Apical Accumulation of the Sevenless Receptor Tyrosine Kinase During *Drosophila* Eye Development Is Promoted by the Small GTPase Rap1

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ABSTRACT The Ras/MAPK-signaling pathway plays pivotal roles during development of metazoans by controlling cell proliferation and cell differentiation elicited, in several instances, by receptor tyrosine kinases (RTKs). While the internal mechanism of RTK-driven Ras/MAPK signaling is well understood, far less is known regarding its interplay with other corequired signaling events involved in developmental decisions. In a genetic screen designed to identify new regulators of RTK/Ras/MAPK signaling during *Drosophila* eye development, we identified the small GTPase Rap1, PDZ-GEF, and Canoe as components contributing to Ras/MAPK-mediated R7 cell differentiation. Rap1 signaling has recently been found to participate in assembling cadherin-based adherens junctions in various fly epithelial tissues. Here, we show that Rap1 activity is required for the integrity of the apical domains of developing photoreceptor cells and that reduced Rap1 signaling hampers the apical accumulation of the Sevenless RTK in presumptive R7 cells. It thus appears that, in addition to its role in cell–cell adhesion, Rap1 signaling controls the partitioning of the epithelial cell membrane, which in turn influences signaling events that rely on apico-basal cell polarity.

EPITHELIAL tissues rely on the complex interplay of diverse signaling events to adopt their appropriate shape, size, and function. Development of the *Drosophila* compound eye is a well-documented example of epithelial morphogenesis. Since its inception as an experimental system over 3 decades ago (Ready *et al.* 1976), its development has been intensively studied (Cagan 2009; Roignant and Treisman 2009). Over the years, this system has produced insights on various signaling pathways and cellular behaviors, and several of these have been found to lead to human diseases or syndromes when dysregulated.

The *Drosophila* eye is composed of hundreds of identical units called ommatidia, each comprising an equal complement of 26 cells [eight photoreceptor neurons (R1–R8), four cone cells, eleven pigments cells, and three mechanosensory bristle cells] (Kumar 2012). Mosaic analysis during eye de-

velopment has revealed the absence of a clonal relationship between differentiating cells (Ready *et al.* 1976). Instead, cell fate specification in each ommatidial cluster occurs in a stereotyped manner by inductive cues from neighboring cells that entail adhesive contacts and an elaborate network of signaling pathways, among which signaling through the EGFR/Ras/MAPK pathway remains one of the best understood events (Roignant and Treisman 2009). Except for the founding R8 photoreceptor cell present in each ommatidium, EGFR activity is required for the differentiation of all other retinal cells. Yet a second receptor tyrosine kinase (RTK), known as Sevenless (SEV), also participates in these developmental decisions, but only in the presumptive R7 cells (Freeman 1997).

In addition to the core members of the Ras/MAPK pathway (*e.g.*, Ras, RAF, MEK, and MAPK), genetic screens based on modulation of the R7 cell specification led to the identification of novel components that act as general regulators of RTK/Ras/MAPK signaling. Kinase Suppressor of Ras (KSR) is one of these and corresponds to a pseudokinase related to RAF family kinases (Therrien *et al.* 1995). KSR has the ability to bridge RAF and MEK together as well as to drive the catalytic activation of RAF through heterodimerization of their respective kinase domain (Udell *et al.* 2011).

Another component identified by genetic means and functionally related to RAF and KSR is Connector eNhancer of KSR (CNK) (Therrien *et al.* 1998). CNK is an evolutionarily conserved protein that comprises multiple protein- and lipid-interaction domains whose function in Ras/MAPK-mediated processes is also conserved (Claperon and Therrien 2007). Previous work has demonstrated the ability of *Drosophila* CNK to bind separately to RAF and KSR/MEK, thereby enabling the formation of a RAF/KSR/MEK complex (Douziech *et al.* 2006). In addition, CNK appears to have a dual role in the Ras/MAPK pathway by keeping RAF inhibited in the absence of an upstream RTK signal and by promoting RAF activation upon RTK signaling (Douziech *et al.* 2003). The mechanism governing these functions of CNK has been partly solved and requires the action of both Ras and the Src family kinase Src42A (Laberge *et al.* 2005). More recently, CNK has been shown to form a complex with Steppke, the fly cytohesin homolog, and collaborate in EGFR-induced MAPK activation by a mechanism that has yet to be characterized (Hahn *et al.* 2013). Whether CNK plays other roles outside the Ras/MAPK pathway in flies is currently not clear, although mammalian CNKs have been found to participate in signaling events regulated by other small GTPases, such as Rho, Rac, Ral, and Arf6 (Lanigan *et al.* 2003; Venkateswarlu 2003; Jaffe *et al.* 2005; Lim *et al.* 2010).

To identify functional partners of CNK that may reveal new RTK/MAPK pathway components or unveil other roles for this scaffolding protein, we conducted a genetic screen in the *Drosophila* eye to isolate dominant modifiers of a CNK-dependent rough eye phenotype. The screen led to the isolation of 24 complementation groups of enhancers including, as expected, several genes encoding *bona fide* components of RTK/Ras/MAPK pathways. Nine novel loci have also been identified. Interestingly, three of those (*Roughened*, *PDZ-GEF*, and *canoe*) encode Rap1-signaling components, which together suggest a functional relationship between CNK and Rap1 signaling. Previous work reported that Rap1 activity is required for adherens junction formation and adhesive contacts between developing photoreceptors (O'Keefe *et al.* 2009). This phenomenon appeared to be critical for maintaining EGFR signaling between cells, although Rap1 activity did not seem autonomously required for the commitment of any cell type. A variation of this model was recently proposed when a detailed genetic analysis of Rap1 requirement demonstrated its essential cell-autonomous role in presumptive R7 cells (Mavromatakis and Tomlinson 2012). However, the mechanism by which Rap1 promotes R7 cell differentiation has not been determined.

Here, we show that Rap1 activity induces and/or maintains the formation of the apical domain of developing photoreceptor cells and that this event correlates with the apical accumulation and activity of SEV in presumptive R7 cells. These results suggest that Rap1 signaling controls cell polarity and thereby influences signaling events that rely on plasma membrane partitioning.

Materials and Methods

CNK C-terminal-dependent screen, mapping, and sequencing

A Kpn1-Not1 PCR fragment encompassing amino acids 382–1557 [CNK C-Terminal, (CCT)] was cloned into the *psE* vector (Dickson *et al.* 1992) and introduced into the w^{1118} background by *P*-element-mediated germline transformation as described previously (Rubin and Spradling 1982). For the mutagenesis, w^{1118} males isogenic for the second and the third chromosomes were fed overnight with a 1% sucrose solution containing 25 mM ethyl methanesulfonate (EMS). Mutagenized males were then crossed with either *CyO, P[sE-CCT]/Adv* or *TM3, P[sE-CCT]/e, ftz, ry* virgin females. Approximately 75,000 F_1 progeny were scored for enhancement of the *sE-CCT* rough eye phenotype using a Leica MZ8 stereomicroscope. Allelism was assessed by complementation tests based on recessive lethality. Complementation groups corresponding to loci encoding components previously linked to the RTK/Ras/MAPK pathway were determined by conducting complementation tests with the following alleles: rl^{E-1171}, S^{E-1602}, cnk^{E-1088}, $Egfr^{E-1063}$, Sos^{P566}, kis^{E-1679}, ksr^{E-1572}, pnt^{E-1265}, $Ras85D^{e1b}$, $dos^{1.46}$, and $mask^{EP3498}$. The remaining groups were mapped using meiotic, deletion, and *P*-element mapping. When possible, genetic identification for these groups was determined by lethal complementation tests using the following alleles: R^{CD3}, R^{rvB1}, $PDZ-GEF^{EP388}$, $PDZ-GEF^{k13720}$, cno^2, $Btk29A^{k00206}$, swm^{37Dh-1}, swm^{F14}, swm^{F15}, $Prp19^{v22146}$, $Prp19^{v22147}$, Dl^1, Dl^{12}, $Bre1^{01640}$, $Nsf2^{A6}$, and $Nsf2^{A15}$. Exon sequencing for a subset of the new loci was performed to ascertain their molecular identity as previously described (Therrien *et al.* 1998).

Genetics, fly stocks, and microscopy

Fly maintenance and genetic interaction studies were conducted according to standard procedures. The following stocks were used: b^1, pr^1, c^1, px^1, sp^1 (second chromosome mapping stock); ru^1, h^1, th^1, st^1, cu^1, sr^1, e^1, ca^1 (third chromosome mapping stock); hypomorphic rl^1 and phl^{12} mutant alleles; several deficiencies including *Df(2L)BSC5/SM6a*, *P*-element insertions (Bloomington Stock Center), *UAS-CNK^{IR}*, *UAS-Rap1^{IR}*, *UAS-PDZ-GEF^{IR}*, *UAS-Ecad^{IR}*, *UAS-Egfr^{IR}*, *UAS-sev^{IR}* (VDRC), and *hs-flp^{122}; Act > CD2 > Gal4; UAS-GFP* (Neufeld *et al.* 1998); *GFP-Rap1* driven by the endogenous Rap1 promoter (Knox and Brown 2002); *sev-lacZ* (Basler *et al.* 1989); and *GMR-sev* (Tomlinson *et al.* 2011).

UAS construct-expressing FLP-out Gal4 clones were induced 72 hr after egg deposition by a 10- to 15-min heat shock at 38°. Third instar larval eye discs were dissected 96 hr after heat shocks. Pupal eye discs were dissected 45 hr after puparium formation.

Scanning electron microscopy and sectioning of adult fly eyes were performed as described by Kimmel *et al.* (1990) and Tomlinson and Ready (1987), respectively. The *UAS-Rap1^{V12}* line was generated by *P*-element-mediated germline

transformation as previously described (Rubin and Spradling 1982).

