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Rap1 maintains adhesion between cells to affect Egfr signaling and planar cell polarity in *Drosophila*

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Abstract

The small GTPase Rap1 affects cell adhesion and cell motility in numerous developmental contexts. Loss of Rap1 in the *Drosophila* wing epithelium disrupts adherens junction localization, causing mutant cells to disperse, and dramatically alters epithelial cell shape. While the adhesive consequences of Rap1 inactivation have been well described in this system, the effects on cell signaling, cell fate specification, and tissue differentiation are not known. Here we demonstrate that Egfr-dependent cell types are lost from Rap1 mutant tissue as an indirect consequence of DE-cadherin mis-localization. Cells lacking Rap1 in the developing wing and eye are capable of responding to an Egfr signal, indicating that Rap1 is not required for Egfr/Ras/MAPK signal transduction. Instead, Rap1 regulates adhesive contacts necessary for maintenance of Egfr signaling between cells, and differentiation of wing veins and photoreceptors. Rap1 is also necessary for planar cell polarity in these tissues. Wing hair alignment and ommatidial rotation, functional readouts of planar cell polarity in the wing and eye respectively, are both affected in *Rap1* mutant tissue. Finally, we show that Rap1 acts through the effector Canoe to regulate these developmental processes.

Keywords

Rap1; adhesion; cadherin; planar cell polarity; canoe; wing vein; ommatidia

Introduction

Members of the Ras superfamily of small GTPases serve as molecular switches in many biological systems, translating environmental signals into specific cellular responses. GTPases of this type cycle between guanosine diphosphate (GDP)-bound and guanosine triphosphate (GTP)-bound states, typically adopting active conformations when associated with GTP (Bourne et al., 1990). GTP-bound Ras-like proteins bind downstream effectors, thereby activating signal transduction pathways. Among factors that regulate the activity of these proteins are GTPase exchange factors (GEFs) that promote the active GTP-bound state, and GTPase activating proteins (GAPs) that promote the inactive state (Vetter and Wittinghofer, 2001). Frequently, Ras-like proteins function as part of intercellular signaling pathways, acting

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downstream of transmembrane receptors to mediate cytosolic or transcriptional effects. The wide variety of biological effects mediated by these proteins includes cell proliferation, differentiation, cytoskeletal remodeling, and vesicular trafficking, to name just a small subset. In *Drosophila*, there are over one hundred genes in the Ras superfamily, roughly divided into the Ras/Rap/Ral, Rho, Rab, Ran and Arf/Sar families. Although a certain amount of crosstalk exists, each family is specialized for particular functions. For example, Rho GTPases generally control actin dynamics important during cell migration, while Rab GTPases are critical for intracellular membrane trafficking (Lundquist, 2006).

The gene *Roughened* (also called *Rap1*) encodes the *Drosophila* Rap1 homologue. Rap1 is the GTPase most highly related to Ras, and was initially identified based on its ability to antagonize Ras function in cell culture, restoring a malignant phenotype of K-Ras-transformed fibroblasts (Kitayama et al., 1989). Canonically, an Egf signal is conveyed through the Egfr/Ras/Raf/MEK/MAPK signal transduction pathway to affect the activity of transcription factors in the nucleus. It has been demonstrated in untransformed fibroblasts that activated Rap1 inhibits Ras signaling through MAP-Kinase (MAPK), potentially inhibiting Ras activation of Raf (Cook et al., 1993). More recent data, however, suggests that Rap1 does not always antagonize Ras signaling. In the *Drosophila* retina, data indicates that Ras and Rap1 function through independent pathways (Asha et al., 1999), while during embryogenesis, Rap1 has been shown to bind Raf and positively regulate the Raf/ERK/MAPK pathway in a Ras-independent fashion (Mishra et al., 2005). Exactly how Ras and Rap1 interact to mediate signaling downstream of receptor tyrosine kinases remains controversial.

A dominant theme emerging from the literature, however, is that Rap1 regulates adhesion between cells, affecting the localization and integrity of cell junctions. In particular, Rap1 has been shown to affect the inside-out activation of integrins (Bos et al., 2003; Caron, 2003; Han et al., 2006) and localization of the apico-lateral adherens junction complex (Hogan et al., 2004; Price et al., 2004; Wang et al., 2006). It is not surprising therefore, that Rap1 is a critical determinant of a cellular morphology and migration. This was first demonstrated when activation of Rap1 signaling, through misexpression of the Rap1-specific GEF C3G, increased cell spreading and attachment to the matrix in culture, while the Rap1-specific GAP Spa1 caused cell rounding and detachment from the matrix (Tsukamoto et al., 1999). Rap1 function has been most extensively characterized in the immune system where it plays a key role in the inside-out activation of integrins, controlling integrin receptor avidity (clustering), and ligand affinity in leucocytes (Kinashi and Katagiri, 2005). In addition, Rap1 polarizes leucocytes, generating a leading edge and stimulating transmigration through the vascular endothelium (Shimonaka et al., 2003).

Our understanding of Rap1 function is less complete in the context of cadherin-based cell adhesion. Cadherins are transmembrane proteins that mediate primarily homophilic cell interactions (Nose et al., 1988). While the extracellular cadherin repeat domains form contacts between neighboring cells, the cadherin intracellular domain binds β-catenin, which in turn complexes with the actin-binding protein α-catenin. In this way, cadherins serve as critical links between the cytoskeletons of adjacent cells. In polarized epithelia, cadherins are part of the adherens junction complex of proteins, localized in a ring around each cell at the boundary between the apical and basolateral membrane domains (apico-lateral). It was initially observed that Rap1 affects adherens junctions in the *Drosophila* wing epithelium (Knox and Brown, 2002). Wild-type wing cells maintain contact with their neighbors as they proliferate (Gibson et al., 2006), resulting in highly cohesive cell clones. In contrast, *Rap1* mutant clones of cells disperse, intermingling with their wild-type neighbors. In these mutant cells, adherens junctions are not evenly distributed around the apical circumference, reducing adhesion to neighboring cells and causing fragmentation of the clone (Knox and Brown, 2002). This result indicates that Rap1 mediates an active process, which during the course of epithelial

development maintains circumferential cell-to-cell contacts. A similar phenotype is seen in the *Drosophila* testes, where Rap1 is essential for stem cell anchoring to the niche (Wang et al., 2006). Since the initial observations in *Drosophila*, it has also been shown that Rap1 activity is required in mammalian cells to maintain E-cadherin-based cell-cell contacts (Hogan et al., 2004; Price et al., 2004).

Here we demonstrate that in developing epithelia the adhesive defects associated with *Rap1* loss of function affect signaling between cells and tissue morphogenesis. In particular, Rap1 activity is critical for differentiation of Epidermal growth factor receptor (Egfr)-dependent cell types in the *Drosophila* wing and eye, as wing veins and photoreceptors are often lost from *Rap1* mutant tissue. Planar cell polarity (PCP) is also impaired in the absence of Rap1, since wing hair formation and alignment, as well as ommatidial rotation are abnormal. Finally, we identify Canoe as a critical Rap1 effector in these developmental processes.

Materials and Methods

Genetics

The $Rap1^{RB3}$ allele contains a point mutation, resulting in a premature stop codon near the N-terminus of the Rap1 protein (Hariharan et al., 1991). $Rap1^{CD3}$ (Asha et al., 1999) and $Rap1^{CD5}$ (Boettner et al., 2003) contain deletions that remove the entire Rap1 coding sequence. All three alleles behave as genetic nulls. Additional genetic reagents include *FRT80B, Ubi-GFP* (Xu et al., 1993), MARCM 80b (Lee and Luo, 2001), UAS-sSpitz, UAS-p35 (Zhou et al., 1997), GMR-p35 (Johnson et al., 2002), *ey-flp* (Newsome et al., 2000), *NP2631-Gal4 (National Institute of Genetics, Japan), UAS-Rap1-IR* (v20761 and v33437, Vienna *Drosophila* RNAi Center), *UAS-*$Rap1^{N17}$ (Boettner et al., 2000), shg^{2} (Tepass et al., 1996), *Actin>CD2>Gal4, UAS-GFP* (Neufeld et al., 1998; Pignoni and Zipursky, 1997; Struhl and Basler, 1993), *sev-Gal4 (K25)* (Wilk et al., 1996), *mδ0.5-Gal4* (Gaengel and Mlodzik, 2003), $Star^{48\text{-}5}$ (Gaengel and Mlodzik, 2003), *ptc-Gal4,* $stan^{192}$ (Rawls and Wolff, 2003), dbt^{ar} (Rothenfluh et al., 2000), mwh^{1} (Wong and Adler, 1993), *UAS-canoe-IR* (*National Institute of Genetics, Japan*), cno^{2} (Miyamoto et al., 1995), ap^{Gal4} (Calleja et al., 1996).

Mitotic recombination was induced using the Flp/FRT system (Xu et al., 1993). Larvae containing the *hs-flp* transgene were heat shocked for 45–60 minutes in a 37°C water bath at 72 hours after egg deposition (AED). Alternatively, *ey-flp* (Newsome et al., 2000) was used to generate clones in the eye. To generate Flp/Gal4 overexpressing clones (Neufeld et al., 1998; Pignoni and Zipursky, 1997; Struhl and Basler, 1993) larvae were heat shocked 8–12 minutes.

Immunohistochemistry

Larval and pupal tissues were fixed in 4% paraformaldehyde/PBS for 20 minutes at room temperature. Samples were placed in PBT (0.1% Triton-X/PBS) containing 4% Normal Goat Serum or 0.1% BSA, and incubated over-night at 4°C or one hour at room temperature. Samples were then incubated with primary antibodies over-night at 4°C. Primary antibodies used were anti-DE-cadherin (1:100, DSHB), anti-DSRF (1:500, Active Motif), anti-dpERK (1:500, Sigma), anti-dpERK (1:100, Cell Signaling Technology), anti-cleaved Caspase-3 (1:100, Cell Signaling Technology), anti-Canoe (1:00, gift from D. Yamamoto), anti-Dlg (1:100, DSHB), anti-DN-cadherin (1:100, DSHB), anti-Elav (1:200, DSHB), anti-Atonal (1:2000, gift from D. Marenda), anti-Senseless (1:1000, gift from H. Bellen), anti-Rough (1:100, DSHB), anti-Prospero (1:10, DSHB), anti-Cut (1:80, DSHB), anti-Stan (1:50, DSHB), anti-Mwh (1:500, gift from P. Adler). Alexa 488-, 568-, and 633-conjugated (Molecular Probes, 1:1500), or Fluorescein-, Rhodamine-, DyLight 649- and Cy5-conjugated (Jackson ImmunoResearch, 1:200) secondary antibodies were used. Rhodamine-conjugated phalloidin (Molecular Probes, 1:1500 in PBS) was used to stain F-actin. Nuclei were stained with Hoechst 33258 (Acros,

1:1500). Discs were mounted in Fluoroguard (Biorad) or 80% Glycerol containing 5% N-propyl gallate. Images were obtained on a Leica TCS SP confocal or a Zeiss Apotome microscope. Stacks of images were compiled and analyzed using Image J and Adobe Photoshop.

Western blots

Western blots were performed as described in Current Protocols in Molecular Biology. Briefly, 30 eye-antennal discs were dissected and lysed in 30 μL RIPA buffer (150mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris pH 8.0, 1mM phenyl methylsulfonyl fluoride). 30 μL 2x SDS-PAGE buffer (Bio-Rad) was added and samples were boiled 10 minutes to denature proteins. 20 μL of each sample were separated by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). Membranes were probed with anti-Rap1 (1:500, BD-Transduction Laboratories), washed and probed with horseradish peroxidase-conjugated antibodies (1:2,500, Jackson ImmunoResearch). Chemiluminescent detection was performed and anaylzed with a ChemiDoc XRX imaging system and Quantity One software (Bio-Rad). Membranes were stripped and re-probed with anti-Ras (1:500, Cell Signaling Technologies) and anti-Tubulin (1:500, Developmental Studies Hybridoma Bank) and analyzed as above.

Quantitative PCR

Thirty *NP2631-Gal4, UAS-Rap1-IR/+* or *NP2631-Gal4/+* eye-antennal imaginal discs were dissected from larvae grown at 25°C. PolyA+ RNA was isolated using a Dynabeads ®mRNA Direct kit™ (Invitrogen). Three independent mRNA isolations were performed for each genotype (biological replicates). The mRNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop). Reverse transcription reactions were performed on 20 ng of mRNA using an iScript™select cDNA synthesis kit (Biorad) following the manufacturer's instructions. cDNA was then diluted 1:10. The PCR quantification was performed using 1μl cDNA per reaction with a ICycler IQ ™multicolored Real Time PCR Detection System (Biorad) using the IQ™ SYBR®Green Supermix (Biorad) as the detection dye. Each cDNA sample was run in triplicate and the average C_T was used to calculate the fold change using the -2^{DDCT} method (Livak and Schmittgen, 2001). Expression levels were normalized to *rp49* expression. A Student's two-tailed t-test was used to assess for statistical significance between control and sample fold change.

The following primers were used:

Gene	Forward Primer	Reverse Primer
Rap1	CAGTGCATCTTCGTTGGAGAA	CGTGATCGAGTAGACCAGCA
Ras85D	AGAGGTGGCCAAACAGTACG	CGCACCAGTGTGTAAAATGC
Ric	CACGAAAACGAATCAAATGC	ACATGGACACGACACATTGC
Rab-RP3	AGCGGTCTTCCTTTTCCAAT	AGGTCTCCATCACGAACAGG
Rap21	GAAAGGAAGTCAGCCAGCAC	GAACGATGGTGGCGAATACT
Rp49	CCAAGGACTTCATCCGCCACC	GCGGGTGCGCTTGTTCGATCC

Adult eye imaging

Prior to sectioning, adult fly heads were fixed in 2% Glutaraldehyde, 2% OsO_4 in phosphate buffer (0.1 M sodium phosphate pH 7.2) for 1 hour on ice, then transferred to 2% OsO_4 in phosphate buffer for 2 hours on ice. Samples were serially dehydrated to 100% ethanol followed by two 10 minute washes in propylene oxide (Electron Microscopy Sciences).

