored). This functional relationship is not due to regulation of *Rho1* expression by Tkv

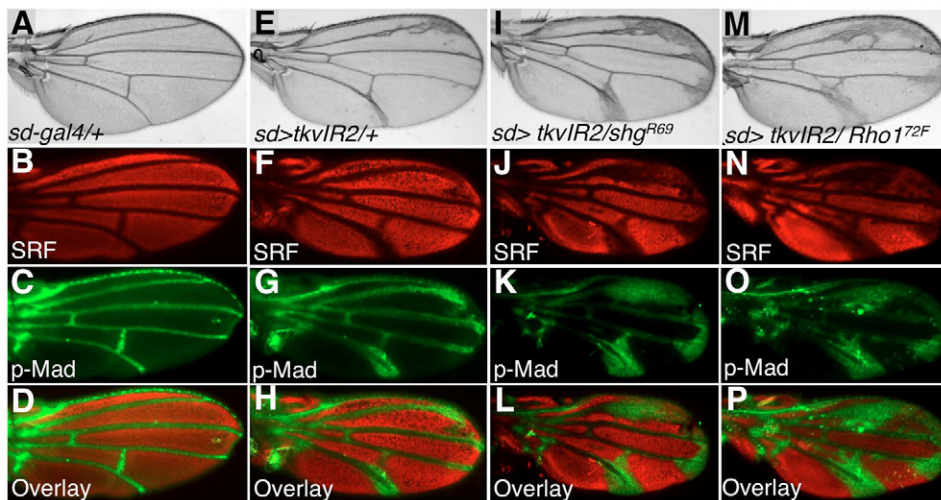


Fig. 9. Dpp signaling works together with DE-Cadherin and Rho1 during *Drosophila* wing patterning. (A,E,I,M) Adult wings. (B-D,F-H,J-L,N-P) 24-36 hours APF pupal wings were dissected and stained with anti-Srf (red; B,F,J,N) and anti-p-Mad (green; C,G,K,O) antibodies to label intervein and vein cells, respectively; (D,H,L,P) overlay. Removing one genomic copy of *shg* (I-L) or *Rho1* (M-P) dramatically enhanced the cell fate defects of *sd>tkv-IR* wings (E-H). The full genotypes were: (A-D) *sd-gal4/+*; (E-H) *sd-gal4, UAS-tkvIR2/+*; *+/+*; (I-L) *sd-gal4, UAS-tkvIR2/+*; *shg^{R69}/+*; (M-P) *sd-gal4, UAS-tkvIR2/+*; *Rho1^{72F}/+*.

activity, as Rho1 protein or transcript levels were not altered in a *tkv* mutant background (see Fig. S2C,D in the supplementary material; data not shown). Conversely, loss of Rho1 (or DE-cadherin) activity did not alter p-Mad levels (data not shown). This effect was specific for *Rho1* because removing one copy of *Cdc42* or a *Rac1*, *Rac2*, *Mtl* triple heterozygote did not detectably modify the *tkv-IR* phenotype (not shown). Together, these results indicate that Dpp signaling cooperates with DE-cadherin and Rho1 to regulate dynamic IPC morphogenesis, movement and cell-cell contacts during morphogenesis of the pupal retina.

Dpp signaling works in conjunction with DE-cadherin and Rho1 during wing patterning

Dpp signaling is a well-known regulator of the cell fate decisions involved in the formation of wing veins (O'Connor et al., 2006). Driving *tkv-IR* using the wing pouch driver *scalloped-gal4* (*sd>tkv-IR*) yielded a classical *tkv* loss-of-function wing vein phenotype (Terracol and Lengyel, 1994) that included thickening of wing veins L2 and L5 and of the intersection between L5 and the posterior cross vein (Fig. 9E-H; data not shown). Removing one genomic copy of either *shg* (Fig. 9I-L) or *Rho1* (Fig. 9M-P) significantly enhanced these wing vein phenotypes to levels comparable to those observed when removing one genomic copy of *tkv* (data not shown). These phenotypes were a result of defects in cell fate determination as assessed with molecular markers specific for vein and intervein cells (Fig. 9). As in the eye, removing one copy of *Cdc42* or a *Rac1*, *Rac2*, *Mtl* triple mutant did not detectably modify the *tkv-IR* phenotype in the wing (not shown). These data suggest that the functional connection between Dpp signaling and DE-cadherin and Rho1 is not exclusive to the eye, but is also relevant to the role of this pathway during cell fate decisions in the wing.