Immunostaining

Eye-antennal imaginal discs from third instar larvae were dissected in plain Schneider medium (Invitrogen), fixed in 1× phosphate-buffered saline (1× PBS); 4% paraformaldehyde for 15 min at room temperature and washed three times with PBT (1× PBS; 0.2% Triton X-100). Primary antibodies were incubated in PBT + 2.5% fat-free dry milk overnight at 4° with gentle rocking. Primary antibodies and dilutions were rat anti-Elav [1/20; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Prospero (1/20; DSHB), mouse anti-dpMAPK (1/1000; Sigma), rat anti-E-Cadherin (1/20; DSHB), mouse anti-armadillo (1/20; DSHB), rabbit anti-aPKC (1/200; Santa Cruz Biotechnology), mouse anti-Dlg (1/20; DSHB), mouse anti-Sevenless (1/20; kindly provided by G. M. Rubin), rat anti-EGFR (1/1000; kindly provided by B.-Z. Shilo), mouse anti-Cut (1/100; DSHB), mouse anti-pTyr (1/20, 4G10), mouse anti-β-Gal (1/2000; Promega), rabbit anti-Runt (kindly provided by A. Brand), mouse anti-CNK (1/200; Douziech *et al.* 2003), rabbit anti-Bazooka (1:1000; kindly provided by A. Wodarz), rabbit anti-Patj (1:1000; kindly provided by E. Knust). Imaginal discs were then washed three times with PBT and incubated with appropriate species-specific Alexa 555-, Alexa 647- (1/1000; Life Technologies), or Cy3-conjugated secondary antibodies (1/1000; Jackson Immuno-Research Laboratories) for 2 hr at room temperature in PBT. Tissues were again washed three times with PBT and then mounted in Mowiol 4-88 (Sigma-Aldrich) and imaged using a Zeiss LSM 510 confocal microscope.

Cell transfection and plasmid constructs

S2 cells were grown in Schneider (Life Technologies) medium supplemented with 10% fetal bovine serum and 0.1% penicillin/streptomycin. A total of 7×10^6 (for total lysates) or 18×10^6 (for immunoprecipitations) cells were transfected using Effectene (Qiagen). Protein expression was induced by adding $CuSO_4$ (0.7 mm) to the medium 24 hr post-transfection. Thirty-six hours post-induction, cells were lysed in NP-40 lysis buffer (20 mM Tris at pH 8.0, 137 mM NaCl, 10% glycerol, 1% Igepal CA-630, 1 mM EDTA, 0.15 units/ml aprotinin, 20 μM leupeptin, and 1× Sigma phosphatase inhibitor cocktail) for 15 min at 4°. Sources and dilutions for antibodies are the following: α-Flag M2 mAb (1:5000; Sigma); α-Pyo mAb (1:2000; Douziech *et al.* 2003); a-HA mAb (1:2000; Douziech *et al.* 2003); and α-pMAPK (1:2000; Sigma).

HA-Ras^{V12}, *HA-MAPK*, *Pyo-RAF*, and *Flag-CNK* have been previously described in (Douziech *et al.* 2003). Standard cloning procedures were used to generate *HA-Rap1^{V12}* and *Pyo-PDZ-GEF^{ΔNT}* constructs. The *HA-Rap1^{V12}* or *Pyo-Rap1^{V12}* constructs encompass the entire *Drosophila* Rap1 open reading frame, harbor a G12V change, and encode a hemagglutinin (HA) or polyoma (Pyo) epitope tag at their N terminus.

The *Pyo-PDZ-GEF^{ΔNT}* encodes an N-terminal truncation of *Drosophila* PDZ-GEF (lacks the first 234 amino acid residues) fused to a Pyo epitope tag at its N terminus. All S2 cell-expressing constructs use the *pMet* backbone (Therrien *et al.* 1998) and are thus copper-inducible. The *Rap1^{V12}* cDNA was also inserted in the *pUAST* (Brand and Perrimon 1993) *P*-element vector.

Results

A screen for modifiers of a CNK-dependent rough eye phenotype

We previously showed that expression of a CCT (amino acids 382–1557) *in vivo* or in *Drosophila* S2 cells exhibited dominant-negative activity with respect to the Ras/MAPK pathway owing to its ability to bind and inhibit the RAF Ser/Thr kinase (Therrien *et al.* 1999; Douziech *et al.* 2003). Consistent with this, expression of *CCT* during eye development impeded Ras/MAPK-dependent differentiation events as exemplified by the loss of photoreceptor cells, which in turn caused a mild roughening of the adult eye surface (Figure 1, A, B, E, and F; Table 1).

To identify novel components that functionally work together with CNK, we took advantage of the *CCT* rough eye phenotype and conducted a genetic screen to isolate heterozygous mutations that acted as dominant enhancers. As *CCT* expression cripples Ras/MAPK signaling, loss-of-function mutations in genes that normally operate in concert with CNK might worsen the phenotype and thereby are expected to emerge as enhancers. Eye roughness was relatively weak, and for this reason suppressors were not sought after. Approximately 75,000 F_1 progeny from EMS-mutagenized parent males were scored for enhanced rough eyes. A total of 407 *Enhancers of CCT* (*EC*) were isolated, of which 276 fell into one of 24 recessive lethal complementation groups (Table 2). Representative examples of rough eye enhancements are shown in Figure 1.

Meiotic-, deletion-, and/or transposon-based mapping were used to position the various groups to specific loci. Consistent with the role of CNK in RTK/Ras/MAPK signaling, 11 groups correspond to genes that had been identified in previous genetic screens targeting this pathway in *Drosophila* (Simon *et al.* 1991; Karim *et al.* 1996; Raabe *et al.* 1996; Therrien *et al.* 2000; Smith *et al.* 2002). These include *rl*, *S*, *cnk*, *Egfr*, *Sos*, and *kis* on the second chromosome and *ksr*, *pnt*, *Ras85D*, *dos*, and *mask* on the third chromosome (Table 2). Moreover, we isolated mutations in three genes encoding members of the Rap1-signaling pathway: PDZ-GEF, a Rap1-specific guanine nucleotide exchange factor also known as Dizzy or Gef26 (Lee *et al.* 2002); Roughened (R), a Rap1 GTPase homolog (Hariharan *et al.* 1991); and Canoe (CNO), a Rap1 effector known as AF6 in mammals (Miyamoto *et al.* 1995; Boettner *et al.* 2003). Finally, we isolated mutations in other loci not previously identified in RTK/Ras/MAPK-dependent modifier screens. They correspond to *Btk29A*, *swm*, *Prp19*, *Dl*, *Bre1*, and *Nsf2* (Table 2).
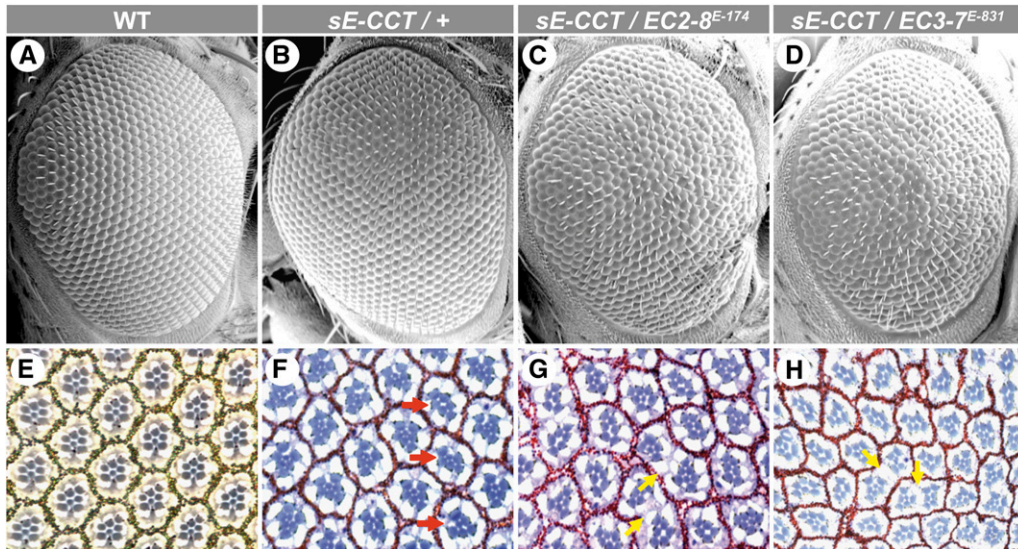
Figure 1 *EC2-8* and *EC3-7* enhance the *sE-CCT* rough eye phenotype. (A–D) Scanning electron micrographs and (E–H) apical sections of adult fly retinae of the indicated genotypes. (A and E) WT. (B and F) *sE-CCT/+*. (C and G) *sE-CCT/EC2-8^{E-174}*. (D and H) *sE-CCT/EC3-7^{E-831}*. The apical section of a WT eye (E) shows the typical trapezoid arrangement of rhabdomeres marking the six outer photoreceptor cells as well as the R7 photoreceptor cell (smaller centrally located rhabdomere). Granules produced by pigment cells can also be seen surrounding individual ommatidia. In F, ommatidia missing photoreceptor cells are indicated with a red arrow. *EC2-8* and *EC3-7* alleles not only enhance the loss of photoreceptor cells induced by *CCT* (G and H), but also lead to the disappearance of pigment cells (yellow arrows). Anterior is to the right.

Additional information on these loci is provided in the *Discussion*.

To confirm that the novel groups were allelic to the aforementioned loci, we isolated genomic DNA from flies heterozygous to those groups noted by table footnote *a* in Table 2 and sequenced their exons. In most cases, specific point mutations introducing either a STOP codon or an amino acid change in conserved residues were found (Figure 2), thus confirming allelism and suggesting that several of the identified alleles are loss-of-functions. One exception to this rule is the *Rap1^{E-722}* allele as no mutation in the *Rap1* open reading frame was detected.

Rap1 signaling is required for RTK-dependent cell differentiation during eye development

The fact that three Rap1 pathway components were identified in the screen strongly suggested a functional relationship between Rap1 signaling and *CCT* activity. We thus decided to further characterize the role of these components in eye development.

To confirm that mutations in Rap1 pathway components genuinely exacerbated the dominant negative activity of *CCT*, we examined whether the *EC2-8* and *EC3-7* alleles (hereafter referred to as *PDZ-GEF* and *Rap1*, respectively) also increased the loss of photoreceptor cells caused by *CCT* expression. As shown in Figure 1, F–H, and quantified in Table 1, heterozygous mutations for both genes considerably augmented *CCT*-mediated photoreceptor loss. The ability of mutations in Rap1 pathway components to enhance *CCT* activity suggests that Rap1 signaling positively influences Ras/MAPK signaling. Consistent with this interpretation, we found that both *PDZ-GEF* and *Rap1* mutant alleles genetically interact with a *Raf* hypomorphic allele during eye development (Supporting Information, Figure S1). Moreover, hypomorphic alleles of *PDZ-GEF* and *Rap1* that show

variable degrees of viability as homozygotes have severe rough eye phenotypes accompanied with a large proportion of ommatidia missing photoreceptor cells (Figure 3, A–H, and Table 1), which phenocopied hypomorphic conditions of Ras/MAPK pathway components such as CNK (Therrien *et al.* 1998). The mutant *PDZ-GEF* and *Rap1* alleles also led to a loss of pigment granules and thereby suggest a failure in the pigment cell differentiation program (Figure 1, F–H). Although this phenotype has not been quantified and its potential link to the loss of photoreceptor cells has not been investigated, it remains consistent with a defect in RTK/Ras/MAPK signaling (Freeman 1997).