Samples were incubated overnight in 50% propylene oxide and Durcapan resin (Electron Microscopy Sciences), followed by 4 hours in 100% resin at room temperature. Samples were oriented in molds in 100% resin and incubated at 60°C overnight. Samples were tangentially sectioned (1μm) at the equatorial region on either a Leica UC6 or a Sorvall MT2 B Ultramicrotome. SEM adult eye images were obtained using a Hitachi TM1000 Table Top Scanning Electron Microscope.

PCP quantifications

Techniques were devised to quantify relative orientation and alignment of wing hairs and ommatidia. In the wing, patched-Gal4 was used to express a *Rap1* RNAi transgene in a stripe between veins L3 and L4. An image of fixed size was taken immediately distal to the posterior cross-vein and anterior to vein L4. 250–300 wing hairs were contained in each image. Using Image J, a line was drawn over each hair delineating the hair orientation. Image J was used to determine an orientation angle for each hair (pointing distally equaled zero). Mean wing hair angle and wing hair angle variance were calculated for each wing with 5–10 wings quantified for each genotype. In wildtype wings, mean wing hair angle was expected to be near zero, and wing hair angle variance low, as hairs generally point distally and are aligned. Deviations in mean wing hair angle indicate a coordinated rotation of hairs away from the distal orientation (hairs could remain aligned). An increase in wing hair angle variance indicates hair misalignment. This calculation can be applied to other aligned epithelial structure as a measure of variation in planar polarity.

In the eye, an image of fixed size was taken at the R7 plane of a tangential section centered at the equator. 50–120 scorable ommatidia were contained in each image. Similar to the wing, using Image J an orientation angle was determined for each ommatidium, and angle means and variances calculated for each eye (3–7 eyes per genotype). The Wilcoxon Rank-Sum two-tailed test was used to determine the statistical significance of measurements.

Results

Rap1 affects epithelial cell adhesion and wing vein differentiation

As has been previously demonstrated, clones of cells lacking *Rap1* activity in the *Drosophila* pupal wing had abnormal adhesive properties (Knox and Brown, 2002). Compared to wild-type controls at 36 hours after puparium formation (APF) (Fig.1A), *Rap1* mutant cells dispersed, scattering into the adjacent wildtype epithelium (Fig.1B). Distribution of the homophilic cell adhesion molecule DE-cadherin (DE-cad) (encoded by the gene *shotgun* (*shg*) in *Drosophila*) was also affected. In *Rap1* mutant cells DE-cad was abnormally concentrated at a single cell-cell interface (Knox and Brown, 2002); Fig.1C), creating asymmetric adhesive contacts and disrupting the regular hexagonal packing of wing epithelial cells. Throughout this analysis, three *Rap1* alleles were analyzed ($Rap1^{RB3}$, $Rap1^{CD3}$, and $Rap1^{CD5}$) resulting in equivalent phenotypes. These results confirm that Rap1 functions to maintain an even distribution of adherens junctions about the apical circumference of epithelial cells.

While it is clear that Rap1 regulates cell adhesion in the wing, we next tested whether manipulations of Rap1 activity would affect differentiation of wing-specific cell types. In particular, the wing blade has a characteristic pattern of veins that add rigidity and are critical for flight (Fig.1F). Vein cell precursors are specified during larval stages (Sturtevant et al., 1993), and by 36 hours APF have adopted a distinct, non-hexagonal shape (Fig.1D). These cells express high levels of DE-cad (compared to surrounding intervein tissue), and this adhesive difference is important for their morphological differentiation (O'Keefe et al., 2007). When *Rap1* loss-of-function clones overlapped vein territories, severe disruptions in

vein cell morphology were observed (Fig.1E), suggesting that Rap1 is required for vein formation. In support of this hypothesis, adult wings containing *Rap1* mutant clones of cells had vein defects never seen in control animals (Fig.1G,H). Wing vein discontinuities were frequently observed, although abnormalities in wing hair formation prevented us from identifying mutant cells in this context. To resolve this issue, pupal wings containing *Rap1* mutant clones were dissected and stained for DSRF/Blistered, a marker of intervein cell fate (Montagne et al., 1996). Similar to the adult phenotype, vein discontinuities in the pupal wing were observed, as DSRF localization frequently expanded into vein territories (Fig.1I,J, arrow). This effect was weakly penetrant, however, as many *Rap1* mutant cells maintained the vein cell fate (DSRF-negative) (Fig.1I, arrowhead).

Rap1 has been shown to affect both Egfr signal transduction (Cook et al., 1993; Mishra et al., 2005), and DE-cad-mediated cell adhesion (Knox and Brown, 2002), processes important for wing vein specification (Brunner et al., 1994; Clifford and Schupbach, 1989; Diaz-Benjumea and Hafen, 1994; Guichard et al., 1999; Karim and Rubin, 1998; Prober and Edgar, 2000; Sturtevant et al., 1993) and morphogenesis respectively. To better understand the role Rap1 plays during wing vein formation, we used the MARCM system (Lee and Luo, 2001) to activate Egfr signaling (via a secreted version of the ligand Spitz) in either wild-type or *Rap1* mutant cells and measured the effect on vein cell fate and cell adhesion. In terms of cell fate, Rap1 did not affect the ability of Spitz to promote vein identity; DSRF levels were downregulated in the presence or absence of Rap1 function (Fig.2E,F, Supplemental Fig.S1C,D). A similar result was obtained when discs were stained for di-phospho-ERK (a more direct readout of MAPK activity in vein cells (Gabay et al., 1997)) (Supplemental Fig.S1A). In terms of cell adhesion, however, Rap1 significantly affected Spitz-expressing cells. Clones of cells expressing Spitz in the pupal wing (36 hours APF) had elevated levels of DE-cadherin, resulting in dramatic apical constriction (Fig.2A,C). Loss of Rap1 in this context did not affect DE-cad levels, nor DE-cad apical/basal localization (Fig.2B,D), however, asymmetric adhesive contacts were prevalent. Taken together these results indicate that in the developing wing epithelium Rap1 does not directly regulate the Egfr signaling cascade to affect vein/intervein identity. Instead, Rap1 affects adhesion between cells, and the effects on Egfr-dependent cell types are a consequence of these adhesive deficits. In Rap1 mutant clones of cells, therefore, vein cells are specified normally, but vein cell fate maintenance and vein differentiation are compromised.

Rap1 affects DE-cad localization and cell shape in the developing eye

To ask whether the cell adhesion phenotypes associated with *Rap1* in the wing are seen in other developmental contexts, we disrupted Rap1 function in the *Drosophila* eye. Using an *ey-flp* transgene, clones of cells lacking *Rap1* function were generated from the earliest stages of eye development. As was seen in the wing, DE-cad localization was affected by the loss of Rap1 activity. In third instar eye imaginal discs cells anterior to the morphogenetic furrow are undifferentiated and proliferating, while behind the furrow cells are exiting the cell cycle and differentiating (Wolff and Ready, 1991). At this stage of development, all epithelial cells in the eye imaginal disc express DE-cad, but DE-cad protein is enriched in cells of the morphogenetic furrow and at cell-cell contacts between photoreceptor precursors (Mirkovic and Mlodzik, 2006) (Fig.3A). DE-cad localization was not affected in *Rap1* mutant clones anterior to the furrow, or in the furrow itself. Posterior to the furrow, however, a dramatic effect on DE-cad localization was observed. While many Elav-expressing photoreceptors were specified in the absence of Rap1 function, DE-cad enrichment between these cells was not observed (Fig.3A). Surprisingly, DN-cadherin (DN-cad) localization in the eye disc was not affected by Rap1 (Supplemental Fig.S2), indicating that Rap1 specifically affects DE-cad based adherens junctions at this stage of development.

At 24 hours APF, DE-cad localization in the wildtype pupal eye reveals a highly organized lattice of cell types (Fig.3B,C). At this stage, loss of Rap1 in the eye had similar, but more dramatic effects on DE-cad localization compared to those seen in the wing. Asymmetries in DE-cad were frequently observed within the clones (Fig.3C, arrows), but more frequent was a diffuse distribution of DE-cad. Cell shapes were also highly disorganized near *Rap1* mutant tissue, with cells both within and immediately adjacent to the clone affected. However, since the loss of Rap1 so dramatically affected DE-cad localization, it was often difficult to delineate cell boundaries within the mutant clones. To circumvent this problem, we visualized Discs large (Dlg) in *Rap1* mutant eye tissue. Dlg is a critical component of the septate junction (Woods et al., 1996), and its localization is not affected by Rap1 activity (Knox and Brown, 2002). At 24 hours APF, Dlg localization reveals a regular pattern of cone and interommatidial cells in wildtype eyes. This pattern was severely disrupted in and around *Rap1* mutant tissue (Fig.3D). These results indicate that in the differentiating eye, Rap1 dramatically affects DE-cad localization and cell shape.

To demonstrate a functional relationship between Rap1 and DE-cad during eye development, we asked whether reduced levels of DE-cad could enhance a hypomorphic *Rap1* phenotype. *NP2631-Gal4* (*NP2631*) expresses in most cells of the third instar eye disc, both anterior and posterior to the morphogenetic furrow (Supplemental Fig.S3A). *NP2631* was used to drive expression of a *Rap1* RNAi transgene (*UAS-Rap1-IR)*, knocking down *Rap1* function in the developing eye (genotype: *NP2631>Rap1-IR*). Controls indicate that *Rap1-IR* specifically affects *Rap1* expression and not other closely related GTPases (Supplemental Fig.S4). In wildtype adult eyes, ommatidia and bristles are arranged in a remarkably regular pattern (Fig. 3E). In comparison, *NP2631>Rap1-IR* eyes were small and rough (Fig.3F). When one copy of the *DE-cad/shg* gene was removed from this genetic background (genotype: *NP2631>Rap1-IR/shg*^{2}) the *Rap1* phenotype was greatly enhanced (Fig.3G). As a control we determined that *shg*^{2} did not interact with the *NP2631* driver alone (data not shown). This genetic interaction strongly suggests that Rap1 and DE-cad function in a common pathway to regulate differentiation and morphogenesis of the *Drosophila* eye.

Egfr-dependent cell types are lost from *Rap1* mutant eye tissue

We next asked whether Rap1 activity is necessary for Egfr-dependent processes in the eye disc, as we had observed in the wing. Egfr signaling has several roles in eye development, but is first required in the morphogenetic furrow for the formation and spacing of ommatidial precursor clusters of cells (Baonza et al., 2001; Brown et al., 2006; Dominguez et al., 1998; Spencer et al., 1998; Yang and Baker, 2001). In differentiating cells posterior to the morphogenetic furrow Egfr signaling is required for proper spacing of the founding R8 photoreceptor, and for subsequent recruitment of all other ommatidial cell types. In the absence of Egfr signaling, therefore, an irregular pattern of R8 photoreceptors is specified, but all other ommatidial cell types are lost (Baonza et al., 2001; Dominguez et al., 1998; Lesokhin et al., 1999; Yang and Baker, 2001). Similarly, a partial decrease in Egfr signaling results in fewer ommatidial cells (Freeman, 1996). In addition, it has been demonstrated that Egfr activity is required for high levels of DE-cad in photoreceptors (Brown et al., 2006), suggesting that cell adhesion plays an important role in photoreceptor specification and differentiation (as in wing veins). Consistent with this hypothesis, *DE-cad/shg* mutants show loss of photoreceptors (Mirkovic and Mlodzik, 2006). As we described above, Rap1 also affects DE-cad levels in photoreceptor precursors (Fig. 2A), our first indication that Rap1 plays a role in Egfr-dependent processes in the eye.

To test whether Rap1 plays a role during the earliest stages of ommatidial development, we stained eye discs containing *Rap1* mutant clones of cells for dpERK and Senseless. In the developing eye, Egfr/MAPK activity is highest in intermediate groups within the

morphogenetic furrow (Baonza et al., 2001; Spencer et al., 1998) (Fig.4C, arrow), and loss of Rap1 did not affect dpERK levels, or the spacing of intermediate groups (Fig.4D, arrow). Similar results were obtained when levels and spacing of Atonal were examined (data not shown). Senseless localizes to a subset of cells within each intermediate group (the R8 equivalence group) (Frankfort et al., 2001) (Fig.4E), and within the furrow its localization was normal in *Rap1* mutant clones (Fig.4F). These results indicate that Rap1 is not required for the patterning events that drive formation and spacing of the intermediate clusters or the R8 equivalence groups. In addition, Rap1 does not prevent the activation of the Egfr signaling pathway, as judged by the normal dpERK levels in *Rap1* mutant tissue.

As ommatidial pre-clusters emerge from the furrow, however, *Rap1* phenotypes become apparent. Posterior to the furrow, Senseless-expressing R8 cells were present in *Rap1* clones, although they were irregularly spaced (Fig. 4F, arrows). This is consistent with a reduction in Egfr function. The spacing phenotype is not due to cell apoptosis, as expression of the cell death inhibitor p35 (Hay et al., 1994) in *Rap1* mutant cells did not restore normal spacing to ommatidia (Supplemental Fig.S5). To examine in more detail why R8 cells were affected, we stained these discs for DE-cad to visualize ommatidial precursors at this stage. In wild-type eye discs, cells in the furrow express high levels of DE-cad and form rosettes, which rearrange to form arcs, which close to form clusters of photoreceptor precursors (Wolff and Ready, 1993) (Fig 4A, arrows). DE-cad is maintained at high levels in clusters, while being downregulated in surrounding cells. Rap1 does not affect DE-cad localization in the furrow (as mentioned previously). However, although arcs and clusters within *Rap1* mutant clones attempt to form, they are structurally abnormal and fail to maintain high levels of DE-cad (Fig 4B, arrows). These early morphological defects, which are likely to result from the effects of Rap1 on cell adhesion, may account for the irregular spacing of R8 cells. Taken together, our results suggest that the morphological changes characteristic of ommatidial precursors as they exit the furrow are regulated by Rap1, but that Rap1 is dispensable for the patterning events that determine and position the rosettes, arcs and clusters.