DISCUSSION

Dpp signaling is a novel, essential regulator of IPC patterning during morphogenesis of the pupal retina

Loss of Dpp pathway activity results in a loss of epithelial integrity (Gibson and Perrimon, 2005; Shen and Dahmann, 2005), but the function of Dpp signaling during maturation of developing epithelia is not fully understood. Here, we show that reducing the activity of components of the Dpp pathway leads to abnormal IPC shape and organization within the ommatidial hexagonal pattern (Fig. 2). This

activity is linked to fine regulation of apical junction components and is required to maintain stable cell-cell contacts during cell movements within the epithelium. The expression of Dpp in primary pigment cells (Fig. 2C,D) and the segregation of its receptors to the neighboring IPCs suggest a model in which Dpp acts in the primaries to organize local IPCs through the dynamic control of apical junctions. This view is supported by the dynamic changes in p-Mad activity in the neighboring IPCs, which is highest during the stage (20-26 hours APF) when IPCs rearrangements are maximal (Fig. 4).

The role of Dpp in cellular morphogenesis during epithelial development is poorly understood. Therefore, we took advantage of the unique stereotyped pattern of the pupal retina to study cell behavior as morphogenesis progresses, focusing on events at the single-cell level. In situ visualization experiments suggest that IPCs with reduced Tkv activity are incapable of maintaining their cell-cell contacts and are subject to aberrant changes in their cell shape (Fig. 3; see Movie 2 in the supplementary material). Further emphasizing the link with cellular adhesion, this function of Dpp signaling involves DE-cadherin and Rho1 (Fig. 8), which are essential regulators of cell adhesion and cell shape (Magie et al., 2002).

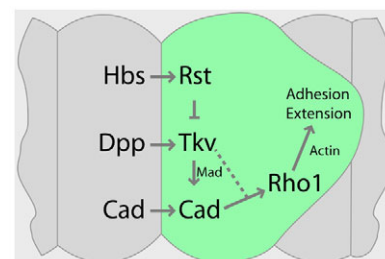


Fig. 10. Dpp signaling regulates IPC morphogenesis in the *Drosophila* pupal retina.

Dpp signaling is required to achieve correct cell-cell contacts, cell positions and cell shape during IPC patterning. This function requires the activity of DE-cadherin (Cad) and Rho1 and is opposed by Rst. This suggests that Dpp signaling acts as an intermediary or 'third force' between the Rst and DE-cadherin adhesion systems, providing a point of fine and dynamic regulation of the adherens junctions during epithelial maturation. A balanced interplay between these three systems is essential to regulate IPC patterning during morphogenesis of the pupal retina. The placement of Rho1 after DE-cadherin is speculative. See text for details.

IPCs require a balance between Rst and Dpp signaling

We provide several lines of evidence indicating that Rst is a negative regulator of Dpp signaling (Figs 5-7). Previous work has demonstrated that Rst directs IPC movements through selective cell adhesion: IPCs seek to maximize their Rst-mediated contacts with primaries while decreasing contacts with their neighbors (Bao and Cagan, 2005) (Fig. 1A-F). Additionally, reducing Rst activity leads to a failure of initial cell movement (D.E.L., S. Bao and R.C., unpublished). Consistent with these results, Rst activity opposes DE-cadherin-mediated cell adhesion (Fig. 1I-P). One model to account for these observations is that cells require a balance between cell movement provided by Hbs-Rst and the stability of cell-cell contacts provided by Dpp signaling. Our live visualization supports the view that reducing Dpp activity leaves cells with an imbalance, as IPCs move toward their proper positions but fail to stabilize cell-cell contacts or lock stably into their final positions (Fig. 3; see Movie 2 in the supplementary material). Furthermore, downregulation of Dpp signaling leads to unstable DE-cadherin IPC-IPC junctions (Fig. 3). Conversely, loss of *rst* results in loss of cell movements, which can be compensated by either reducing cell adhesion (Fig. 1M-P) or Dpp signaling activity (Fig. 5), again supporting the importance of maintaining a balance between the Hbs-Rst and the Dpp–DE-cadherin systems. Perhaps Dpp (and, by extension, BMP) activity is utilized in the adult for similar functions – for example, as a ‘proof-reading’ mechanism to remove aberrant cells from an epithelium.