A number of studies have reported a link between Rap1 and RTK signaling during *Drosophila* development, but the underlying molecular connection is unclear. In two of these, Rap1 is suggested to serve as a direct RAF activator, which would explain how it influences RTK-dependent MAPK signaling (Lee *et al.* 2002; Mishra *et al.* 2005). In contrast, a third study failed to detect such a role downstream of EGFR during eye and wing development (O'Keefe *et al.* 2009). Instead, the authors reported that Rap1 activity is required for cadherin-based adherens junction formation and that elimination of Rap1 impedes EGFR signaling as a consequence of reduced adhesive contacts between differentiating cells.

Given that RAF has been proposed to work as a direct effector of Rap1 in *Drosophila* (Lee *et al.* 2002; Mishra *et al.* 2005), we verified whether ectopic expression of a constitutively activated form of Rap1 (Rap1^{V12}) during eye development stimulated RAF activity by assessing the levels of the dually phosphorylated (active) form of MAPK (pMAPK). Surprisingly, unlike Ras^{V12}, clonal Rap1^{V12} expression in third instar eye discs had no significant effect on pMAPK levels (Figure S2) despite the fact that it considerably altered eye development and produced a few extra R7 cells

Table 1 Summary of photoreceptor cell counts

Genotype	% of ommatidia missing R7 cell	% of ommatidia missing at least one outer photoreceptor cell	No. of ommatidia analyzed
sE-CCT/+	3	7	617
sE-CCT/PDZ-GEF^{E-174}	25	25	490
sE-CCT/ PDZ-GEF^{E-696}	13	25	686
sE-CCT/+ ; Rap1^{E-722}/+	9	10	509
sE-CCT/+ ; Rap1^{E-831}/+	63	58	631
PDZ-GEF^{E-174}	40	10	505
PDZ-GEF^{E-174}/Df(2L)BSC5	54	11	612
PDZ-GEF^{E-696}/Df(2L)BSC5	66	62	253
^{a}Rap1^{E-814}	21	20	321

^{a} Only recognizable ommatidia/rhabdomeres were scored.

(Figure S3, A–C) as reported previously (Mirey *et al.* 2003; Mavromatakis and Tomlinson 2012) as well as extra cone cells and pigment cells (Figure S3D). Likewise, no activation of MAPK was detected when Rap1^{V12} was overexpressed in S2 cells (Figure S4A). Furthermore, in contrast to Ras, depletion of Rap1 by RNA interference (RNAi) in S2 cells did not compromise EGFR- or SEV-induced MAPK activation (Figures S4, B and C). Interestingly, Rap1^{V12} associated with RAF, but significantly more Rap1^{V12} was required compared to Ras^{V12} (Figure S4D). Together, these results suggest that RAF is not a direct effector of Rap1 in these biological settings. However, considering the genetic data supporting a role for Rap1 in MAPK signaling (Lee *et al.* 2002; Mishra *et al.* 2005; Mavromatakis and Tomlinson 2012), it is possible that the mechanism is indirect.

In third instar eye discs, activation of MAPK primarily depends on EGFR activity, and the resulting pMAPK levels are initially detected in groups of cells within the morphogenetic furrow that give rise posteriorly to clusters of maturing photoreceptors (Gabay *et al.* 1997) (Figure S5). Although less prominent, pMAPK staining is also detected in developing photoreceptor cells posterior to the furrow. The signal is detectable up to 12 rows behind the furrow and encompasses the R7 cell differentiation region, which requires inputs from both the EGFR and the SEV RTKs. As expected, depletion of EGFR by clonally expressing an *Egfr* dsRNA during eye development abrogated pMAPK levels in the two zones described above (Figure S5A). In contrast, depletion of SEV affected only pMAPK levels in the R7 differentiation region (Figure S5B). We used the same experimental paradigm to compare the effect of depleting CNK, Rap1, or PDZ-GEF by RNAi on pMAPK levels. Consistent with its role as a Ras/MAPK pathway component, reducing the dose of CNK severely decreased pMAPK levels in the morphogenetic furrow as well as in the R7 differentiation region (Figure 4A). Interestingly, depletion of either Rap1 or PDZ-GEF in the morphogenetic furrow had no impact on pMAPK induction. However, the second wave of pMAPK signal patterning the R7 cell differentiation area was greatly reduced (Figure 4, B and C). Similar observations were made in homozygous *Rap1* or *PDZ-GEF* mutant clones (Figure S6 and Figure S7). These findings suggest that Rap1 signaling is not generally required for RTK-mediated

MAPK induction, but can influence it in a region-specific manner.

Given the critical importance of MAPK activation for cell differentiation during eye development, the ability of Rap1 depletion to affect MAPK activation specifically in the R7 differentiation zone should have detrimental consequences on R7 photoreceptors. To verify this, we stained the third instar eye disc using an anti-Elav antibody to highlight differentiated photoreceptor neurons in combination with an anti-Prospero (Pros) antibody, which specifically marks non-neuronal cone cells and R7 cells (Kauffmann *et al.* 1996). In agreement with their effect on MAPK activity, *Rap1* and *PDZ-GEF* RNAi severely impeded the number of

Table 2 Groups of dominant enhancers of *sE-CCT* on second and third chromosomes

Groups	Genes	Cytological position	No. of alleles
		Chromosome *II*	
EC2-1	rl	h41	10
EC2-2	S	21E4	21
EC2-3	cnk	54B7	9
EC2-4	Egfr	57E9-F1	35
EC2-5	Sos	34D1	44
EC2-7	kis	21B4-B5	4
EC2-8	Gef26/PDZ-GEF^a	26C2-C3	6
EC2-9	Btk29A^a	29A1-A3	2
EC2-10	swm^a	37E4	4
EC2-11	Prp19^a	55C9-D1	3
EC2-12	ND	43A4-C1	3
		Chromosome *III*	
EC3-1	ksr	83A5	13
EC3-2	pnt	94E10-E13	33
EC3-3	Ras85D	85D19	25
EC3-4	dos	62E7	9
EC3-5	Dl	92A1-A2	27
EC3-6	mask	95F3-F5	6
EC3-7	R/Rap1^a	62B7	5
EC3-8	Bre1^a	64E8	2
EC3-9	ND	68A7-B1	2
EC3-10	cno^a	82F4-F6	3
EC3-11	Nsf2^a	87F15	5
EC3-12	ND	ND	3
EC3-13	ND	98B6-E5	2

^{a} Mutations reported in Figure 2.
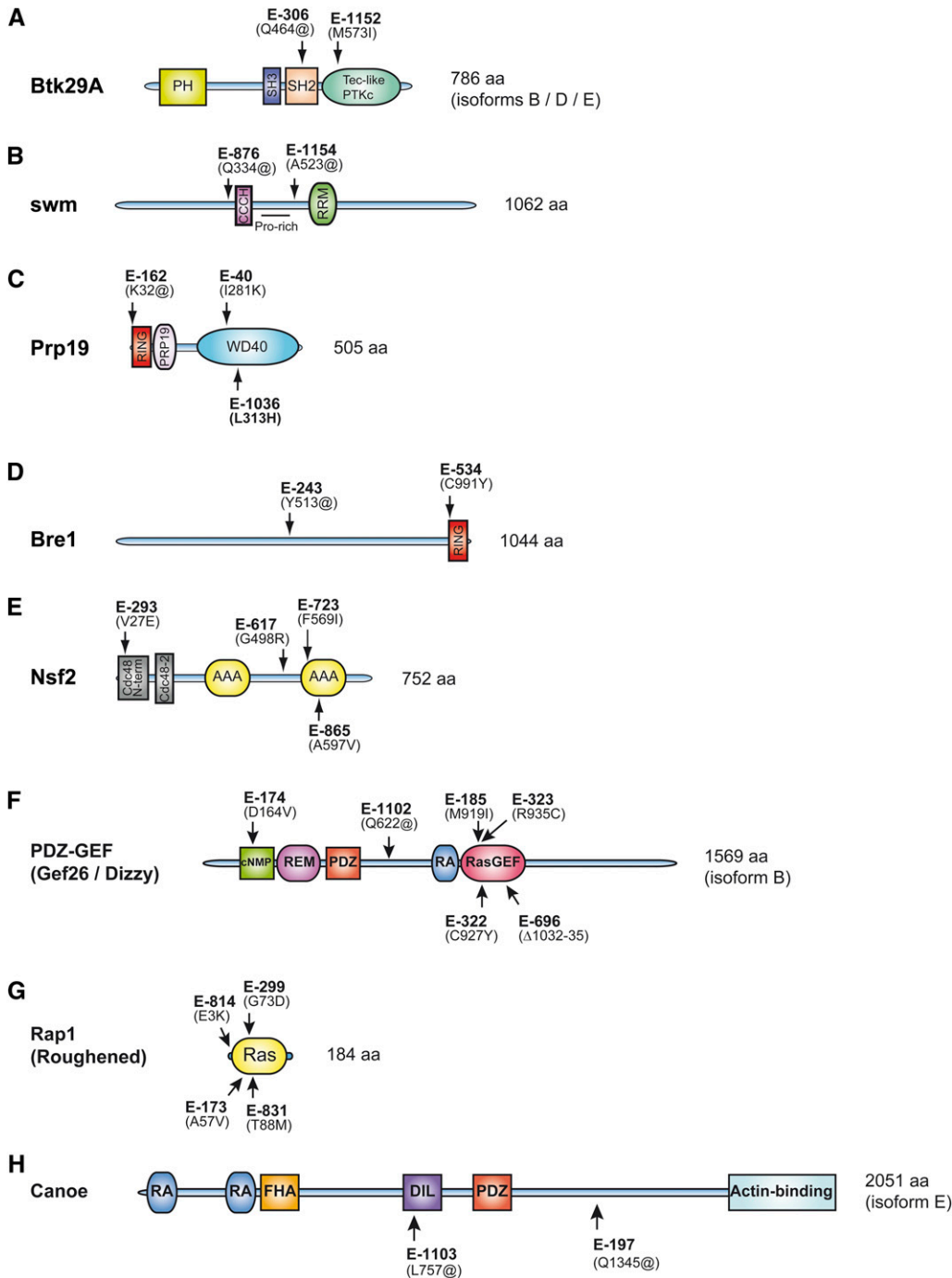ND, not determined.