With R8 present, we next asked whether each ommatidium contained the regular complement of photoreceptor precursors. Elav is expressed by post-mitotic neurons, and posterior to the furrow labels regularly spaced clusters of photoreceptors (Fig.4G). In *Rap1* mutant clones the Elav clusters were variable in size, suggesting a loss of photoreceptor cell types (Fig.4H, arrows). To determine which cells were affected we first examined Rough (R2/R5) and Prospero (R7/cone) expression in *Rap1* mosaic eyes. Both Rough and Prospero cell types were frequently absent from *Rap1* mutant ommatidial clusters (Fig.4I and data not shown). Cone cells (labeled by the Cut protein (Blochlinger et al., 1993)) require Egfr signaling for their specification and were often lost from eye discs containing *Rap1* mutant clones (data not shown), revealing another cell type that requires Rap1 function.

We also examined Cut localization in pupal eyes (40 hours APF). In some cases, ommatidia associated with *Rap1* mutant cells contained a full complement of photoreceptors, but were missing cone cells (Fig.4J). This indicates that cone cell loss is not a secondary consequence of missing photoreceptors. Interestingly, the arrangement of photoreceptors in the pupal eye can also be affected by *Rap1* loss-of-function clones, as demonstrated by the ommatidium with one photoreceptor surrounded by the seven others (all eight photoreceptors are normally arranged in a circle). This is consistent with improper maintenance of adhesive contacts in ommatidia containing *Rap1* mutant cells.

Effects of *Rap1*- clones on photoreceptor development do not result from apoptosis

During the course of the experiments described above we noticed occasional Elav-positive nuclei in ectopic locations. As photoreceptors are specified their nuclei migrate to the apical surface of the eye epithelium (Wolff and Ready, 1993). Optical sections through *Rap1* mutant

clones, however, revealed Elav nuclei at the basal surface. They were consistently located well behind the furrow in a region where developing ommatidia are fairly mature. Whether these are cells that are being eliminated from the epithelium, or simply cells with mis-positioned nuclei remains to be determined. It is important to point out that *Rap1* mutant ommatidia with photoreceptor loss are often detected in regions where basal nuclei are not present. Thus, despite these basal nuclei, the primary effect of *Rap1* on photoreceptors is likely to be a failure of recruitment.

Nevertheless, to confirm that *Rap1* is necessary for photoreceptor recruitment, and that photoreceptor loss from *Rap1* mutant clones does not primarily result from apoptosis, we expressed the apoptosis inhibitor p35 in *Rap1* mutant cells using the MARCM system or GMR-p35 (Johnson et al., 2002). Acridine orange stainings were used to confirm that p35 inhibited apoptosis (not shown). In both cases we still observed loss of Elav-positive cells from developing ommatidia, and basally localized Elav-positive nuclei (Supplemental Fig.S5). These results indicate that the loss of photoreceptors observed in *Rap1* loss-of-function clones does not result from apoptosis.

In summary, Rap1 has no effect on early patterning events that occur ahead of and in the furrow. Instead, Rap1is required for proper morphology of ommatidial preclusters, for proper spacing of R8 precursors, and for recruitment of photoreceptors and cone cells after R8 specification. The fact that Rap1 clones do not affect dpERK levels indicates that, as in the wing, Rap1 is not required for Egfr signaling per se. Rather, our results are consistent with the idea that the role of Rap1 in Egfr-dependent developmental events results from effects of Rap1 on cell adhesion.

Rap1 function is required for ommatidial rotation

In addition to apical/basal polarity, cells within a developing tissue are often polarized within the plane of the epithelium. Termed planar cell polarity (PCP), this process allows for a large number of cells to coordinately differentiate asymmetric structures. In the *Drosophila* retina each ommatidium is polarized since the R3 and R4 photoreceptors occupy asymmetric positions. Within each ommatidium, the PCP network of proteins (Frizzled and Dishevelled for example) determines which cell adopts the R3 versus R4 fate; thus determining ommatidial chirality (Adler, 2002; Mlodzik, 1999; Strutt and Strutt, 1999). Following specification of R3/R4, each ommatidial precluster undergoes a 90-degree rotation toward the midline of the developing eye field. This rotation involves a complex rearrangement of cell-cell contacts, and requires input from planar cell polarity, although the mechanisms linking the PCP complex of proteins to the rotation process remain unclear (Seifert and Mlodzik, 2007; Wolff et al., 2007). In this way, ommatidial rotation is a readout of PCP signaling in the eye. It has been demonstrated that Egfr activity and cadherin-based cell adhesion both affect ommatidial rotation. Hypomorphic mutations in the Egfr pathway that allow for normal specification of ommatidial cell types frequently have rotation phenotypes, but proper chirality (Brown and Freeman, 2003; Gaengel and Mlodzik, 2003; Strutt and Strutt, 2003), and DE-cad has been shown to promote ommatidial rotation (Mirkovic and Mlodzik, 2006). Since Rap1 affects Egfr-dependent processes and cadherin-based cell adhesion, we asked whether Rap1 was necessary for ommatidial rotation in the developing eye.

The protein Rough is strongly expressed in photoreceptors R2 and R5 that, under wildtype circumstances, are located on opposite sides of each ommatidium. This pattern allows one to track the degree of rotation for ommatidia in the larval eye disc (Fig.5A). Using an *ey-flp* transgene, clones of cells lacking *Rap1* function were generated and late third instar discs were stained for Rough. In these discs, the ommatidial rotation pattern was disrupted, as adjacent ommatidia were frequently misaligned (Fig.5B); only complete ommatidia were included in the analysis. Based on the scattered nature of the clones, it was not possible to determine

whether this was a cell-autonomous effect. We therefore examined sections through adult eyes containing *Rap1* mutant clones. In these retinas, most mutant cells did not survive, leaving behind scar-like tissue devoid of recognizable cell types (Fig.5C). A few mutant cells did survive, however, and were typically found at the edges of clones in incomplete or fused ommatidia. These phenotypes have been described before (Asha et al., 1999). When ommatidial orientation was scored in these eyes (again, only complete ommatidia were included), those containing *Rap1* mutant cells were sometimes misaligned (Fig.5C, white arrow). In addition, ommatidia adjacent to *Rap1* mutant territories, but containing entirely wildtype photoreceptors, were also affected (Fig.5C, black arrows). This is not surprising since the rotation process involves the coordinated making and breaking of cell-cell contacts, so a cell lacking normal adhesive properties would likely affect its neighbors. Rap1 therefore plays an important role in ommatidial rotation in the eye.

In order to generate adult retinas in which Rap1 function had been compromised to a lesser degree, *sevenless-Gal4* (*K25*) was used to drive expression of *Rap1-IR*, knocking down *Rap1* gene function in a subset of retinal cell types (R3, R4, R7 and cone cells, Supplemental Fig.S6). At the gross level, a rough eye phenotype was obtained (compare Fig.5D and 5E). When sections through the *sev-Gal4>Rap1-IR* adult eye were examined, a small number of ommatidia contained an irregular complement of photoreceptors, similar to the *Rap1* loss-of-function result (Fig.5E). Ommatidial orientation was scored in these eyes, examining only those ommatidia containing a wildtype set of photoreceptors. Compared to controls, *Rap1-IR* expressing eyes had significant defects in ommatidial rotation. In order to quantify this phenotype, an orientation angle (with respect to the dorsal/ventral axis) was measured for each ommatidium (normal orientation equals 90 degrees). Mean orientation angle and angle variance was then calculated for each eye, and these values were used to compare between genotypes. In control eyes, ommatidia were essentially aligned resulting in a low orientation variance (mean angle=91.9; median angle variance 17.6, n=3). Knocking down *Rap1* function with *sev-Gal4* had little effect on orientation angle, but significantly ($p<.05$) increased angle variance (mean angle=92.4; median angle variance 282.2, n=6), indicating that Rap1 activity is essential for proper ommatidial rotation.

Similar albeit stronger results were obtained when the dominant negative $Rap1^{N17}$ was expressed in the eye. The phenotype obtained with *sev-Gal4* was too severe for analysis, so a weaker driver, *mδ0.5-Gal4* was used. *mδ0.5-Gal4* is expressed at highest levels in R4, but also more weakly in R3 and R7 (Supplemental Fig.S3). Reduction of Rap1 signaling in this subset of cells resulted in a rough eye phenotype, many ommatidia with abnormal numbers of cells, and severe effects on ommatidial rotation (Fig.5G). Thus, results from three independent methods of reducing Rap1 function demonstrate that Rap1 has a role in ommatidial rotation.

Rap1 genetically interacts with Egfr pathway components in the eye

To more firmly establish a role for Rap1 in ommatidial rotation, we asked whether *Rap1* genetically interacts with genes known to affect this process. Star is a transmembrane protein located in the endoplasmic reticulum that facilitates the trafficking and activation of Egf ligands, positively regulating signaling through the Egfr pathway (Lee et al., 2001). It has been demonstrated that *Star* is haploinsufficient in the eye (Heberlein et al., 1993), and *Star* heterozygotes have subtle effects on ommatidial formation and rotation (Brown and Freeman, 2003; Gaengel and Mlodzik, 2003) (Fig. 6A,D). The adult eyes of *Star* heterozygous flies ($Star^{48\text{-}5}/+$) are mildly rough with misarranged ommatidia, and occasional ommatidial fusions (Fig. 6A). We first asked whether reduction of *Star* activity could affect the *NP2631>Rap1-IR* rough eye phenotype (Fig. 6B) by generating *NP2631>Rap1-IR/*$Star^{48\text{-}5}$ animals. Adult eyes from these individuals demonstrated a clear genetic interaction between *Rap1* and

Star$^{48\text{-}5}$ in this context (Fig.6C), but the dramatic nature of the phenotype precluded an analysis of ommatidial rotation.

To generate a subtler phenotype involving *Rap1* and *Star*, double heterozygotes were generated (genotype: *Star*$^{48\text{-}5}$*/+; Rap1*^{RB3}*/+*). As mentioned above, when tangential sections of *Star/+* adult eyes were examined, mild ommatidial rotation defects (mean angle=85.0; median angle variance=262.4) and occasional loss of photoreceptors (median % aberrant=5.2%) were observed (Fig.6D). Importantly, *Rap1/+* animals had normal ommatidial rotation (mean angle=89.6; median angle variance=48.1), and very few ommatidia with loss or gain of photoreceptors (median % aberrant=1.1%) (Supplemental Fig.S6). *Star/+;Rap1/+* doubly heterozygous flies showed a statistically significant ($p<0.05$) increase in ommatidial misalignment (mean angle=101.3; median angle variance=1657.2) (Fig.6E,F), and in the number of ommatidia containing aberrant numbers of photoreceptors (median % aberrant=22.2%) (Fig.6G).

We also tested whether sensitized genotypes involving other Egfr pathway members could be modified by *Rap1* loss-of-function alleles. Kekkon 1 (Kek) is a transmembrane protein that negatively regulates Egfr activity (Ghiglione et al., 1999). Expression of *kek* under *sev-Gal4* control resulted in weak rotation defects (mean angle=94.4; median angle variance=55.3) and some photoreceptor loss (median % aberrant=13.7%), (Supplemental Fig.S6). Removing a single copy of *Rap1* significantly ($p<0.05$) enhanced the effects of *sev>kek* on both ommatidial rotation and photoreceptor loss (mean angle=89.9; median angle variance=293.1; median % aberrant=25.5%) (Supplemental Fig.S6). Expression of GFP under *sev-Gal4* control had no effect on rotation (mean angle=92.0; median angle variance=17.5), and no photoreceptor loss/gain was detected.

Expression of an activated Egfr (λ-top) (Queenan et al., 1997) under *sev-Gal4* control also resulted in ommatidial rotation and photoreceptor defects (mean angle=94.8; median angle variance=645.2; median % aberrant=2.2%) (Supplemental Fig.S6). Although *Rap1* appears to have no effect on the *sev-λ-top* photoreceptor gain/loss phenotype (median % aberrant=1.0%), it slightly but significantly ($p<0.05$) suppresses the ommatidial rotation defects (mean angle=95.5; median angle variance=503.3) (Supplemental Fig.S6). Thus, with respect to ommatidial rotation, reducing *Rap1* levels by a single gene copy dominantly enhanced the effects of Egfr loss (*Star* and *sev>kek*), and dominantly suppressed the effects of Egfr overactivation (*sep>λ-top*). These results demonstrate a clear role for Rap1 in ommatidial rotation, an Egfr/DE-cad-dependent process.

Rap1 is required for PCP signaling in the wing

To explore in more detail whether Rap1 affects PCP signaling (as our data in the eye suggested) we returned to the wing. Each cell in the wing blade generates a single hair located at its distal edge that points distally. PCP signaling is required for the proper formation and orientation of these wing hairs. Adult wings containing *Rap1* loss of function clones had patches of disorganized wing hairs that were never seen in control wings (Fig.7A). These hairs were not distally oriented and often more than one hair formed from a single wing cell (Fig.7A', arrow).

