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 morphology, 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...
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-lacZP10638, tkv16713, Rho172F and putP flies were kindly provided by the Bloomington Drosophila Stock Center, dppE90 by Kristi Wharton (Brown University, Providence, RI), UAS-tkvQ253D (constitutively active) by Michael O’Connor (University of Minnesota, Minneapolis, MN), tkv4 by Nick Baker (Albert Einstein College of Medicine, Bronx, NY), tkv8 and Mad12 by Laurel Raftery (Massachusetts General Hospital, Charlestown, MA), shgR69 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 rstCT by Karl Fischbach (Albert-Ludwigs-Universitaet, Freiburg, Germany). The rst 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’/H11032 half; and tkv-IR2, directed towards the 3’/H11032 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, dppE90 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, dppE90 pupae were held at the permissive temperature until dissection.
Whole eye mutants for \textit{punt} were generated using the EGUF system (Stowers and Schwarz, 1999) using pupae with the following genotypes: (1) control: \textit{ey-Gal4}, UAS-\textit{FLP}; FRT82B, cl, GMR-hid/FRT82B lacZ; (2) experimental: \textit{ey-Gal4}, UAS-\textit{FLP}; FRT82B, cl, GMR-hid/FRT82B \textit{punt}.

Discrete \textit{tkv} and Mutant 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 \textit{tkv} 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) \textit{tkv} clones: hs-\textit{FLP}; \textit{tkv}\textsuperscript{\textit{B}} \textit{FRT40}\textit{ub-nGFP} \textit{FRT40}; (2) \textit{tkv}\textsuperscript{\textit{B}} clones: hs-\textit{FLP}; \textit{tkv}\textsuperscript{\textit{B}} \textit{FRT40}\textit{ub-nGFP} \textit{FRT40}; (3) \textit{Mad}\textsuperscript{\textit{B}} clones: hs-\textit{FLP}; \textit{Mad}\textsuperscript{\textit{B}} \textit{FRT40}\textit{ub-nGFP} \textit{FRT40}; (4) \textit{tkv}\textsuperscript{\textit{B}} single-cell clones: UAS-\textit{CD4};GFP, hs-\textit{FLP}; \textit{GAL80}, \textit{FRT40}\textit{tkv}\textsuperscript{\textit{B}} \textit{FRT40}; \textit{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 Axioshot microscope equipped with a Quantix CCD camera (Photometrics) and Image Pro Plus software. Images were processed with Photoshop (Adobe). Confocal zxy 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.
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’ (IPC) 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 pattern formation, 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 *DE-cadherin* with *Dpp* resulted in an abnormal hexagonal 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.

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-aCatenin-GFP/tkvΔ; UAS-tkv-IR1(2X)/UAS-tkv-IR1(2X)] (Fig. 3; see 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*Δ (E-H) and *Mad*12 (I,J) dissected at 25 hours APF and stained with anti-DE-cadherin (red; E-I). Clonal tissue is marked by the absence of GFP (green; F,H,J) or outlined by dotted lines (E,G,I). Arrows point to IPC-IPC junctions with abnormally low-to-undetectable DE-cadherin staining.
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°/3°s similar to those observed when Dpp activity was reduced (Fig. 2B). Additionally, we observed cases of ectopic 2°/3°s. Whole eye clones of genotypically punct tissue showed patterning defects that were similar to, but milder than, those seen in dpp and tkv mutants, and discrete punct clones showed infrequent defects (data not shown). Weaker phenotypes of punt versus tkv clones have 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).
Interestingly, when two tkv 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/H11032 region and tkv-IR2 within the 3/H11032 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 scaled-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-IR2 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...
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 Mad12 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/Egfr or wingless 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 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)/Rho172F; 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 rstCT 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>tkvQ253D+) 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. 6-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 rstΔ7 showed uniform IPC patterning defects accompanied by general upregulation of cell surface Tkv (Fig. 7G-I). Mutants for the hypomorphic allele rstΔ8, 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; TePas 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 shgR69 (Fig. 8A-C). This genetic enhancement is consistent with our observation that DE-cadherin and Rst act in opposition (Fig. 1I-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 (Rho1Δ28) 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.
Dpp regulates epithelial patterning

**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).
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. 11-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 Rh1-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 Rh1 (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 BMPR1A) 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 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 Rh1 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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