Figure 2 Identification of the molecular lesions of eight *Enhancers of CCT*. (A–H) Protein domain composition (according to the Conserved Domains tool at the National Center for Biotechnology Information) of proteins encoded by loci not previously identified in RTK/RAS-dependent modifier screens is shown. To confirm allelism between the complementation groups and the assigned loci, exon sequences for at least two heterozygous mutations for each group were determined. Mutations in the expected locus were found for each group. The position of the resulting amino acid lesion for each sequenced allele is shown on the protein schematics. "@" denotes STOP codons.

Elav/Pros double-positive cells, thus indicating a failure of R7 cells to differentiate (Figure 5, A and B). Except for the general lack of R7 cells, only a few ommatidia appear to be missing several photoreceptor cells (Figure 5). This latter observation is consistent with previous work reporting that elimination of Rap1 activity has partial effects on photoreceptor cell differentiation (O'Keefe *et al.* 2009). Together, while these findings indicate that Rap1 signaling plays a role during photoreceptor cell differentiation, this role is particularly critical for setting up the R7 cell differentiation program.

Compromised Rap1 signaling perturbs apical domain formation of developing photoreceptor cells

The results presented above suggest that Rap1 does not influence MAPK signaling through a direct action on RAF. We therefore wanted to address the mechanism by which Rap1 impinged on R7 cell differentiation. Rap1 activity is now well recognized for its role in cadherin-based cell–cell adhesion, and various Rap1 pathway components, including Rap1 itself, have been found to localize to adherens junctions (Knox and Brown 2002) (Figure S8). Consistent with this, depletion of either Rap1 or PDZ-GEF by RNAi
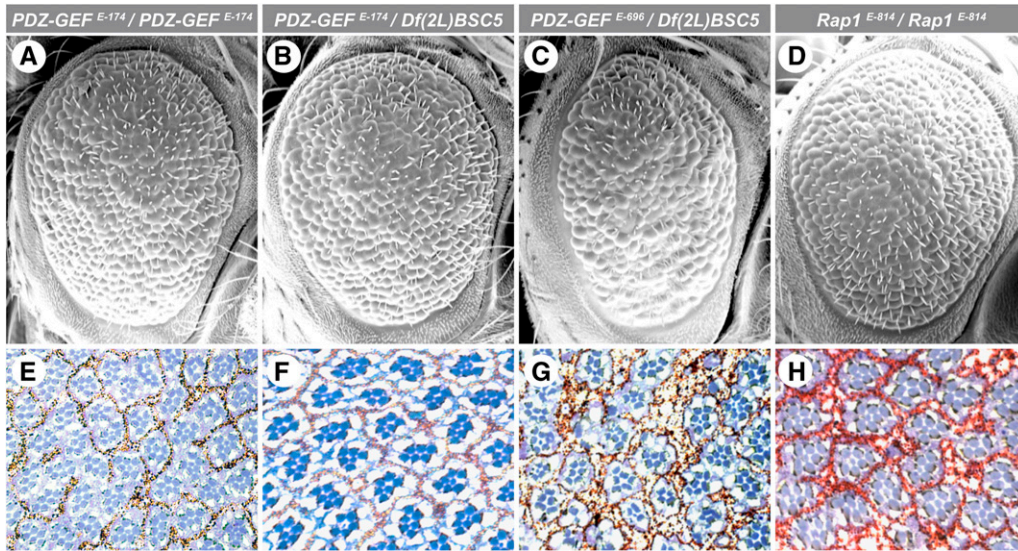
Figure 3 PDZ-GEF and Rap1 are required for photoreceptor cell differentiation. (A–D) Scanning electron micrographs and (E–H) apical sections of adult fly retinae of the indicated genotypes. (A and E) *PDZ-GEF^E-174^/PDZ-GEF^E-174^*. (B and F) *PDZ-GEF^E-174^/Df(2L)BSC5*. (C and G) *PDZ-GEF^E-696^/Df(2L)BSC5*. (D and H) *Rap1^E-814^/Rap1^E-814^*. Anterior is to the right.

significantly reduced the epithelial cadherin (Ecad) and Armadillo (Arm; fly β-catenin) levels that normally define the adherens junctions of developing ommatidia (Figure 6, A and C). Despite the clear impact on adherens junctions, no major cell adhesion defects could be observed in these RNAi-based knockdown conditions (GFP-positive cells do not significantly intermingle with non-GFP-positive cells), which is consistent with the fact that most outer photoreceptor cells differentiated normally. The reason for this could be that there are still sufficient levels of adherens junctions to support a relatively normal cell–cell adhesion. Another proposed role for adherens junctions is the establishment/ maintenance of apicobasal polarity in the fly embryo, whereby adherens junctions participate in the maintenance of the apical domain and may also serve to physically separate antagonistically acting apical determinants from basolateral determinants (Bilder *et al.* 2003; Tanentzapf and Tepass 2003; Kaplan *et al.* 2009). To verify this possibility, we examined the effect of disrupting adherens junctions on the polarity of developing photoreceptor cells. For this, *Ecad* dsRNA-expressing clones in third instar eye discs were stained with anti-aPKC and anti-DLG, which, respectively, mark the apical and basolateral membranes (Woods *et al.* 1996; Wodarz *et al.* 2000). *Ecad* RNAi clearly reduced aPKC levels, but did not affect DLG levels (Figure S9A). This observation is consistent with an impaired apical compartment. We next verified whether reducing Rap1 activity would have similar consequences. As shown in Figure 6, both *Rap1* and *PDZ-GEF* RNAi diminished aPKC staining in neuronal clusters, but did not reduce DLG levels and, if anything, appeared to slightly elevate DLG levels (Figure 6, B and D). A disrupted apical accumulation of aPKC was also seen in *PDZ-GEF* mutant clones (Figure S7). The impact of reduced Rap1 signaling was not restricted to aPKC as it also impeded the accumulation of two other apical markers, namely, Bazooka (BAZ) and PATJ (Figure S10), which further supports the idea of a perturbed apical domain. Together,

these observations suggest that adherens junctions play a role in apical domain formation/maintenance, as observed in fly embryos (Bilder *et al.* 2003), and that Rap1 signaling also participates in this event.

Given the functional link between CNK and Rap1 pathway components, we examined whether CNK also played a role in adherens junction and/or apical domain formation in differentiating photoreceptor cells. As shown in Figure S11, CNK is expressed within the morphogenetic furrow as well as a few rows posterior to it, but its expression rapidly declines more posteriorly. Unlike for Rap1 or PDZ-GEF, knockdown of CNK by RNAi had no impact on the levels of adherens junction (Ecad) or apical domain (aPKC) markers within recruited photoreceptor cells (Figure S11, A–C). Nonetheless, owing to its role as a Ras/MAPK pathway component (Therrien *et al.* 1998), the loss of CNK impacted cell differentiation and thereby reduced the size of ommatidial clusters (Figure S11B).

Rap1 signaling controls the levels of apically localized tyrosine-phosphorylated proteins of developing photoreceptor cells

A number of signaling proteins mediating RTK signals, such as DRK and DOS, have been found to localize to the apical region of differentiating eye cells (Olivier *et al.* 1993; Raabe *et al.* 1996). Since several of these proteins, including the RTKs, are tyrosine-phosphorylated in response to EGFR and SEV activation, we stained third instar eye discs with an anti-pTyr antibody as a readout for RTK signaling. As shown in Figure 7, the knockdown of either Rap1 or PDZ-GEF greatly reduced pTyr levels that are normally associated with the apical surface of developing ommatidia (Figure 7, A and C). These findings demonstrate the ability of Rap1 signaling to control RTK signaling in developing photoreceptor cells by possibly influencing the formation of the apical domain.

Next, we examined whether Rap1 signaling was also involved in the proper localization of EGFR, which has been found to localize to both apical and basolateral membranes
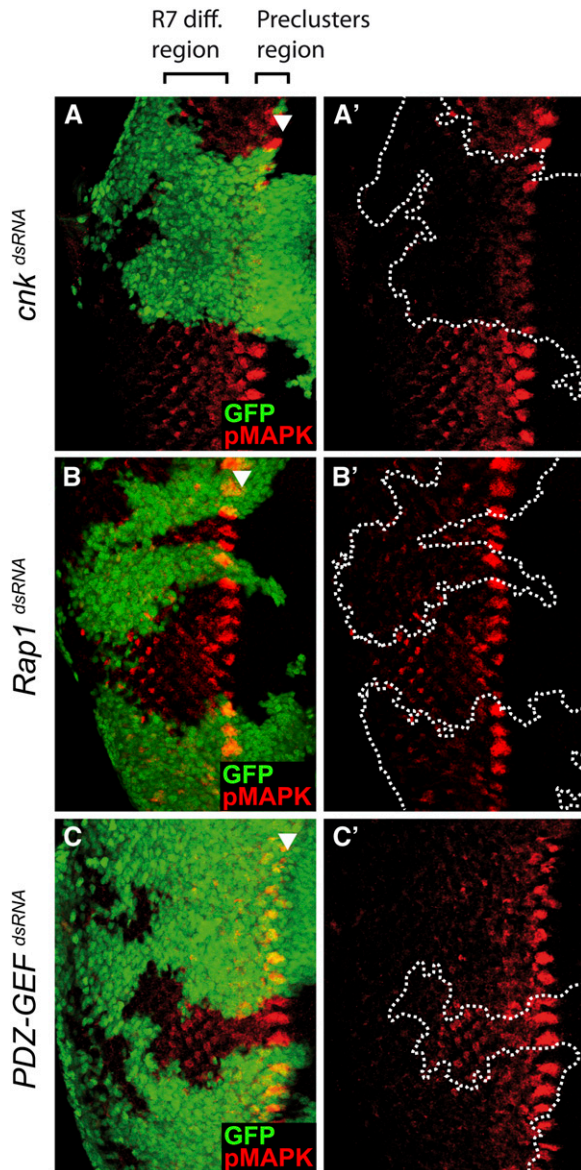
R7 diff. region   Preclusters region

Figure 4 Knockdown of Rap1 or PDZ-GEF attenuates pMAPK levels in the R7 cell differentiation region of third instar larval eye discs. (A–A′ to C–C′) Random clones (GFP-positive cells) expressing the indicated dsRNAs in third instar eye discs were induced using the FLP-out/Gal4 system. pMAPK levels were monitored by immunofluorescence. The arrowhead denotes the position of the morphogenetic furrow. Anterior is to the right.

of epithelial cells (Dumstrei *et al.* 2002). EGFR expression is the highest within the morphogenetic furrow and in developing clusters of photoreceptors two to three rows behind the furrow and then sharply declines, but localizes throughout the cell membranes (Figure S12). However, neither its expression nor its subcellular distribution was affected upon depleting Rap1 or PDZ-GEF activity (Figure S12).