To determine whether this phenotype was associated with *Rap1* mutant cells, we examined pupal wings of the same genotype. At 32 hours APF, F-actin staining reveals the position of the wing pre-hair. Compared to the surrounding wild-type tissue, *Rap1* mutant cells had an abnormal pattern of F-actin distribution (Fig.7B,C, Supplemental Fig.S7A). Pre-hair formation was clearly delayed in these cells, as F-actin foci were less defined, or absent altogether. When prehairs were evident, however, they were found at the distal edge of *Rap1* mutant cells (arrows Fig.7C), suggesting that these cells were polarized appropriately, but unable to coordinate changes in the actin cytoskeleton necessary for wing hair formation. Consistent with this

interpretation, localization of the PCP protein Starry Night (Stan) was essentially normal (concentrated at proximal/distal cell junctions) in *Rap1* mutant cells (Supplemental Fig.7C). These experiments also indicated that Rap1 acts cell-autonomously in this process, as the development of wing hairs was unaffected in wild-type cells immediately adjacent to, or surrounded by, *Rap1* mutant tissue (Fig.7C). By 36 hours APF, most *Rap1* mutant cells had generated a wing hair, although they were disorganized compared to surrounding wild-type tissue (Fig.7D). Based on these results, we conclude that Rap1 is not involved in propagation of the PCP signal through the wing epithelium, but instead functions as a cell-autonomous PCP effector, organizing the polarized formation of wing hairs.

Genes involved in PCP signaling worsen a mild *Rap1* phenotype

To understand more clearly the role played by Rap1 during PCP signaling in the wing, we asked whether known PCP genes could modify a mild *Rap1* phenotype. *ptc-Gal4* was used to drive expression of *Rap1-IR* in the wing, knocking down *Rap1* function in a stripe of cells between veins L3 and L4 (Supplemental Fig.S3D). At 25°C wing hair alignment in this area of the wing was severely disrupted (compare Fig.8A and 8B). To quantify this effect, an orientation angle (with respect to the proximal/distal axis) was calculated for each wing hair in the affected region. Mean hair angle (zero degrees points distally) and angle variance were then calculated for each wing, and these values were used to compare between genotypes. In control wings, the hairs uniformly pointed distally resulting in low mean angle and low hair angle variance (mean angle=−1.1; median angle variance=36.6) (Fig.8G,H). *Rap1-IR* expression resulted in a slight posterior deflection of wing hairs (mean angle=−5.4), and more random wing hair orientation, and therefore, a significantly larger wing hair angle variance (median angle variance=545.8). To generate a more moderate phenotype for use in genetic interaction experiments, *ptc>Rap1-IR* animals were raised at 18°C to reduce *Rap1-IR* expression (Fig.8C), lowering wing hair angle variance (median angle variance=80.0). For these wings, and all subsequent genotypes, wing hairs were deflected posteriorly a few degrees (Fig.8G), so our analysis will focus on angle variance.

starry night (*stan*), also called *Flamingo*, encodes for an atypical cadherin that functions as a core PCP gene (Chae et al., 1999). When a loss-of-function allele of $stan^{192}$ was crossed into the *ptc>Rap1-IR* background, wing hair angle variance was significantly more affected (median angle variance=204.4) (Fig.8D,H). Similarly, a loss-of-function allele of *double time* (dbt^{ar}) genetically interacts with the *ptc>Rap1-IR* phenotype (median angle variance=220.4) (Fig.8H). *dbt* is not a core PCP gene, but instead regulates the activity of the core component Dishevelled (Cong et al., 2004; Klein et al., 2006; Strutt et al., 2006; Tsai et al., 2007). Finally, the gene *multiple wing hairs* (mwh^{1}) severely affected wing hair alignment in combination with reduced *Rap1* function (median angle variance=380.1) (Fig.8E,H). *mwh* encodes a novel GTPase-binding domain/formin homology 3 (GBD/FH3) domain protein, and functions as a cell-autonomous, downstream effector of the PCP signaling pathway (Peyer and Hadorn, 1965; Strutt and Warrington, 2008; Yan et al., 2008). Importantly, *ptc-Gal4* did not interact with *stan*, *dbt*, or *mwh* on its own (data not shown). *Rap1* genetically interacts, therefore, with genes at every level of the PCP hierarchy: core components, regulators, and effectors. The strong interaction with *mwh*, however, combined with the nature of the *Rap1* phenotype, suggests that Rap1 functions together with *mwh* as a PCP effector in the wing; coordinating cytoskeletal components to differentiate a single, polarized wing hair.

We examined whether Mwh localization was altered in *Rap1* mutant clones of cells. At 32 hours APF, Mwh is concentrated at growing wing hairs (Strutt and Warrington, 2008; Yan et al., 2008). Loss of Rap1 had a dramatic effect on Mwh localization, but the pattern resembled that seen with F-actin: delay in prehair formation and loss of focused Mwh accumulation, but correct localization to the distal edge of *Rap1* mutant cells (compare Supplemental Fig.S7A

and B). This suggests that in the absence of Rap1, Mwh still localizes appropriately to F-actin foci at this timepoint. Whether Rap1 affects Mwh activity, however, remains an open question.

We also asked whether *DE-cad/shg* could modify the *ptc>Rap1-IR* phenotype (genotype: *ptc>Rap1-IR;shg*^{R69}/+). DE-cad regulates ommatidial rotation in the eye (Mirkovic and Mlodzik, 2006), as mentioned previously, and evidence suggests that PCP genes regulate DE-cad localization during the hexagonal packing of wing epithelial cells (Classen et al., 2005). Wing hairs were significantly more disorganized in these animals (median angle variance= 496.64) (Fig.8F). The fact that this interaction is stronger than with *stan*, *dbt* or *mwh* confirms that the critical link is between Rap1 and DE-cad during this process, as it is in other contexts.

The Rap1 effector Canoe affects PCP signaling, cell adhesion and epithelial cell shape in the developing wing

To understand the molecular mechanisms underlying Rap1 function in the wing, we asked whether the GTPase effector Canoe plays a role in this developmental context. Canoe is a scaffolding protein containing several protein-protein interacting motifs, and has been shown to act downstream of both Ras and Rap1 (Boettner et al., 2003; Carmena et al., 2006; Gaengel and Mlodzik, 2003). In the eye, *canoe* phenotypes include photoreceptor loss and ommatidial rotation defects (Gaengel and Mlodzik, 2003; Matsuo et al., 1997; Miyamoto et al., 1995); effects very similar to loss of *Rap1*. In the wing epithelium it has been demonstrated that loss of Rap1 results in Canoe mislocalization (similar to DE-cad) (Knox and Brown, 2002).

The *FRT/Gal4* system was used to generate clones of cells expressing a *canoe* RNAi transgene (*cno-IR*), and adult or pupal wings analyzed. Adult wings containing *cno-IR* clones of cells frequently had patches of disorganized wing hairs (Fig.9A) and wing vein discontinuities (arrow Fig.9A´). When pupal wings were examined at 32 hours APF, prehair formation (revealed by F-actin staining) was defective in *cno-IR* expressing cells (Fig.9C). The effects were subtler than those seen in *Rap1* loss of function cells (prehair formation was not delayed), but *cno-IR* expression likely results in a hypomorphic phenotype. In addition, compared to control GFP-expressing clones, *cno-IR* clones were fragmented. *cno-IR* clones did not form a single cohesive unit, but instead intermingled with surrounding wild-type cells (Fig.9C,D). This phenotype is remarkably similar to that observed with *Rap1* null clones.

To analyze Canoe's effect on epithelial cell shape and adherens junction localization, 36 hour APF wings containing *cno-IR* clones were dissected and stained for DE-cad. In these wings, DE-cad asymmetries were frequently observed, with abnormally high levels of DE-cad found between pairs of cells (Fig.9D, arrows). The regular hexagonal packing of wing epithelial cells was also disrupted by *cno-IR* expression in these wings. Based on these experiments, it is clear that in this developmental context *canoe* and *Rap1* phenocopy one another to a high degree. Since this combination of phenotypes is so unique (clone dispersal and DE-cad asymmetry in particular), this strongly suggests that Canoe functions together with Rap1 to control cell adhesion in the wing epithelium.

To demonstrate a more direct role for Canoe in Rap1 developmental processes we next asked whether reducing *canoe* function could worsen a hypomorphic *Rap1* phenotype. When a single copy of *canoe* was removed from the *ptc>Rap1-IR* genetic background, wing hair misalignment significantly increased (median angle variance=333.1) (compare Fig.8A and Fig. 9B). *canoe* did not interact with the Gal4 driver alone (data not shown).

Although Canoe has been previously linked to Ras in cone cell development and ommatidial rotation (Gaengel and Mlodzik, 2003; Matsuo et al., 1997), our results led us to ask whether Canoe could also be linked to Rap1 in the eye. We therefore stained discs containing *Rap1*- clones of cells with anti-Canoe. In wildtype ommatidia Canoe is enriched at junctions between

photoreceptors in a pattern similar to that of DE-cad. When *Rap1* mutant clones were generated in the developing eye disc, Canoe enrichment at these cell junctions was eliminated (Fig.9E). Taken together, these data suggest that Canoe functions as a critical effector of Rap1 signaling in both the *Drosophila* wing and eye to regulate epithelial cell shape, cellular differentiation and tissue morphogenesis.

Discussion

Rap1 affects Egfr-dependent developmental processes

In *Drosophila* imaginal discs, low levels of Egfr signaling are required for growth and survival of developing epithelial cells (Clifford and Schupbach, 1989; Diaz-Benjumea and Hafen, 1994; Prober and Edgar, 2000), while high pathway activity is found in spatially restricted patterns and directs the differentiation of specific cell types (Freeman, 1996; Guichard et al., 1999; Kumar et al., 1998; Martin-Blanco et al., 1999; Yang and Baker, 2001). In the experiments presented here, we have demonstrated that *Rap1* loss-of-function phenotypes resemble *Egfr* hypomorphic mutations in both the wing and eye. Following these initial observations, we investigated in greater depth the nature of these defects, asking first whether Rap1 was directly affecting Egfr signal transduction. Our data indicates that *Rap1* mutant cells are fully capable of responding to an Egf signal, as Spitz expression in *Rap1* clones results in high Egfr/Ras/MAPK pathway activity (as measured by dpERK levels, and loss of DSRF expression). Moreover, in wing and eye imaginal discs, loss of Rap1 does not affect the pattern of dpERK localization. At this early stage of development, therefore, vein cells and ommatidial precursors in the furrow (intermediate groups) are specified appropriately in the absence of Rap1 function. Interestingly, in these contexts Rap1 does not have dramatic effects on cell adhesion; *Rap1* clones in the wing disc do not scatter, and DE-cad localization is unaffected in the eye furrow. At later stages of development, however (posterior to the furrow, and the pupal wing), adhesive deficits associated with *Rap1* loss-of-function are manifest and Egfr-dependent cell types are lost. In the pupal wing, *Rap1* mutant cells scatter, the Egfr signal is not maintained, and wing veins fail to differentiate. Similarly in the eye, abnormal adhesive contacts between ommatidial precursors are observed as they exit the furrow. As a result, cells within a developing ommatidium likely fail to maintain their relative positions, photoreceptor/cone cell recruitment is impaired, and an incomplete ommatidium differentiates. In these contexts, therefore, Rap1 does not activate the Egfr/Ras/MAPK signaling cascade to specify cell fate, but instead regulates and/or maintains adhesive contacts necessary for the continued receipt of an Egfr signal.

Why are Egfr-dependent cell types particularly sensitive to Rap1 loss? In several developmental contexts, including the *Drosophila* wing, eye and trachea, it has been demonstrated that Egfr/Ras signaling up-regulates DE-cad levels (both transcriptionally and post-translationally) (Brown et al., 2006; Cela and Llimargas, 2006; O'Keefe et al., 2007). Loss of Egfr signaling reduces DE-cad levels, often leading to the loss of epithelial integrity. When DE-cad levels are manipulated, Egfr-dependent cell types are often affected (even when epithelial integrity is maintained). In particular, reduction of DE-cad results in photoreceptor loss, ommatidial rotation defects (Mirkovic and Mlodzik, 2006), and wing vein discontinuities (O'Keefe et al., 2007). These data suggest that DE-cad functions to stabilize or maintain Egfr/Ras/MAPK signaling between cells. In the *Drosophila* eye this makes intuitive sense as cells with highest levels of MAPK activity (ommatidial precursors) form tightly adhesive clusters of cells, segregating themselves from the rest of the epithelium as differentiation proceeds. In *Rap1* mutant cells the ability of Egfr/Ras/MAPK signaling to positively affect DE-cad-mediated adhesion is clearly compromised. As a result, Egfr signaling between cells is weakened, and/or not maintained, and Egfr-dependent cell types are lost. In this way, Ras and

Rap1 act in parallel to regulate DE-cad-mediated adhesion, facilitating consistent responses to developmental signals, and the appropriate differentiation of cell types within an epithelium.

Rap1 is necessary for planar cell polarity

PCP signaling orients large groups of cells within the plane of an epithelium allowing for the coordinated differentiation of asymmetric structures. For example, in the wing epithelium each cell generates a single hair at its distal edge that points distally, and mutations in the planar cell polarity network of proteins result in wing hair misalignment, and often problems in wing hair formation. Briefly, a core set of PCP proteins (including Frizzled, Disheveled, Prickle, Strabismus, Starry night and Diego) becomes asymmetrically distributed along the proximal/distal axis of each wing cell. While the precise mechanisms by which these protein asymmetries are established remain unclear, a number of effector proteins have been identified that translate the asymmetric PCP signal into the polarized formation of actin-based wing hairs (Klein and Mlodzik, 2005; Seifert and Mlodzik, 2007; Zallen, 2007). These same genes direct ommatidial alignment in the eye.