Is Dpp signaling a general regulator of cell adhesion and cell shape?

Our results in the wing raise the interesting possibility that regulation of DE-cadherin and Rho1-dependent cell shape and cell adhesion might be a characteristic of Dpp pathway activity common to other biological systems. Similar to the pupal retina, epithelial cells in the wing disc with reduced Dpp signaling displayed abnormal morphologies and were unable to maintain their positions. In the case of the wing, these defects were manifested as viable cysts of mutant cells that were basally excluded from the epithelium (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). The mechanisms involved in such cell behaviors remain unknown. Our results suggest that the role of Dpp signaling during wing patterning also involves DE-cadherin and Rho1 (Fig. 9). Our experiments do not distinguish whether the defects in wing cell fates are a direct or a secondary effect of altered cell adhesion, although altering DE-cadherin activity by itself was not sufficient to cause such defects (data not shown). Cell adhesion and cell fate have been related previously: for example, Rho-dependent cell shape changes can influence fate decisions in stem cells (McBeath et al., 2004). Despite the commonalities observed, tissue-specific factors are likely to regulate Dpp-dependent epithelial patterning: for example, Rst does not appear to have a role in wing development, and we did not observe changes in retinal Tubulin distribution reported for the wing (Gibson and Perrimon, 2005) (see Fig. S2E-H in the supplementary material).

Dpp is the closest ortholog of vertebrate BMP2/4, and it appears to be active during cellular morphogenesis in a number of contexts including the developing vertebrate eye (Belecky-Adams et al., 2002; Furuta and Hogan, 1998). Interestingly, and similar to our observations for IPCs (Fig. 4), fiber cells in the developing vertebrate lens show high levels of p-SMAD activity during the period of cell elongation. Loss of the Type I receptor

ALK3 (also known as BMPRI A) or expression of the inhibitor noggin led to abnormal morphogenesis of these fiber cells including mispositioning and failure to elongate (Beebe et al., 2004); requirements for E-cadherin (also known as cadherin 1) and RHOA function have not been explored.

Finally, Rst does regulate developmental processes other than IPC patterning. For example, Rst is expressed in retinal axons and is required for correct targeting of those axons into the larval brain lobes (Schneider et al., 1995). Interestingly, Dpp signaling also has a role in this process (Yoshida et al., 2005). We have observed genetic interactions between *rst*³ and members of the Dpp pathway in the arrangement of these descending axons (L. Wickline and R.C., unpublished), raising the intriguing possibility that the two systems act together in axon targeting as well.

Summary and future directions

Our results provide evidence to support a model in which the Dpp pathway acts as an intermediary between the Rst and DE-cadherin adhesion systems. A balanced interplay between these three systems is essential to regulate epithelial cell movements, cell shape and cell-cell contacts during morphogenesis of the pupal retina (Fig. 10).

Several questions emerge from our study. For example, our data suggest that Rst acts on Dpp signaling by regulating surface-associated Tkv. Immunoprecipitation experiments failed to identify a physical interaction between Rst and Tkv (not shown), suggesting intermediate steps remain to be identified. Also, the transcription factor Mad is required to regulate IPC patterning (Fig. 2P; Fig. 4; Fig. 5; Fig. 8B), but the transcriptional targets that link Dpp signaling to DE-cadherin and Rho1 are unknown. A better understanding of the links between these three pathways should help shed light on the mechanisms that regulate the fine cellular events required during patterning of developing epithelia.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/10/1861/DC1>

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