Rap1 signaling is required for the apical accumulation of SEV

The SEV RTK localizes to the apical domain of presumptive R7 cells, and this subcellular distribution is likely critical for

interacting with the ligand BOSS presented at the apical surface of the apposing R8 cells (Banerjee *et al.* 1987; Tomlinson and Ready 1987; Kramer *et al.* 1991). Because R7 cells are particularly sensitive to Rap1 depletion, we wondered whether this could be due to a failure to properly localize SEV to the apical membranes. Even though SEV is required only for R7 cell differentiation, it is detected on the apical surface of several cells within each developing ommatidium where it is first expressed in differentiating R3/R4 photoreceptor cells (Banerjee *et al.* 1987; Tomlinson *et al.* 1987) (Figure 7). Strikingly, reducing the dose of either Rap1 or PDZ-GEF by RNAi or eliminating their presence in homozygous mutant clones greatly affected the accumulation of SEV at the apical surface of the cells (Figure 7, B and D; Figure S6; and Figure S7). Interestingly, a similar observation was made upon depleting Ecad by RNAi (Figure S9B). This suggests that the effect of Rap1 signaling on SEV apical accumulation is related to its impact on adherens junction formation. We verified whether this was accompanied by a relocalization of SEV to basolateral membranes, but did not find any evidence for this (Figure S9, C–E), which indicated that the overall levels of SEV were reduced.

Given that Rap1 modulates MAPK signaling in the R7 cell differentiation area, we verified whether SEV levels were controlled by a MAPK-dependent positive feedback mechanism. To verify this, we compared SEV levels in third instar eye discs between WT and a hypomorphic allele of *mapk* (rl^1), which produces a mild rough eye phenotype (Figure S13) owing to moderate cell differentiation failures (Biggs *et al.* 1994). In this context, no difference in overall SEV levels could be detected (Figure S13), thus suggesting that SEV levels are not influenced by MAPK-mediated signaling.

Next, we examined whether Rap1 signaling controlled *sev* gene expression. To this end, we took advantage of an enhancer trap line carrying a *LacZ* reporter construct inserted in the *sev* locus that recapitulates a normal *sev* expression pattern during eye development (Basler *et al.* 1989). The impairment of Rap1 signaling had no impact on *sev-lacZ* expression and therefore indicated that Rap1 influences SEV levels post-transcriptionally (Figure 7E). Further supporting this model, overexpressed SEV using a *pGMR-sev* transgene also failed to localize to the apical domain of developing ommatidial cell clusters depleted of Rap1 activity and, accordingly, did not rescue the loss of R7 cells (Figure 7, F and G). Finally, we tested whether Rap1^{V12} overexpression modulated SEV levels, but did not find any evidence for this (Figure S14), which also supports the conclusion that Rap1 does not directly control SEV expression. Interestingly, however, Rap1^{V12} expression elevated adherens junction formation in cells surrounding nascent ommatidia next to the morphogenetic furrow and thereby raised the apparent number of cells per cluster (Figure S14). Whether this phenomenon explains the supernumerary eye-specific cells seen in more mature Rap1^{V12} clones (Figure S3) will require further investigation.
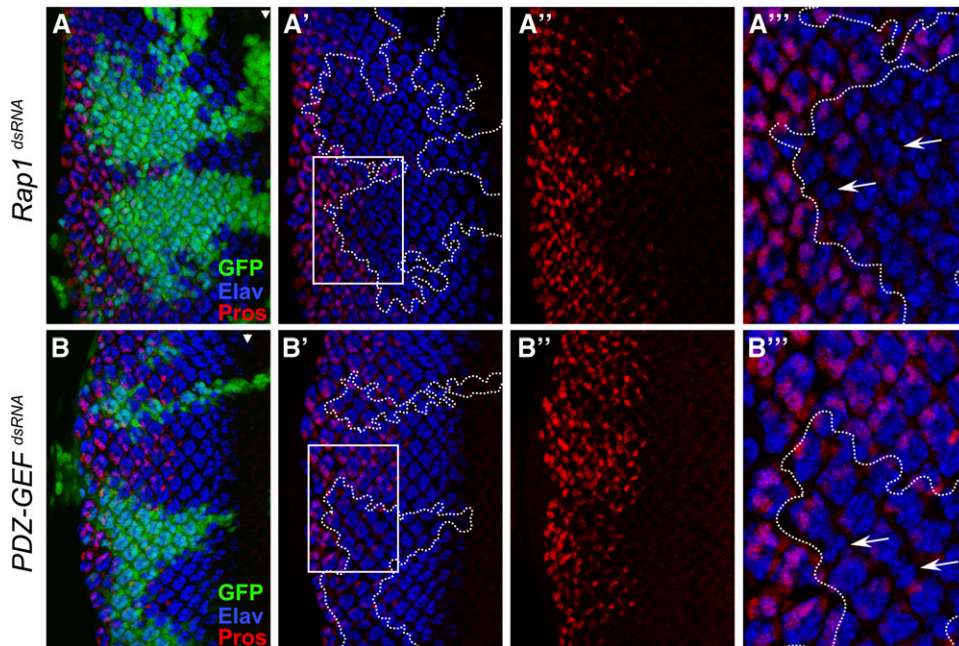
Figure 5 Knockdown of Rap1 or PDZ-GEF impedes R7 cell fate commitment. (A–A''' to B–B''') Random clones (GFP-positive cells) expressing the indicated dsRNAs in third instar eye discs were induced using the FLP-out/Gal4 system. The Elav and Prospero (Pros) markers were then revealed by immunofluorescence. Enlarged areas (A''' and B''') correspond to the boxed regions shown in A' and B' and highlight (arrows) the less frequent examples of ommatidia lacking several Elav-positive cells in *Rap1* or *PDZ-GEF* dsRNA-expressing areas. Arrowheads denote the position of the morphogenetic furrow. Anterior is to the right.

Discussion

In this report, we describe a genetic screen in *Drosophila* for dominant modifiers of a CNK-dependent rough eye phenotype. We then characterize two of those modifiers, *Rap1* and *PDZ-GEF*, to further shed light on the mechanism by which Rap1-mediated events influence photoreceptor cell development.

Modifiers of dominant-negative CNK

Given the role CNK plays in RTK-elicited Ras/MAPK signaling, mutations in loci encoding general components of this pathway in flies were recovered in the *CCT* screen. They correspond to *Star* [*S*; trafficking factor for the EGFR ligand Spitz (Mayer and Nusslein-Volhard 1988; Lee *et al.* 2001; Tsruya *et al.* 2002)], *Egfr*, *daughter of sevenless* (*dos*; Gab2 homolog; Herbst *et al.* 1996; Raabe *et al.* 1996), *Son of sevenless* (*Sos*; RasGEF; Simon *et al.* 1991), *Ras85D*, *rl/mapk*, *ksr*, and *pointed* (*pnt*; ETS domain transcription factor mediating MAPK activity; Brunner *et al.* 1994; O'Neill *et al.* 1994) (Table 2). Mutations in two genes previously identified in RTK-dependent screens, but of unclear function, were also isolated. These are *kismet* [*kis*; chromatin remodeler (Therrien *et al.* 2000)] and *multiple ankyrin repeats single KH domain* [*mask*; putative RNA-binding protein (Smith *et al.* 2002)].

Mutant alleles not identified in classical RTK/MAPK-dependent genetic screens, but for which a functional link to RTK signaling in flies or in other organisms had been established were also recovered. These include *Btk family kinase at 29A* [*Btk29A*; the single representative of Tec family kinases (Readinger *et al.* 2009)]; *Delta* (*Dl*; Baker and Rubin 1992); and three Rap1 pathway loci, *PDZ-GEF*, *Rap1*, and *canoe* (*cno*) (Li *et al.* 1997; Lee *et al.* 2002; Gaengel and Mlodzik 2003; O'Keefe *et al.* 2009). Finally, mutations were

isolated in four additional loci not previously reported to influence RTK/MAPK signaling: *Pre-mRNA-processing factor 19* (*Prp19*), *BREFeldin A sensitivity 1* (*Bre1*), *NEM sensitive factor 2* (*Nsf2*), and *second mitotic wave missing* (*swm*).

The *Prp19* locus encodes a core spliceosome component (Chan *et al.* 2003). We recently unveiled a specific role for these proteins in the Ras/MAPK pathway. Indeed, we identified several splicing factors, including Prp19 and Prp8, in a genome-wide RNAi screen in S2 cells for modulators of Ras-induced MAPK activation (Ashton-Beaucage *et al.* 2014). Incidentally, a single mutant allele of *Prp8* was also recovered in the *CCT* screen (reported in Ashton-Beaucage *et al.* 2014). Characterization of their implication in the pathway revealed that they specifically regulate MAPK protein levels by controlling the alternative splicing of selected introns of the *mapk* pre-mRNAs. It is thus likely that *Prp19* alleles were recovered in the *CCT* screen because of their impact on endogenous MAPK levels during eye development.