Here we identify Rap1 as a novel effector of the PCP signaling pathway in *Drosophila*. In the eye, PCP signaling determines chirality (based on R3/R4 cell fate specification) as well as the direction of ommatidial rotation. *Rap1* mutant cells cause very subtle defects in chirality (data not shown), but dramatically affect ommatidial rotation. In other words, transmission of the PCP signal through the eye epithelium is essentially unaffected in Rap1 mutant tissue, but the adhesive/cytoskeletal response to PCP is compromised. In the eye, therefore, Rap1 primarily acts as a PCP effector. What are the mechanisms by which Rap1 affects PCP in the eye? Current evidence suggests that additional signaling inputs are required downstream of the PCP genes to mechanically drive the rotation process (*nemo* for example) (Choi and Benzer, 1994). One signaling pathway that acts downstream of PCP (or in parallel) to control ommatidial rotation is the Egfr pathway, as Egfr signaling has previously been shown to affect ommatidial rotation (Brown and Freeman, 2003; Gaengel and Mlodzik, 2003; Strutt and Strutt, 2003). It is possible that *Rap1* mutant cells mis-rotate because Egfr signaling is compromised, however this is not the only possibility. Rap1 also regulates DE-cad/ adherens junction localization in the developing eye and it has been demonstrated that DE-cad promotes ommatidial rotation (Mirkovic and Mlodzik, 2006). It is most likely, therefore, that Rap1 maintains/regulates DE-cad-mediated adhesive contacts necessary for ommatidial rotation, and in this way effects PCP in the eye.

The mechanisms by which Rap1 affects PCP in the wing, however, are less clear, as Egfr (data not shown) and DE-cad do not play clear roles in this process. Adult wings containing *Rap1* mutant cells often contained disorganized patches of wing hairs, and when pupal wings were examined, prehair formation was disrupted. It has been previously shown that many PCP genes (including the core components) have non-autonomous effects on wing hair formation, however this was not the case for *Rap1*. In the pupal wing, wildtype cells immediately adjacent to *Rap1* mutant clones had normal prehairs. Loss of Rap1 also did not affect localization of the core PCP protein Stan, and when prehairs formed in *Rap1* mutant cells, they were found at the distal edge. These data indicate that *Rap1* mutant cells are polarized appropriately. As in the eye, therefore, Rap1 does not affect propagation of the PCP signal through the wing epithelium, but instead acts as a local, cell-autonomous PCP effector, translating cell polarity into a coordinated cytoskeletal response.

We demonstrated genetic interactions between a hypomorphic *Rap1* phenotype in the wing (*ptc>Rap1-IR*) and several PCP genes. Loss of a single copy of *starry night* (a core PCP gene), *double time* (a PCP regulator), or *multiple wing hairs* (a PCP effector) all exacerbated wing hair misalignments associated with the *ptc>Rap1-IR* genotype. Among these factors, the strongest interaction was seen between *Rap1* and *multiple wing hairs* (*mwh*), consistent with

Rap1 acting as a PCP effector. In adult and pupal wings containing *Rap1* mutant clones, multiple wing hairs were frequently observed (similar to the *mwh* phenotype). Interestingly, *mwh* has recently been cloned (Strutt and Warrington, 2008; Yan et al., 2008), and found to encode a GTPase-binding domain/formin homology 3 (GBD/FH3) domain containing protein, functioning to inhibit actin filament formation. Although our data suggests that Rap1 does not directly affect Mwh localization, it will be interesting to see whether Rap1 regulates Mwh activity to control actin dynamics essential for the polarized formation of wing hairs. Ommatidial rotation is also perturbed in mutants for the *Drosophila* Myosin II, Zipper (Fiehler and Wolff, 2007), also implicating actin dynamics in this process. Thus, the effects of Rap1 on PCP readouts in both the wing and eye could be connected at the level of the actin cytoskeleton.

The most striking genetic interaction with *ptc>Rap1-IR*, however, was seen with *DE-cad/shg*, suggesting that the adhesive and cell shape phenotypes associated with *Rap1* disrupt PCP signaling in the wing. In fact, it has recently been demonstrated that subtle defects in epithelial cell packing (associated with the posterior crossvein, or loss of PTEN) exacerbate polarity phenotypes associated with *fat* mutant clones (Ma et al., 2008). It is not surprising, therefore, that extreme cell shape defects associated with *Rap1* loss-of-function clones affect wing hair alignment. Rap1 PCP phenotypes are more severe than those described by Ma et al., however, as wing hairs associated with *Rap1* mutant cells are not swirled, but instead are randomly oriented.

Deciphering the mechanisms by which Rap1 regulates DE-cad localization, therefore, is critical to understanding Rap1 function in this context and others (cancer cell metastasis in particular). An intriguing link between Rap1 and components of the exocyst has recently been established (Frische et al., 2007). Since the exocyst is involved in recycling E-cad from endosomes to the plasma membrane (Langevin et al., 2005), it is possible that Rap1 interacts with exocyst components to control recycling of DE-cad-based adherens junctions, promoting the making and breaking of cell contacts necessary for hexagonal packing of wing epithelial cells and ommatidial rotation. In the pupal wing, evidence suggests that PCP factors regulate the polarized trafficking of DE-cad-containing exocyst vesicles to coordinate epithelial cell shape (Classen et al., 2005). Therefore, Rap1 may serve as the critical link between PCP factors and the exocyst in this developmental context, a hypothesis to be tested in the future.

Canoe is a Rap1 effector in the *Drosophila* wing and eye

The protein encoded by AF-6/Canoe includes two N-terminal Ras association (RA) domains, a phospho-peptidebinding Forkhead-Associated domain (FHA), a DIL domain found in myosins that likely mediates protein-protein interactions, and a PDZ domain involved in protein-protein interactions. In developing epithelia, it has been demonstrated that AF-6/Canoe functions as a scaffolding protein (linking proteins together via its multiple protein-protein interaction domains) to regulate adhesion between cells and the actin cytoskeleton. Both AF-6 and Canoe bind the junctional protein ZO-1/Polychaetoid, and localize in vivo to tight or adherens junctions (Takahashi et al., 1998; Yamamoto et al., 1997). Consistent with this role, mouse embryos lacking AF-6 are not viable due to severe defects in cell adhesion and cell polarity (Zhadanov et al., 1999).

It has been demonstrated that localization of the AF-6/ZO-1 complex to cell junctions is a regulated process. The GTP-bound form of Ras binds the RA domains of AF-6 (Kuriyama et al., 1996), causing dissociation of the AF-6/ZO-1 complex, and loss of both proteins from cell junctions (Boettner et al., 2000; Yamamoto et al., 1997). In this way, AF-6 acts as an effector of the Egfr/Ras signaling pathway at points of cell contact. This hypothesis is supported in vivo as Canoe plays a critical role in Egfr/Ras-dependent developmental processes in the *Drosophila* eye (Gaengel and Mlodzik, 2003; Matsuo et al., 1997). While this data suggests

that AF-6/Canoe acts downstream of Ras, it has subsequently been demonstrated that AF-6/Canoe binds more effectively to Rap1 (Boettner et al., 2000; Boettner et al., 2003; Linnemann et al., 1999; Su et al., 2003). In several contexts, including dorsal closure of the *Drosophila* embryo (Boettner et al., 2003), activity-dependent remodeling of dendritic spines in neurons (Xie et al., 2005), and integrin-based adhesion in cultured cells (Su et al., 2003), functional connections between Rap1 and AF-6/Canoe have been demonstrated. It seems, therefore, that AF-6/Canoe functions downstream of multiple GTPases, to control cell adhesion. Finally, GTPases such as Ras and Rap1 are not the only signal transducers to affect AF-6/Canoe. In various developmental systems it has been shown that Canoe interacts with the Notch, JNK and Wg signaling pathways as well (Carmena et al., 2006; Miyamoto et al., 1995; Takahashi et al., 1998). These results suggest that AF-6/Canoe functions as a critical scaffolding protein at cell junctions, binding multiple signal transduction effectors and integrating a complex array of signals to affect adhesion and actin dynamics of developing cells.

In the studies described here, we identify two novel roles for *canoe* in the *Drosophila* wing epithelium, both of which involve Rap1. First, *canoe* loss-of-function cells had adhesive defects. As opposed to wildtype cells that maintain adhesive contacts with their neighbors as they grow and divide, *canoe* mutant cells dispersed, resulting in highly fragmented clones of cells. The adherens junctions of these cells were not evenly distributed, but instead concentrated at one cell-cell interface. In this way, pairs of *canoe* mutant cells adhered tightly to one another, scattered into the adjacent wildtype epithelium, and disrupt the characteristic, hexagonal packing of wing epithelial cells. This phenotype is very unusual and phenocopies *Rap1* in this context. These results strongly suggest that Canoe acts as a Rap1 effector in the developing wing, controlling the even distribution of adherens junctions about the apical circumference of epithelial cells. Secondly, we demonstrate a role for *canoe* in the planar cell polarity pathway. Signaling through the planar cell polarity pathway is necessary for appropriate wing hair formation and alignment. Adult wings containing *canoe* mutant clones of cells often had disorganized patches of wing hairs, and at pupal stages, *canoe* mutant cells were defective in actin-rich prehair formation. In this context *Rap1* and *canoe* genetically interacted, as removal of a single copy of *canoe* dramatically worsened wing hair alignment in a hypomorphic *Rap1* background. Canoe, therefore, acts together with Rap1 as an effector of the planar cell polarity pathway, likely affecting actin dynamics essential for wing hair formation and alignment.

Supplementary Material

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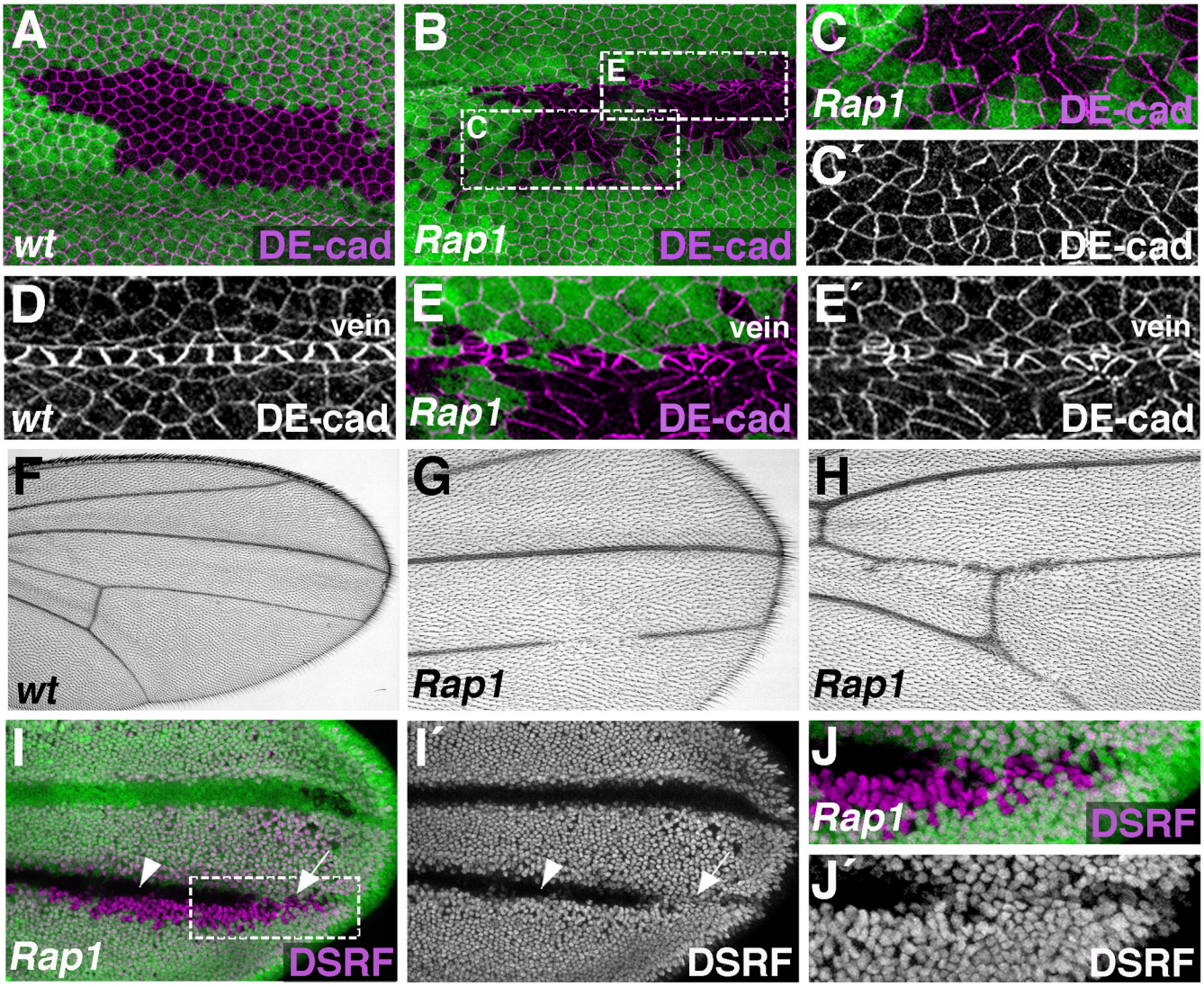

Figure 1. Rap1 regulates epithelial cell shape, DE-cad localization, and wing vein integrity

(A–J) The *Flp/FRT* system was used to generate either wildtype (A,D,F), or *Rap1* mutant (B,C,E,G–J) clones of cells. (A–E) Pupal wings (36 hours APF) stained for DE-cad. (A) In wildtype clones (GFP-negative), cells are generally hexagonal, and form a single cohesive unit. Green marks GFP-positive cells. (B) *Rap1* mutant cells (GFP-negative) are irregularly shaped and intermingle with neighboring wildtype cells. In addition, DE-cad is asymmetrically distributed in *Rap1* mutant cells. (C) Enlarged view of boxed region in B is shown. (D) Wildtype vein cells are morphologically distinct from surrounding intervein cells. (E) Loss of Rap1 severely disrupts vein cell morphology. Enlarged view of boxed region in B is shown. Compared to control adult wings (F), wings containing *Rap1* mutant clones of cells have loss of wing vein material (G,H). (I) Pupal wing (36 hours APF) stained for DSRF, a marker of intervein cell fate. *Rap1* mutant clones of cells (GFP-negative) are associated with ectopic DSRF expression (arrow), however many *Rap1* mutant cells remain DSRF-negative (arrowhead). (J) Enlarged view of boxed region in I is shown.