The association that the last three genes (*Bre1*, *Nsf2*, and *swm*) might have with respect to *CCT* activity is less clear. *Bre1* encodes a RING finger-containing E3 ligase mediating histone H2B monoubiquitination (Bray *et al.* 2005). This modification contributes to specific histone epigenetic changes such as histone H3K4 and H3K79 methylation that correlate with transcriptional activation (Wright *et al.* 2011). A role for Bre1 in Notch- and Wingless-dependent gene expression has been reported (Bray *et al.* 2005; Mohan *et al.* 2010), but whether it acts similarly downstream of RTK signaling is not known. Given the concerted, yet distinct role Notch and EGFR signaling play in morphogenetic furrow progression and thereby in eye development (Doroquez and Rebay 2006), it could well be that, as for *Dl*, *Bre1* was recovered primarily for its function in Notch signaling.
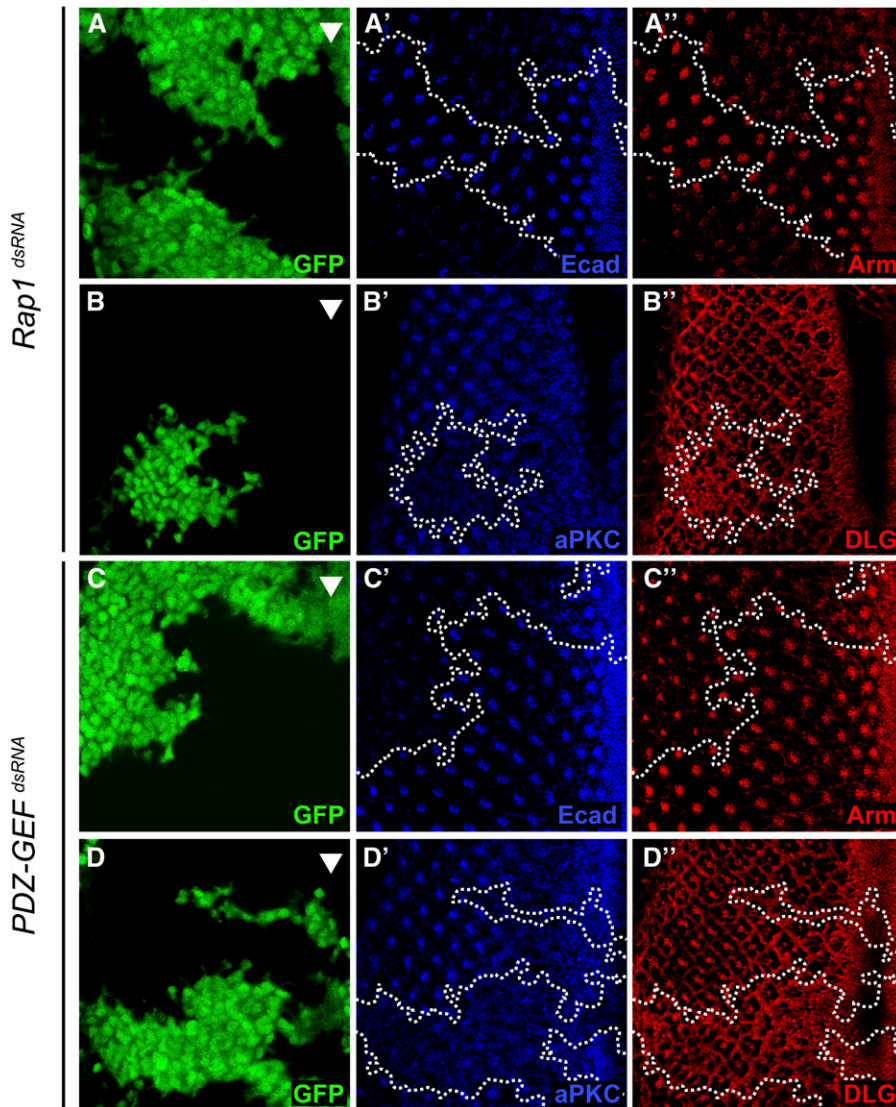
Figure 6 Rap1 and PDZ-GEF dsRNAs impair the expression of adherens junction and apical markers in third instar eye discs. (A–A'' to D–D'') Random clones (GFP-positive cells) expressing the indicated dsRNAs in third instar larval eye discs were induced using the FLP-out/Gal4 system. Eye discs were then immunostained with (A' and C') anti-Ecad;, (A'' and C'') anti-Arm, (B' and D') anti-aPKC, and (B'' and D'') anti-DLG antibodies. The arrowhead denotes the position of the morphogenetic furrow. Anterior is to the right.

Nsf2 encodes an AAA ATPase involved in vesicular trafficking and synaptic vesicle release (Boulianne and Trimble 1995; Pallanck *et al.* 1995). Previous genetic studies have also associated this gene with Notch and Wingless signaling (Stewart *et al.* 2001), and thus this could be the basis for the isolation of *Nsf2* alleles. Alternatively, Nsf2 activity could be required in trafficking events directly involved in RTK signaling.

The *swm* gene [aka *Su(Rux)2B* (Dong *et al.* 1997)] encodes a novel protein that comprises a CCCH zinc finger and a RNA recognition motif (Casso *et al.* 2008). SWM localizes to the nucleus and was found to play multiple roles during *Drosophila* development (Casso *et al.* 2008), although its precise molecular function is not known. During eye development, Swm regulates the proliferation of undifferentiated cells by controlling their G1/S transition (Dong *et al.* 1997). In particular, third instar eye discs deprived of SWM activity are reduced in size and, as epitomized by the gene name, they lack the second mitotic wave, which corresponds to

a row of cells located at a few cells distance posterior to the morphogenetic furrow that undergo a unique and synchronous round of cell division (Baker 2001). This event increases the pool of uncommitted cells used for completing ommatidial assembly. Both Notch and EGFR signaling are essential for cell cycle progression of the uncommitted cells in the second mitotic wave, but act at distinct steps (Baonza *et al.* 2002; Firth and Baker 2005). Whether the *swm* alleles were recovered because of their impact on the second mitotic wave or for another role of SWM in differentiating cells remains to be investigated.

Rap1 signaling is required upstream of SEV for MAPK activation in the R7 cell precursors

The ability of the *CCT* screen to identify mutations in three loci linked to Rap1 signaling strongly suggests a functional relationship between CNK and Rap1 activity. Yet, we did not find evidence for physical association between CNK and Rap1 or PDZ-GEF (Figure S4E), and thus the molecular
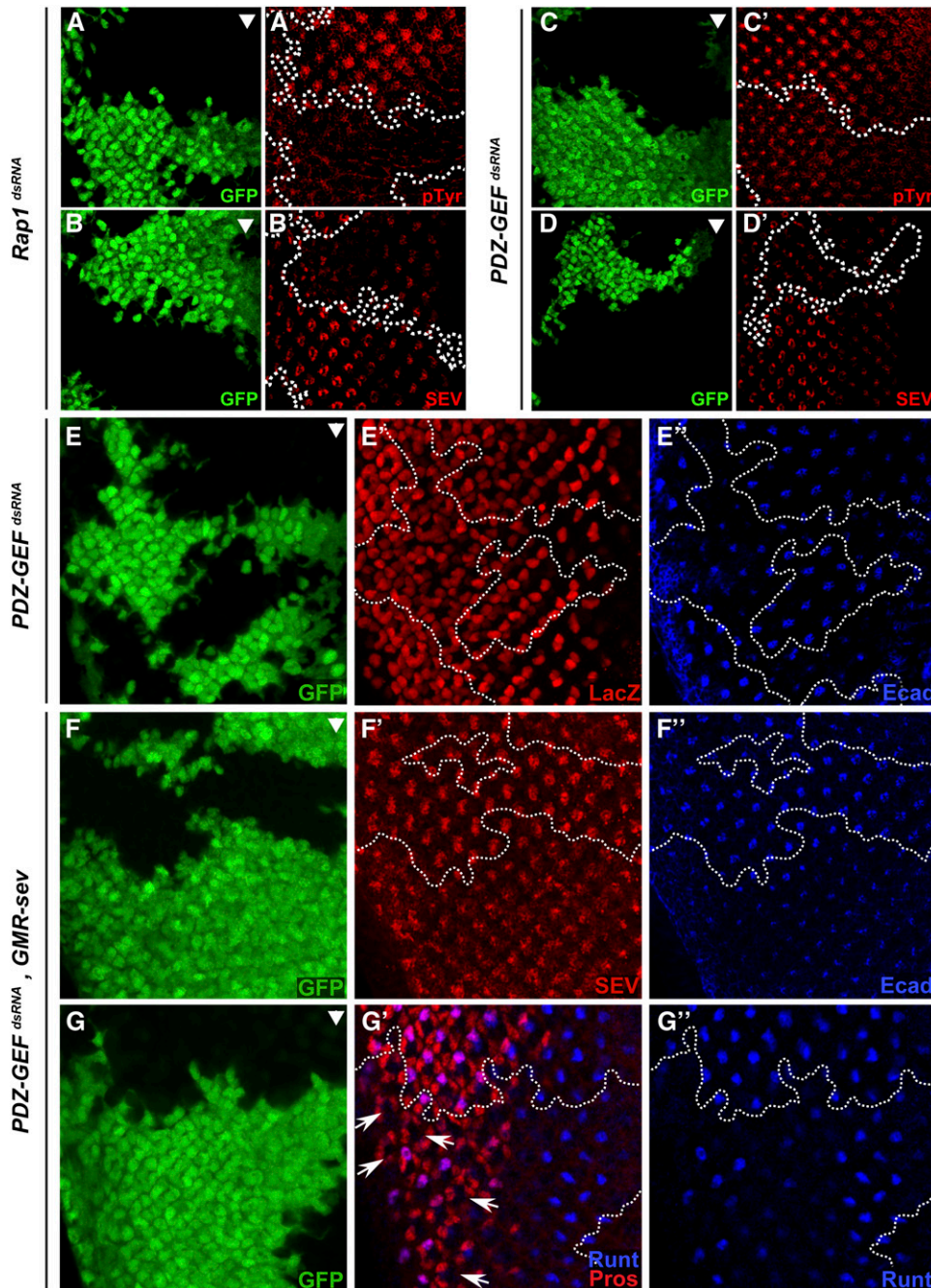
Figure 7 Rap1 signaling controls SEV levels through a post-transcriptional mechanism. (A–G) Random clones (GFP-positive cells) expressing the indicated dsRNAs in third instar eye discs were induced using the FLP-out/Gal4 system and immunostained with (A′ and C′) anti-pTyr, (B′, D′ and F′) anti-SEV, (E′) anti-β-Gal, (E′′–F′′) anti-Ecad, and (G′ and G′′) anti-Runt and anti-Prospero (Pros) antibodies. (A′–D′) *Rap1* and *PDZ-GEF* dsRNAs impede SEV and pTyr levels in developing photoreceptor cells. However, reducing Rap1 activity, as achieved using *PDZ-GEF* dsRNA (E–E′′), does not prevent *sev-LacZ* expression. On the other hand, SEV overexpression using a *pGMR-sev* transgene (F and G) does not restore SEV levels to the apical domain (F′), nor does it rescue R7 cell differentiation as assessed by the loss of Runt/Pros double-positive cells (G′; arrows point to several examples) in the *PDZ-GEF* dsRNA-expressing area. (F′′–G′′) Anti-Ecad staining was performed as a control to demonstrate the activity of the *PDZ-GEF* dsRNA. Arrowheads mark the position of the morphogenetic furrow. Anterior is to the right.

underpinning of this relationship is currently not known. One possibility for their genetic interactions could be through their separate roles in RTK-mediated events. Rap1 signaling promotes adherens junction formation in differentiating photoreceptor cells, which contributes to their clustering (O'Keefe *et al.* 2009). This phenomenon, in turn, is thought to enable the cells to respond to extracellular cues promoting differentiation. By lowering Rap1 activity, cohesive contacts between differentiating cells would be suboptimal and thereby would impede ommatidial assembly to some degree. In this scenario, the impact of *CCT* expression on photoreceptor cell differentiation would be exacerbated by heterozygous mutations in Rap1-signaling components as

these would reduce the sensitivity of developing cells to differentiation cues.

Interestingly, it has been noted that loss of Rap1 activity does not prevent EGFR-induced MAPK activation *per se*, and thus Rap1 does not appear to work like Ras as a direct RAF activator (O'Keefe *et al.* 2009). However, the data for this conclusion were based on small Rap1 mutant clones that were close or within the morphogenetic furrow. We extended this work by producing larger clones depleted in Rap1 or PDZ-GEF activity. These clones covered the zone where R7 cell commitment normally occurs. Markedly, we found that reduced Rap1 signaling in this area considerably decreased MAPK activity as well as global pTyr levels (Figure

4 and Figure 6) and thereby mimicked the loss of RTK activity. Consistent with this, a strong impairment in R7 cell fate specification was observed (Figure 5).

In agreement with our findings, Mavromatakis and Tomlinson (2012) recently showed by genetic means that R7 cell fate specification had an absolute requirement in Rap1 activity. According to their model, R7 cell precursors sense higher Notch signaling owing to their position in the developing ommatidium, which at this stage is antagonistic to Ras/MAPK-mediated neuronal differentiation. To counteract Notch signaling, Mavromatakis and Tomlinson (2012) proposed that presumptive R7 cells turn on two RTKs (EGFR and SEV) to produce higher MAPK activity. Intriguingly, their work suggested that Rap1 was required downstream of SEV, although they could not distinguish whether Rap1 acted through the canonical MAPK pathway or parallel to it. We investigated this and found that Rap1 does not seem to work directly through the MAPK pathway since ectopic expression of Rap1^{V12} during eye development or in cultured S2 cells did not promote MAPK phosphorylation (Figure S2 and Figure S4). Moreover, depletion of Rap1 or PDZ-GEF by RNAi in S2 cells had no consequence on MAPK activation induced by SEV or EGFR (Figure S4). Although the precise mechanism by which Rap1 influences signaling downstream of SEV remains to be delineated, our combined data suggest that Rap1 works at two distinct levels in SEV-mediated signaling, that is, upstream of SEV by modulating the apical localization of SEV and downstream of SEV by a mechanism that has yet to be characterized.

Rap1 signaling, adherens junction, and apico-basal polarity

Adherens junctions form a belt-like microdomain that encircles epithelial cells apically and that play a major role in cell–cell adhesion, motility, and polarity (Etienne-Manneville 2011). One of the core structural components of adherens junctions is Ecad, which is a transmembrane glycoprotein that forms Ca^{2+}-dependent homophilic interactions between adjacent cells. The intracellular portion of Ecad is complexed to the catenins that, in turn, mediate linkage to the actomyosin cytoskeleton (Yonemura 2011). Studies conducted over the past 10 years in both vertebrate and invertebrate organisms demonstrate the critical role that Rap1 signaling plays in modulating the connections of adherens junctions to the actomyosin network, which then influence cell–cell adhesion, cell shape, and cell migration (Frische and Zwartkruis 2010).

Although our data are consistent with this view, they also hint at a new role for Rap1 signaling that is to control apical domain formation in developing photoreceptor cells. Given that adherens junctions may act as physical barriers between apical and basolateral membrane compartments, the influence of Rap1 on adherens junction dynamics could represent the mechanism by which Rap1 exerts its effect on the apical domain compartment. A more exciting alternative would be that Rap1 activity directly controls the formation of the apical domain. Work conducted in fly embryos by Peifer and colleagues recently provided evidence supporting this model (Choi et al. 2013). Indeed, not only did they find that Rap1 activity is essential for establishing the apico-basal polarity of cellularizing embryos, but their data also suggest that it has a direct impact on the apical localization of Bazooka, a member of the Par complex, which then orchestrates apical domain assembly. Whether Rap1 signaling has a direct influence on cell polarity during eye development is still unclear. Nonetheless, further characterization of the impact that Rap1 signaling has on apical domain formation/maintenance should reveal novel aspects by which cell compartmentalization is brought about and regulated as well as how it connects to downstream signaling events in epithelial cells.

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GENETICS

Apical Accumulation of the Sevenless Receptor Tyrosine Kinase During *Drosophila* Eye Development Is Promoted by the Small GTPase Rap1