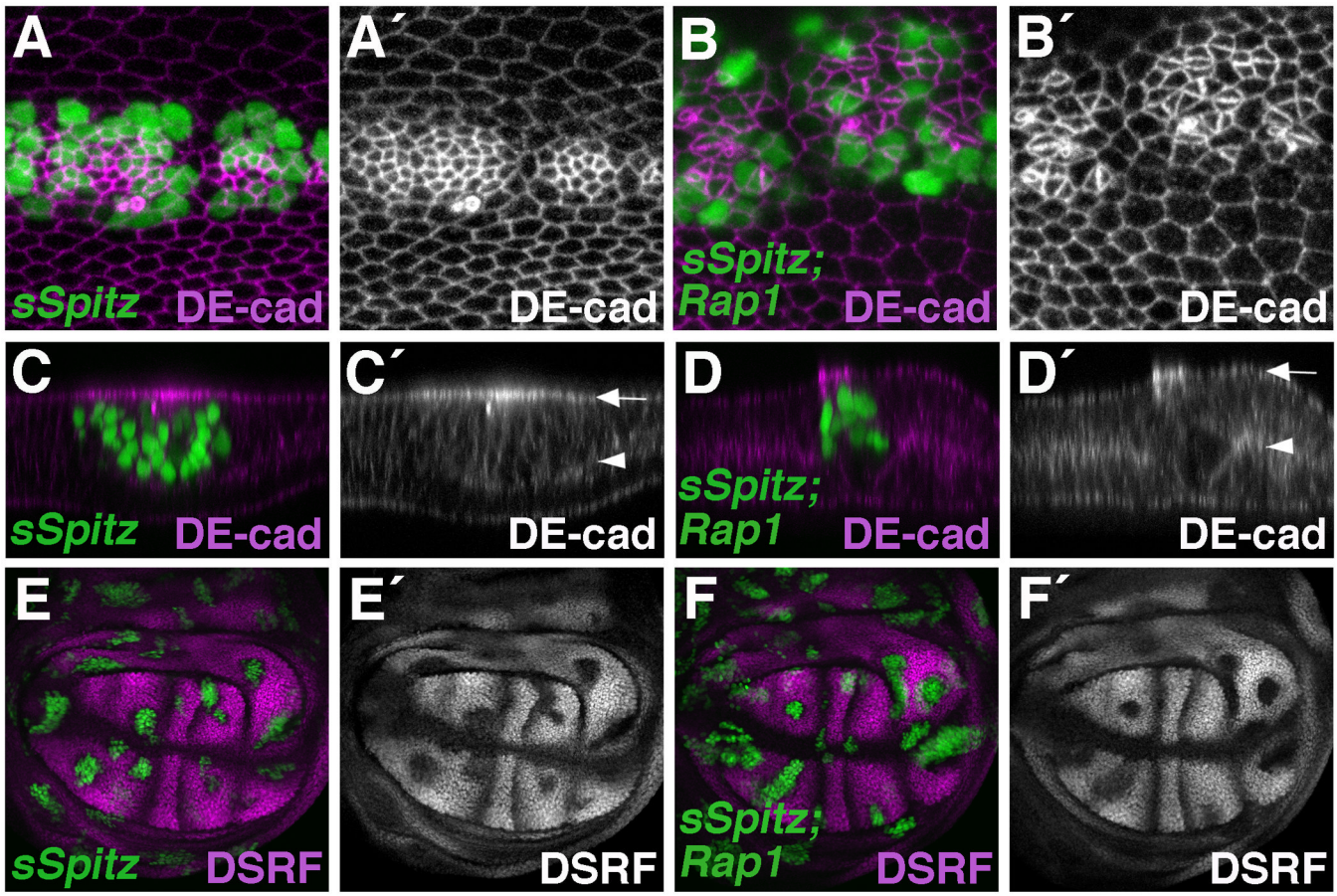

Figure 2. Rap1 and Egfr signaling affect different aspects of DE-cad localization

(A–F) The MARCM system was used to express a secreted version of Spitz (sSpitz) in wildtype (A,C,E) or *Rap1* mutant (B,D,F) cells. (A–D) Pupal wings (36 hours APF) stained for DE-cad are shown. (A) sSpitz expressing cells (GFP-positive) have high levels of DE-cad and are apically constricted compared to their wildtype neighbors. (B) Loss of Rap1 dramatically affects DE-cad localization in sSpitz expressing cells. (C,D) Optical cross-sections through the bi-layered wing epithelium are shown. Arrows and arrowheads indicate apical and basal surfaces of the dorsal wing epithelia respectively. (C) In sSpitz expressing cells, DE-cad is concentrated near the apical cell surface (apico-laterally). (D) Loss of Rap1 does not affect the apical/basal distribution of DE-cad in sSpitz expressing cells. (E,F) Wing imaginal discs stained for the intervein marker DSRF are shown. (E) sSpitz expressing cells downregulate DSRF indicating a switch to vein cell fate. (F) Loss of Rap1 does not affect the ability of sSpitz to downregulate DSRF.

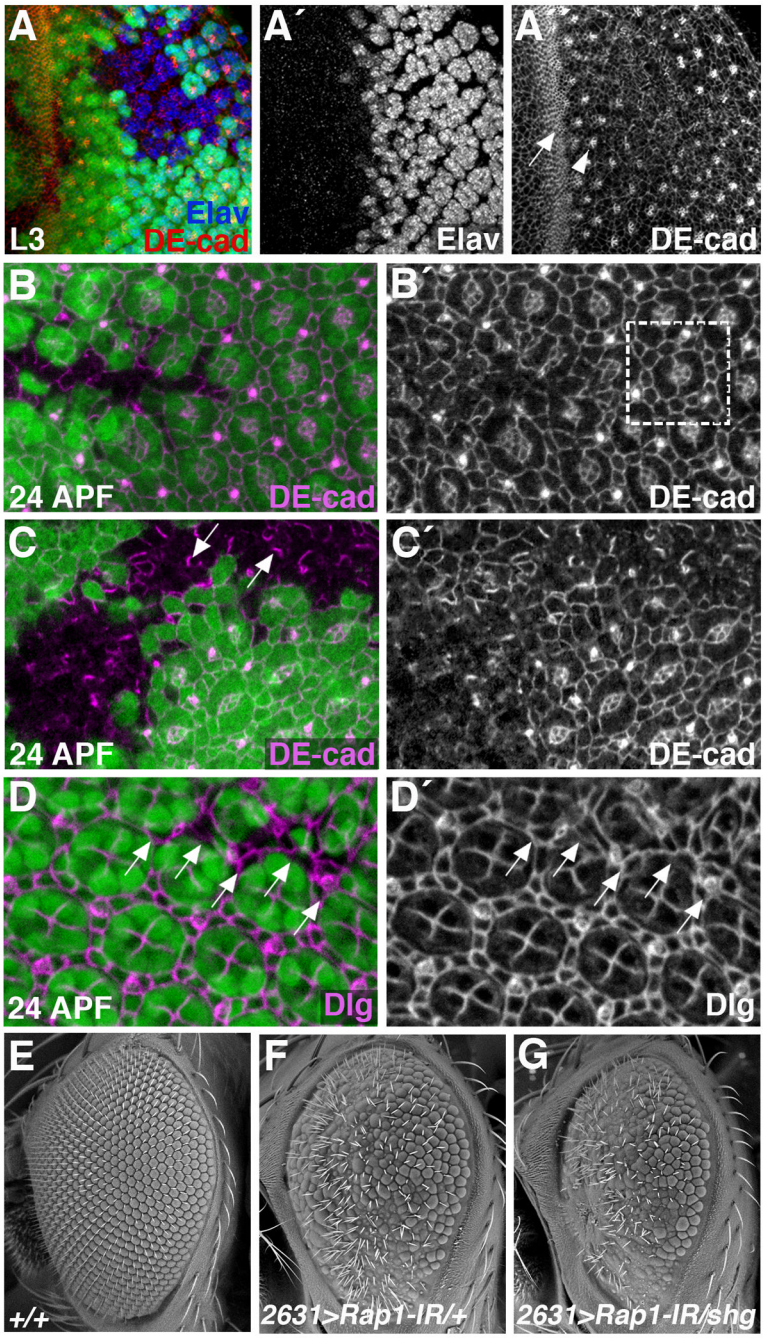

Figure 3. Rap1 affects cell adhesion and cell shape in the developing eye

(A–D) Clones of cells lacking *Rap1* (GFP negative) were generated using the *Flp/FRT* system. Eye tissue from late L3 larvae (A) or 24 hour APF pupae (B–) were stained for DE-cad (A–C) or Discs Large (Dlg) (D). (A) During larval stages, DE-cad levels are highest in the morphogenetic furrow (arrow) and at junctions between photoreceptor precursors (arrowhead). In *Rap1* mutant cells, photoreceptors are still specified (A´), but the pattern of DE-cad localization is altered (A´´). (B,C) At 24 hours APF, DE-cad localization in the eye demarcates a regular lattice of cone and pigment cells. Boxed region in B´ indicates a single ommatidial unit. Loss of *Rap1* disrupts DE-cad localization and the regular pattern of cell types. (C) Asymmetrical distributions of DE-cad are frequently observed in *Rap1* mutant tissue (arrows).

(D) Dlg localization similarly reveals a regular pattern of cone and pigment cells in wildtype 24 APF pupal eyes. Arrows point to *Rap1* mutant cells, which are morphologically defective. (E–G) SEM images of adult eyes are shown. *NP2631-Gal4*, when crossed to a *Rap1*-RNAi transgene (*Rap1-IR)*, knocks down *Rap1* function in most eye cells. Compared to wildtype eyes (E), *NP2631>Rap1-IR* eyes are rough, typically indicating a defect in cell-type specification or differentiation (F). (G) The *NP2631>Rap1-IR* phenotype is significantly worsened when a single copy of the *DE-cad/shg* gene is eliminated.

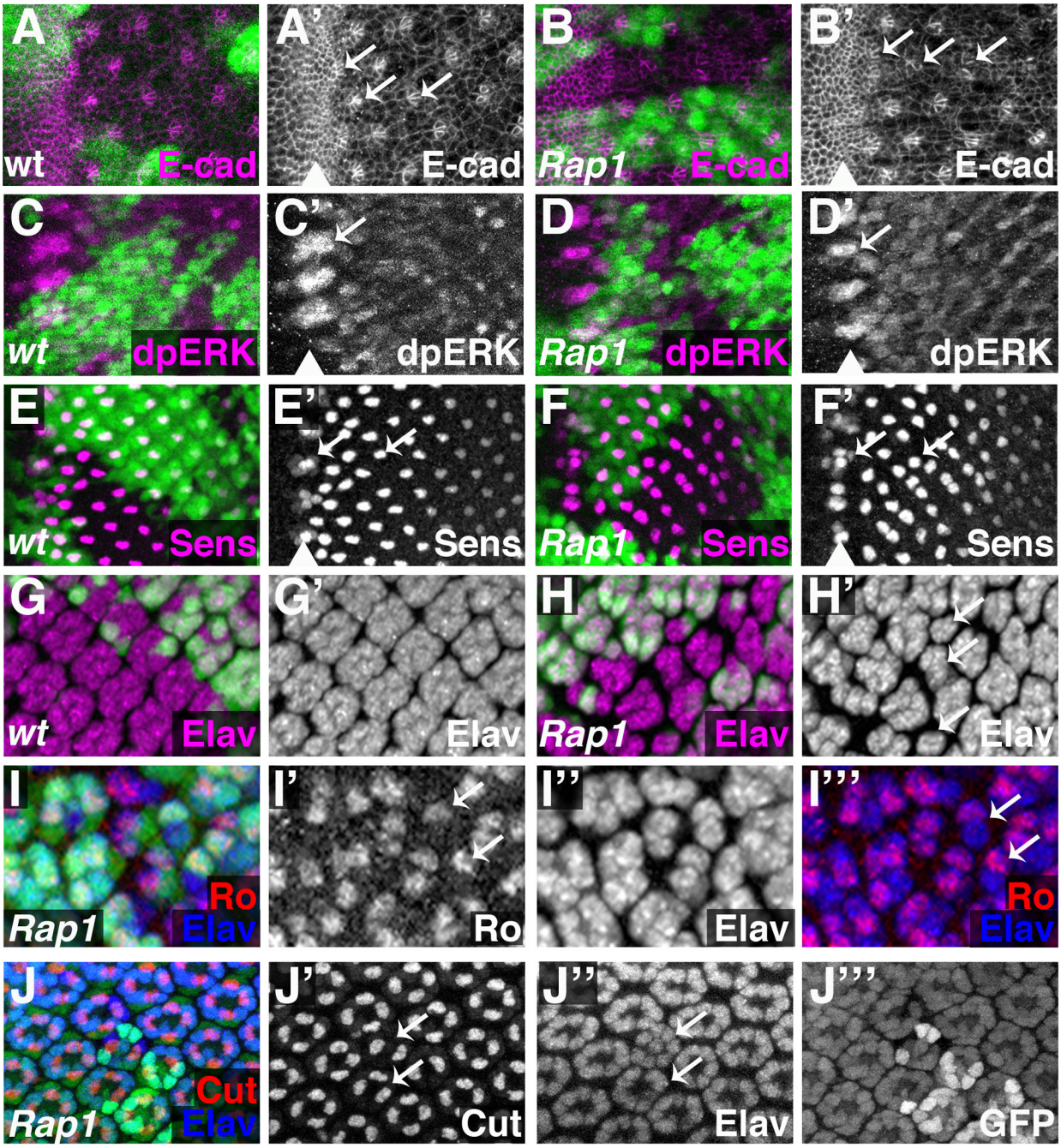

Figure 4. Rap1 is required for proper ommatidial cell development

Wildtype (A,C,E,G) or *Rap1* mutant (B,D,F,H–J) clones of cells (GFP negative) were generated using the *Flp/FRT* system. An *ey-flp* transgene was used to generate clones from the earliest stages of eye development. Larval eye imaginal discs were stained for E-cadherin (E-cad) (A,B), dpERK (C,D), Sens (E,F), Elav (G,H), Ro/Elav (I). 40-hour APF pupal disc was stained for Cut/Elav (J). An arrowhead marks the position of the morphogenetic furrow (A'–F'). (A) E-cadherin is expressed at high levels in the furrow. Cells in the furrow form rosettes (left arrow), which reorganize into arcs (middle arrow); the arcs close to form clusters, each of which is the precursor of an ommatidium. E-cadherin is maintained at high levels in arcs and clusters, but not in intervening cells. (B) E-cadherin staining reveals that cells in *Rap1*-

tissue form morphologically abnormal arcs and clusters (arrows). (C) In wild-type eye discs, high levels of di-phosphorylated MAPK (dpERK) are present in intermediate groups, with lower levels behind the furrow. (D) dpERK levels and spacing intermediate groups is not affected by *Rap1*- clones. (E) Senseless is expressed in R8 equivalence groups (left arrow) and at high levels in R8 (an Egfr-independent cell type). (F) Senseless expression in R8 equivalence groups is normal, and Senseless-expressing R8 cells are present in *Rap1* mutant clones, although their spacing is altered. (G) Elav marks post-mitotic neurons, normally found in clusters posterior to the morphogenetic furrow. (H) In *Rap1* mutant clones, Elav clusters are often irregularly shaped (arrows), suggesting a loss of photoreceptor precursors. Rough most strongly labels R2 and R5, and ommatidia containing a single Rough positive cell are frequently found in and around *Rap1* mutant tissue (I, arrows). Cut localizes to cone cells, which are frequently lost from *Rap1* clones (J), including from ommatidia containing a full complement of photoreceptors (arrows).

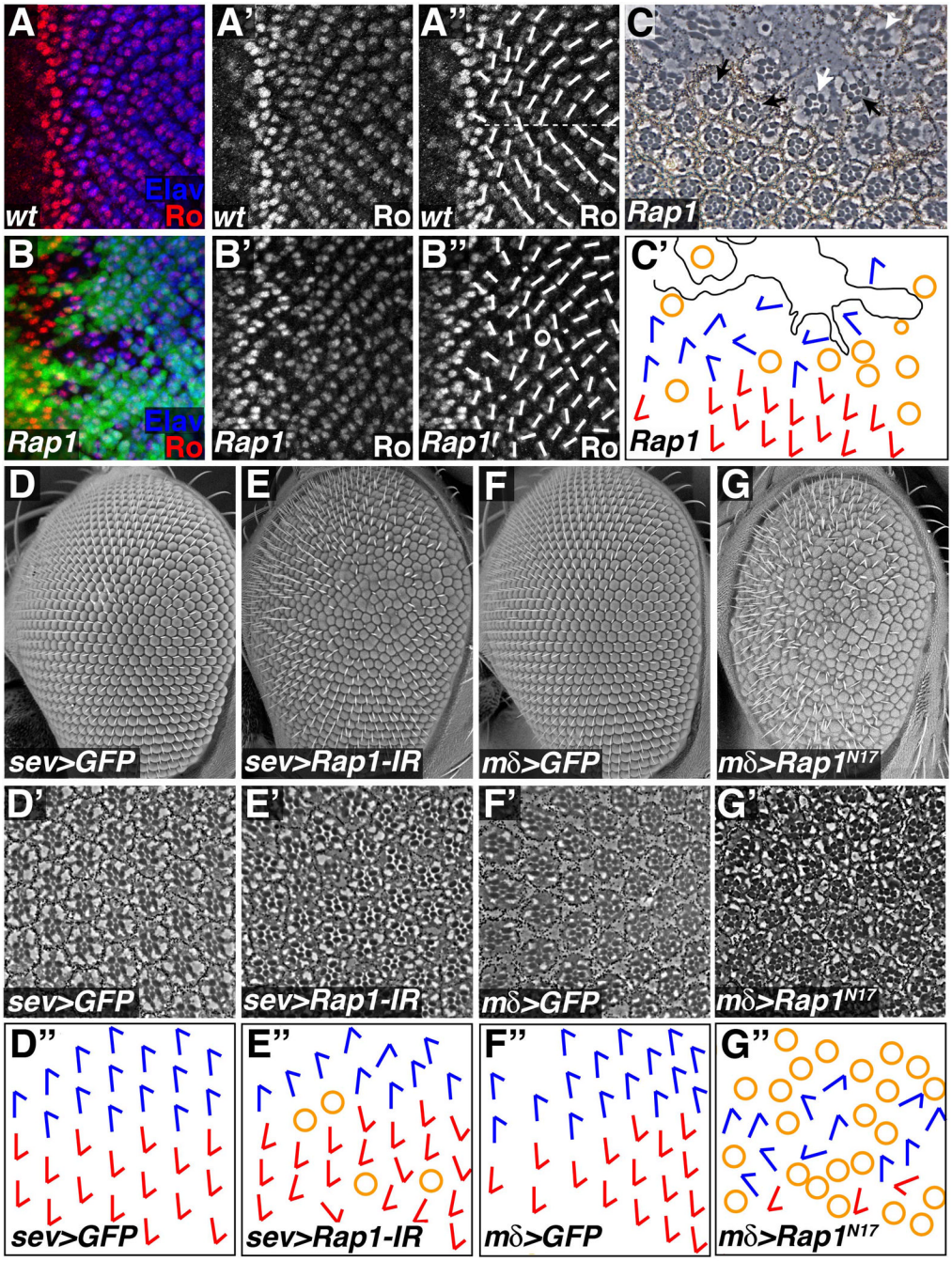

Figure 5. Rap1 affects ommatidial rotation in the developing eye

(A,B) Eye imaginal discs were stained for Rough (Ro) and Elav. Short white lines indicate ommatidial orientation, while the horizontal dotted line marks the equator. (A) In wildtype discs a regular pattern of ommatidial orientation is apparent. (B,C) Clones of cells lacking *Rap1* (GFP negative) were generated using the *Flp/FRT* system. (B) *Rap1* mutant cells disrupt the overall pattern of ommatidial rotation. Circle denotes an ommatidium with abnormal numbers of photoreceptors. (C) In tangential sections of adult eyes *Rap1* mutant cells lack pigment. Scar-like tissue often formed in *Rap1* mutant clones. At the edges of clones misaligned ommatidia containing *Rap1* mutant cells (white arrow), or entirely wildtype cells (black arrows) could be found. White arrowhead indicates an ommatidium containing Rap1

mutant cells that has a normal alignment. (C′) Schematic representation of the section in C is shown. Blue and red arrows indicate ommatidial orientation; yellow circles indicate incomplete ommatidia; black line demarcates scar tissue. (D–G) Loss and gain of *Rap1* function in subsets of cells also disrupts ommatidial rotation. SEM micrographs (D–G), tangential sections (D′–G′), and schematic representations of ommatidial orientation (D′′–G′′) are shown for each genotype. (E) *sev-Gal4* in combination with *Rap1-IR* was used to knock down Rap1 function in a subset of differentiating photoreceptors (R3, R4, R7) and cone cells. (G) Similarly, *mδ0.5-Gal4* was used to express $Rap1^{N17}$ in R4 and weakly in R3 and R7. In both cases, reduction of Rap1 activity significantly increased ommatidial misalignment when compared to controls (D and F respectively).

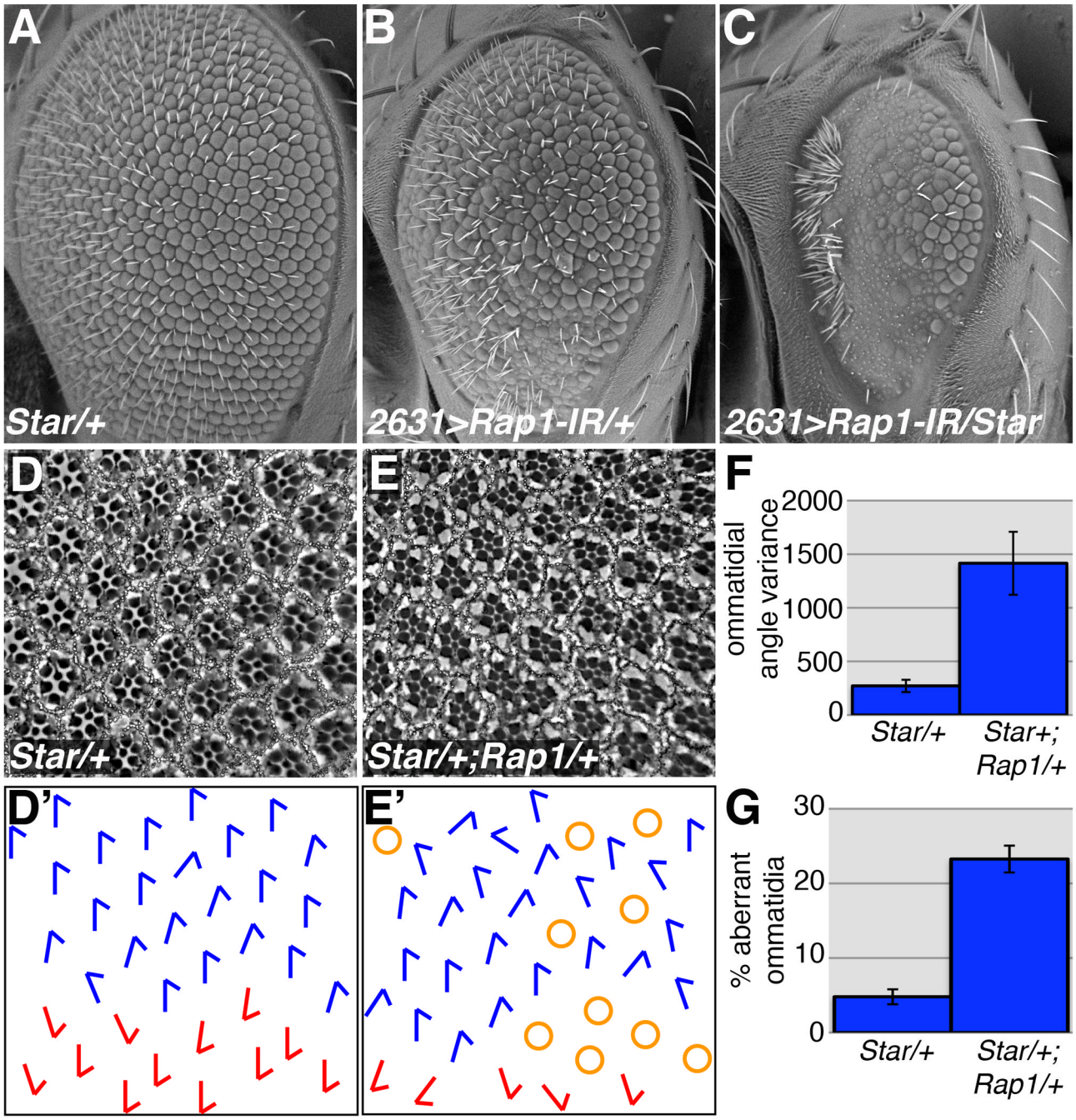

Figure 6. *Rap1* and *Star* genetically interact

Star is a positive regulator of Egf signaling, and $Star^{48\text{-}5}$ heterozygotes have a subtle effect on adult eye architecture (A). The rough eye phenotype associated with *NP2631>Rap1-IR* (B) is dramatically worsened when a single copy of $Star^{48\text{-}5}$ is eliminated (C). (D) When sections through adult $Star^{48\text{-}5}$/+ eyes are examined, ommatidia are slightly misaligned compared to wildtype. Loss of one copy of *Rap1* significantly enhances the $Star^{48\text{-}5}$ adult eye phenotype (E). Tangential sections through adult eyes (D,E) and schematic diagrams of ommatidial orientation (D´,E´) are shown for each genotype. (F,G) Quantifications of the *Rap1, Star* genetic interaction are shown. Loss of one copy of *Rap1* significantly increases ommatidial

misalignment (F), and the percentage of aberrant ommatidia associated with *Star*$^{48\text{-}5}$ heterozygotes ($p<0.05$).

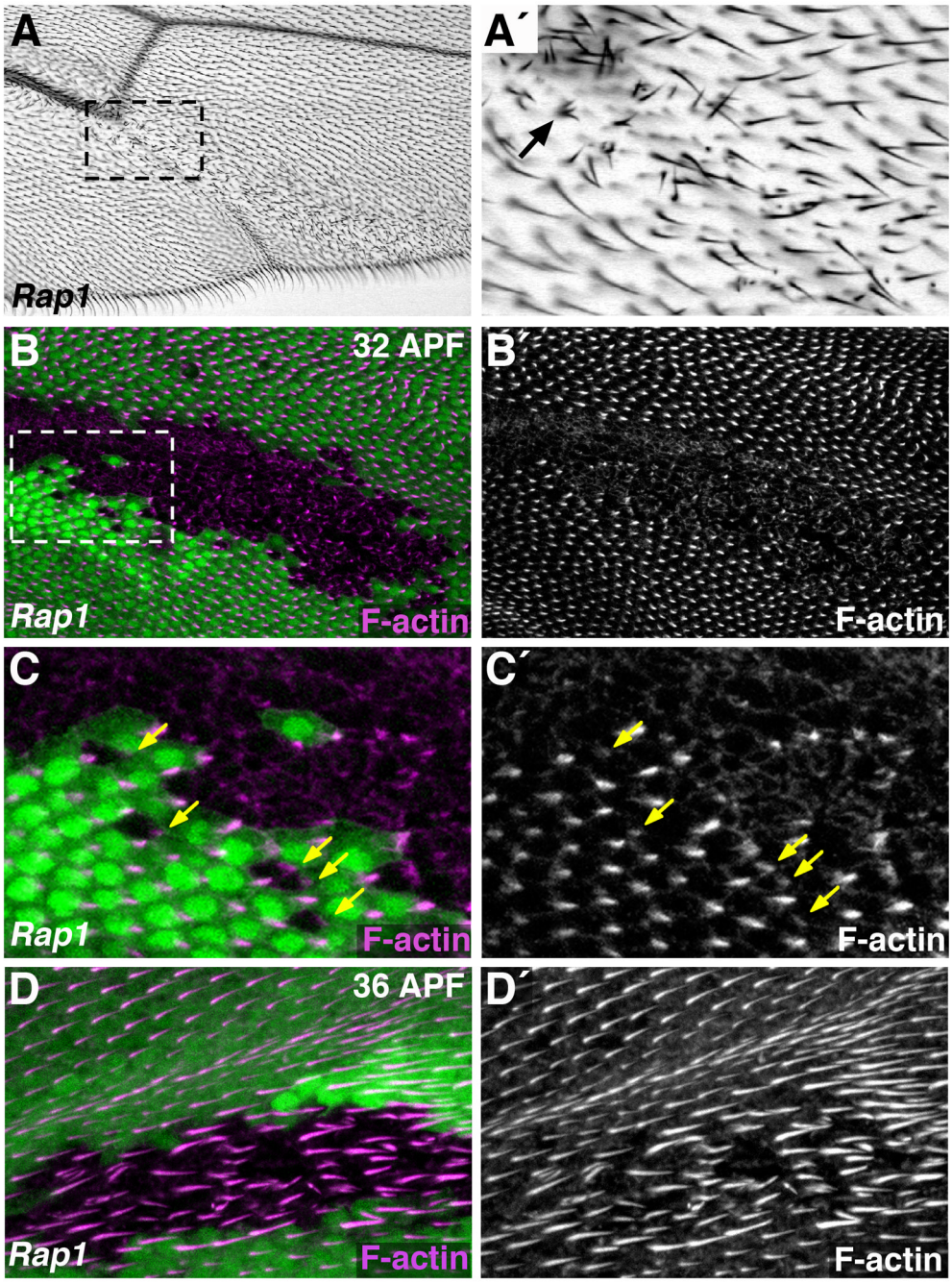

Figure 7. Rap1 is necessary for the polarized formation of wing hairs

(A–D) The *Flp/FRT* system was used to generate *Rap1* mutant clones of cells. (A) Adult wings containing *Rap1* mutant clones of cells often have patches of disorganized wing hairs. These hairs do not point distally, as in wildtype cells, and multiple hairs seem to form from single wing cells (arrow, A´). (A´) Magnified view of boxed region in A is shown. (B–D) Fluorescently labeled phalloidin was used to visualize F-actin (highly enriched in wing hairs) during pupal stages. (B) At 32 hours APF *Rap1* mutant cells (GFP-negative) are defective in pre-hair formation. (C) Magnified view of boxed region in B is shown. Wildtype cells adjacent to *Rap1* mutant clones form normal prehairs, indicating a cell autonomous role for *Rap1*.

Arrows indicate defective prehairs of *Rap1* mutant cells. (D) At 36 hours APF irregular wing hair structures are associated with *Rap1* mutant cells (GFP-negative).

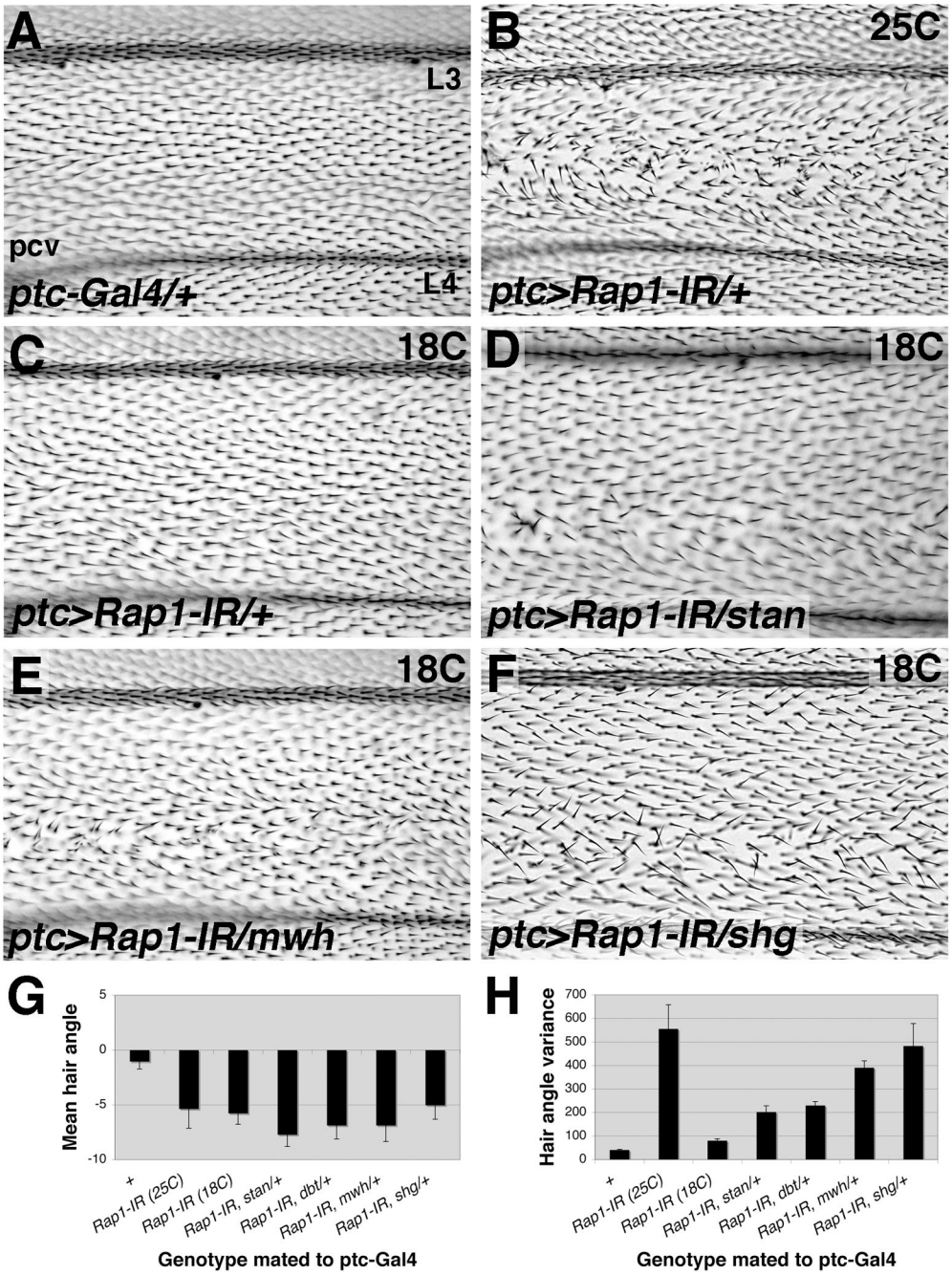

Figure 8. Genes involved in PCP signaling worsen a mild Rap1 phenotype

Compared to control wings (A) expression of Rap1-IR via ptc-Gal4 at 25°C results in wing hair misalignment between veins L3 and L4 (B). (C) At 18°C ptc>Rap1-IR results in a mild misalignment phenotype. Wing hair orientation becomes significantly more variable if one copy of stan (D), mwh (E), or DE-cad (encoded by the gene shg) is removed (F). For each image vein L3 (top), vein L4 and the posterior cross vein (pcv) are shown. (G) Mean wing hair angle quantification is shown. Zero degrees points to the right (distally), while negative values point down (posteriorly). (H) Quantification of wing hair angle variance is shown. Error bars indicate SEM.

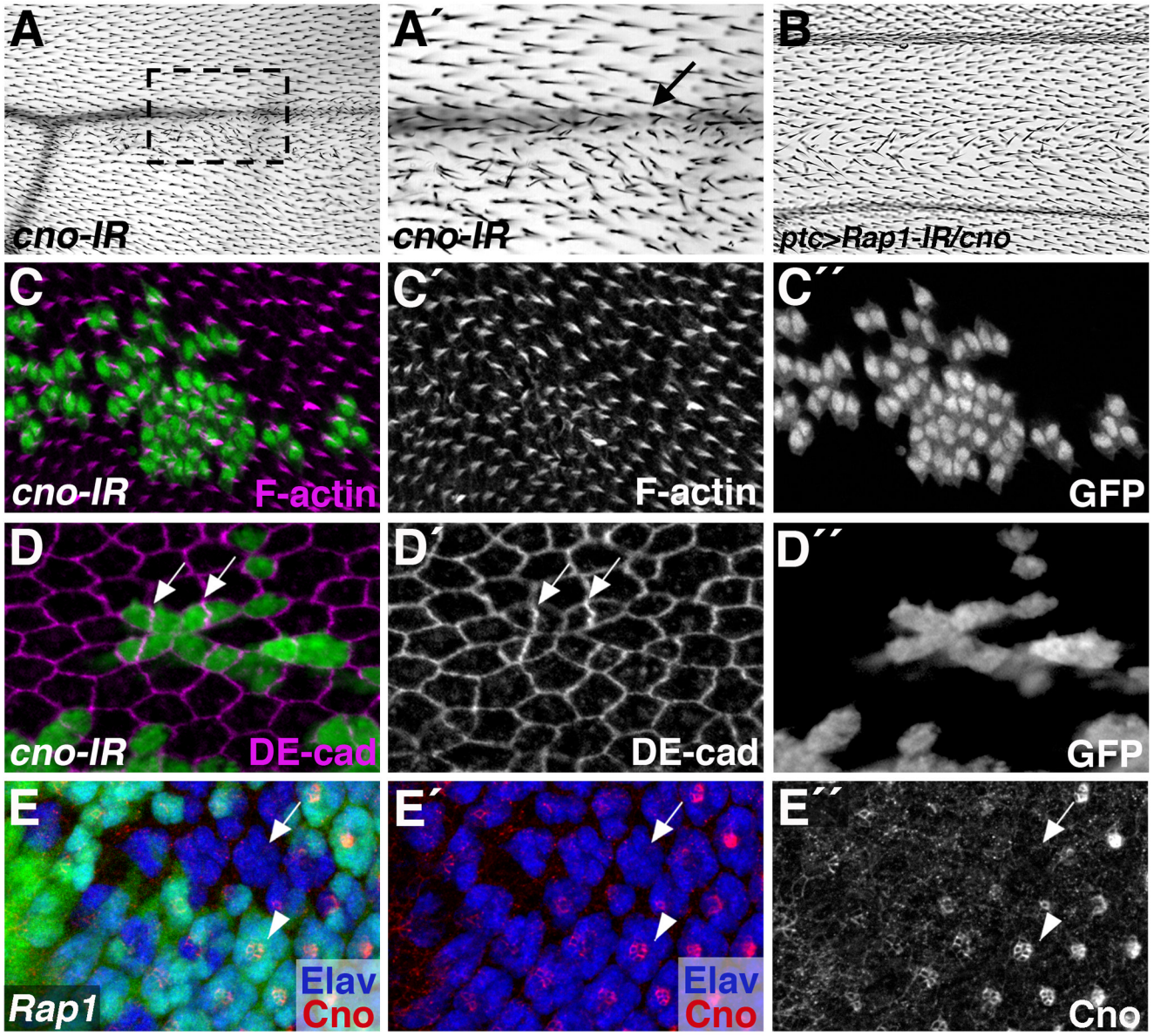

Figure 9. *canoe* exhibits a *Rap1*-like phenotype in the wing

(A,C,D) The *FRT/Gal4* system was used to express a *canoe*-RNAi transgene (*cno-IR*) in clones of cells. (A) Adult wings lacking *canoe* in clones of cells often have patches of disorganized wing hairs, and loss of wing vein material (arrow, A´). (A´) Magnified view of the boxed region in A is shown. (B) *canoe* genetically interacts with *Rap1*. A subtle PCP phenotype associated with *ptc>Rap1-IR* is exacerbated when one copy of *canoe* is removed. (C,D) In pupal wings, clones of cells expressing *cno-IR* are fragmented. (C) Wing prehair formation is abnormal in *cno-IR* cells (32 hours APF). (D) *cno-IR* expressing cells exhibit cell shape and DE-cad mislocalization phenotypes similar to *Rap1* loss of function (36 hours APF). Arrows indicate high levels of DE-cad found at the interface between two *cno-IR* expressing cells. (E) The *Flp/FRT* system was used to generate *Rap1* mutant clones of cells (GFP-negative) in the larval eye. Cno localization is disrupted in *Rap1* mutant cells. Arrow points to an ommatidium lacking Rap1, while arrowhead indicates a wildtype ommatidium.