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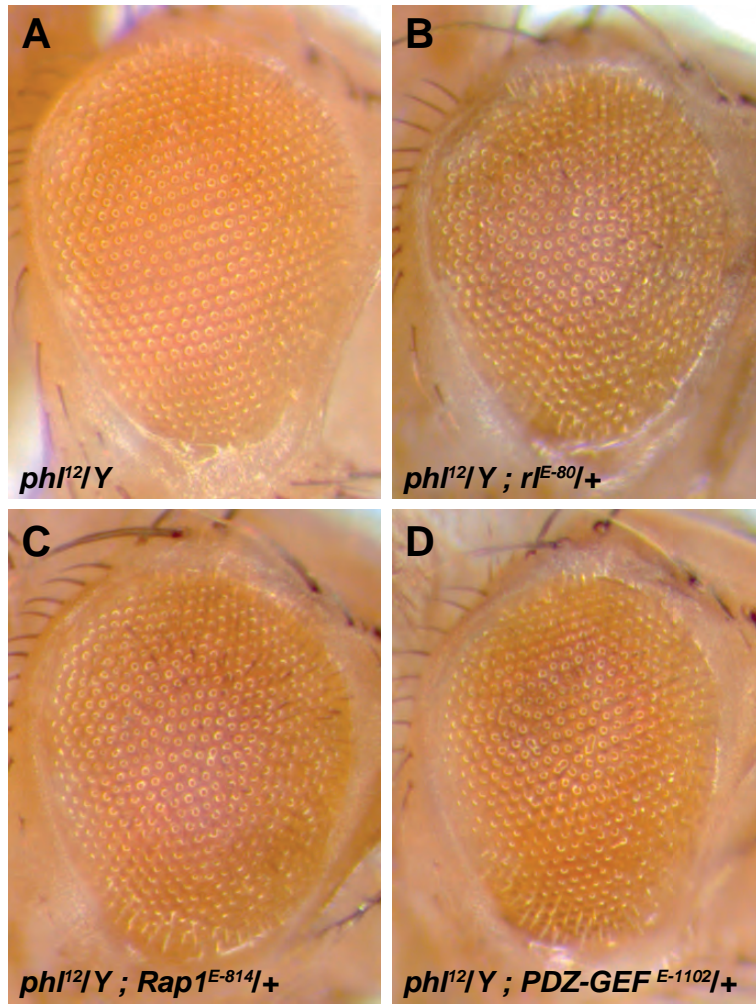
Figure S1 *Rap1* and *PDZ-GEF* alleles genetically interact with a hypomorphic allele of *raf* (*phl^{12}*). (A-D) Micrographs of adult eyes of the indicated genotypes. A *rolled* (*rl*) loss-of-function allele (B) was used as reference for enhanced eye roughness. Anterior is to the right.
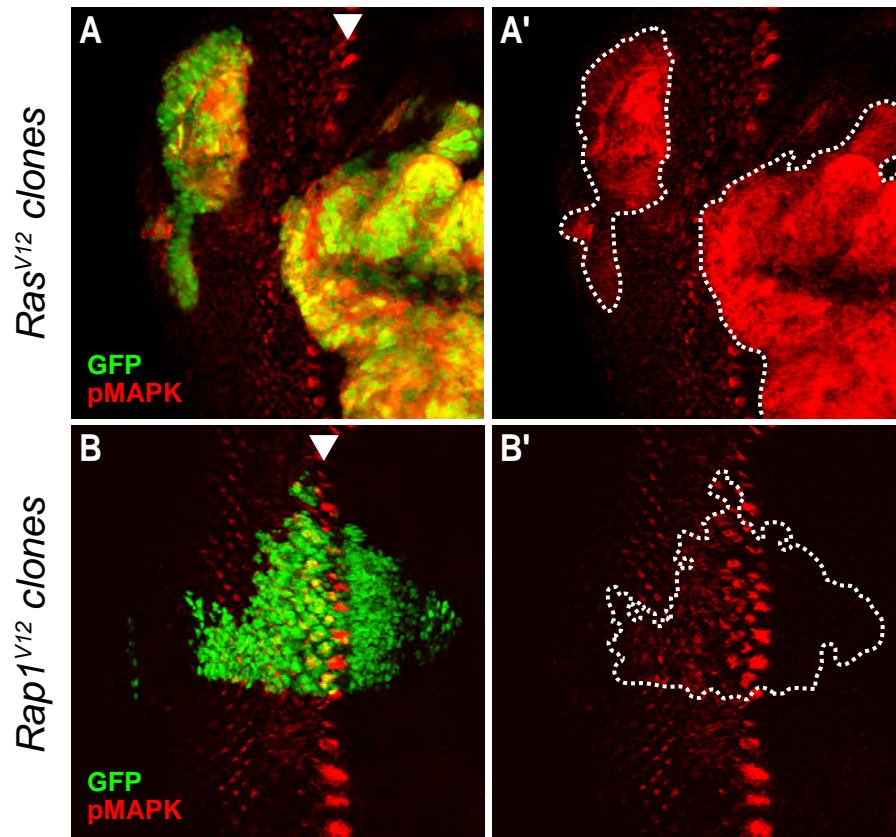
Figure S2 Unlike *Ras^V12^*, clonal overexpression of *Rap1^V12^* during eye development does not promote ectopic activation of MAPK (pMAPK). (A-A') *UAS-Ras^V12^* and (B-B') *UAS-Rap1^V12^* were clonally expressed in third instar eye discs and visuallized using the FLP-out / Gal4 system (GFP-positive cells). The arrowhead marks the position of the morphogenetic furrow. Anterior is to the right.
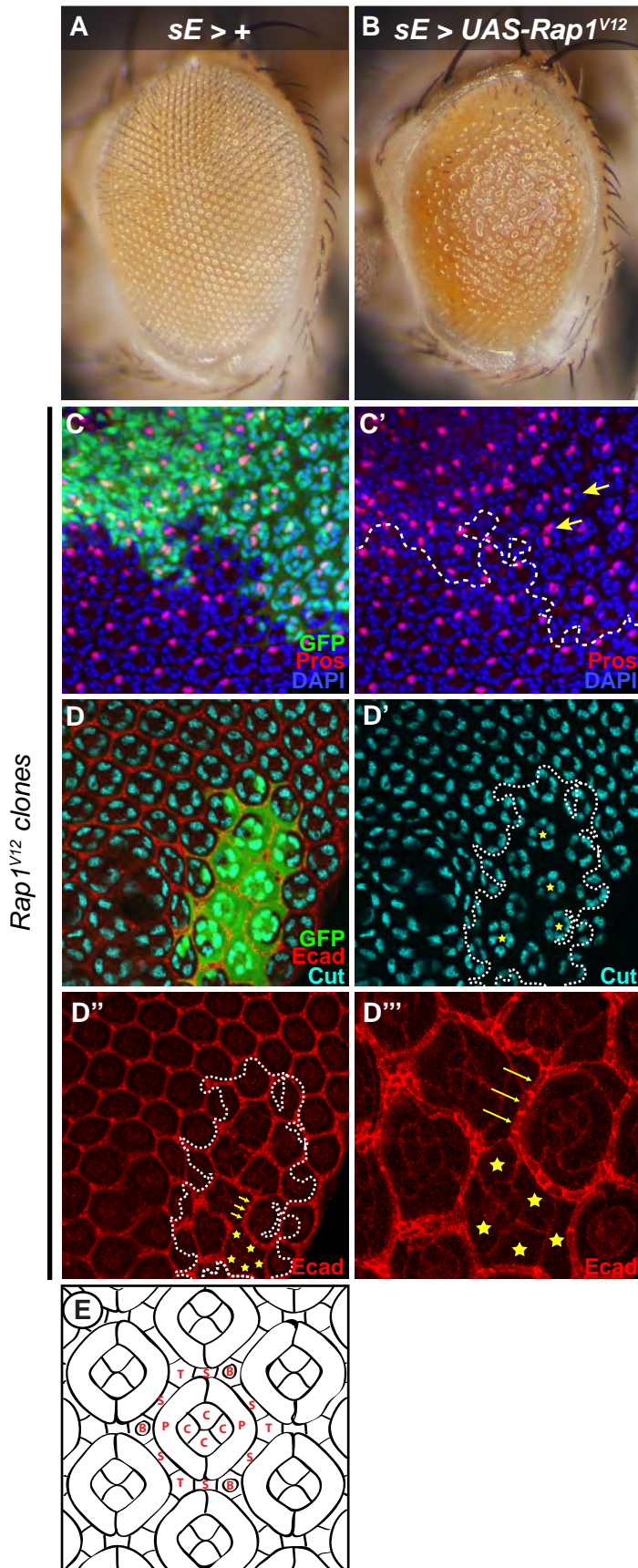
Figure S3 Rap1^{V12} is constitutively active. Adult retina expressing (A) *sE-Gal4* alone or (B) *sE-Gal4* combined with *UAS-Rap1^{V12}*. (C-D''') Random clones (GFP-positive cells) expressing Rap1^{V12} in pupal eye discs (45h after pupal formation) were induced by heat shock using the FLP-out/Gal4 system. Pupal eye discs were then immunostained with (C-C'') anti-Prospero to reveal R7 (Pros-positive) cells (nuclei outlined by DAPI) or (D-D''') anti-Ecad and anti-Cut to reveal cell outlines and cone cells, respectively. Dotted lines (C'-D'') mark the Rap1^{V12}-expressing clonal areas. (C') Arrows point to examples of ommatidia containing extra R7 cells. Rap1^{V12} expression also leads to extra cone cells (examples of five cone cells instead of the normal four cone cells per ommatidium are marked by a star in D'), and extra primary and secondary pigment cells (stars and arrows, respectively, in D''). (D''') Enlarged area encompassing the ommatidia where extra primary and secondary pigment cells are respectively highlighted by stars and arrows in D''. (E) Schematic representation of the apical cell outlines of a 45h pupal eye disc. Typically, at an apical focal plane, cell outlines reveal the two primary (P), six secondary (S), and three tertiary (T) pigment cells as well as the three mechano-sensory bristle (B) cells and the four cone (C) cells composing a normal ommatidium (Wolff and Ready, 1991).
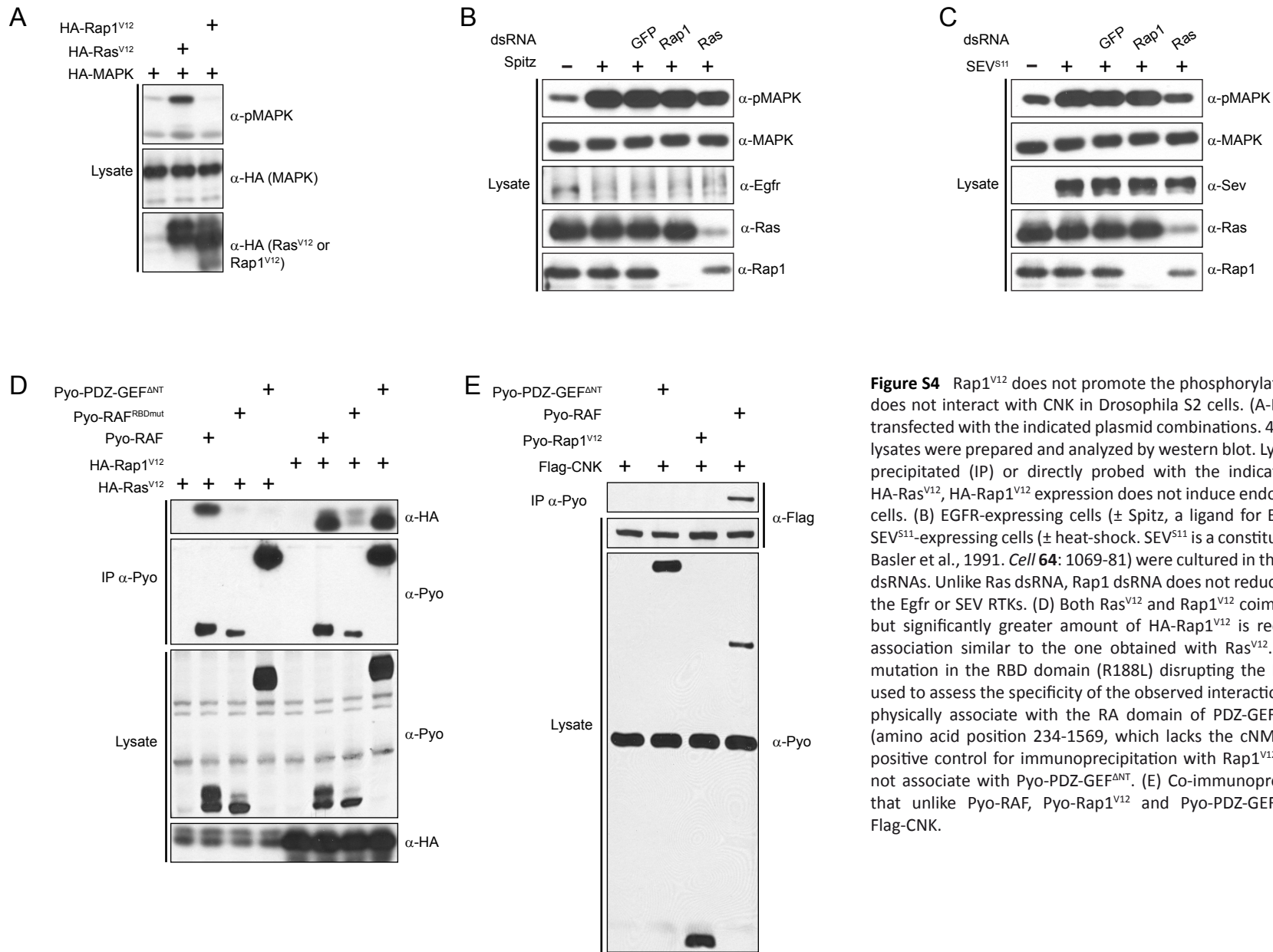
Figure S4 Rap1[V12] does not promote the phosphorylation of MAPK (pMAPK) and does not interact with CNK in Drosophila S2 cells. (A-E) S2 cells were transiently transfected with the indicated plasmid combinations. 48 hrs post-transfection, cell lysates were prepared and analyzed by western blot. Lysates were either immuno-precipitated (IP) or directly probed with the indicated antibodies. (A) Unlike HA-Ras[V12], HA-Rap1[V12] expression does not induce endogenous pMAPK levels in S2 cells. (B) EGFR-expressing cells (± Spitz, a ligand for EGFR) or (C) heat-inducible SEV[S11]-expressing cells (± heat-shock. SEV[S11] is a constitutively active variant of SEV; Basler et al., 1991. *Cell* **64**: 1069-81) were cultured in the presence of the indicated dsRNAs. Unlike Ras dsRNA, Rap1 dsRNA does not reduce pMAPK levels induced by the Egfr or SEV RTKs. (D) Both Ras[V12] and Rap1[V12] coimmunoprecipitate with RAF, but significantly greater amount of HA-Rap1[V12] is required to reach a level of association similar to the one obtained with Ras[V12]. RAF[RBDmut] harbors a point mutation in the RBD domain (R188L) disrupting the Ras-RAF association and is used to assess the specificity of the observed interactions. Since Rap1 is known to physically associate with the RA domain of PDZ-GEF, we used Pyo-PDZ-GEF[ΔNT] (amino acid position 234-1569, which lacks the cNMP domain; Figure 2F) as a positive control for immunoprecipitation with Rap1[V12]. Interestingly, Ras[V12] does not associate with Pyo-PDZ-GEF[ΔNT]. (E) Co-immunoprecipitation assays revealed that unlike Pyo-RAF, Pyo-Rap1[V12] and Pyo-PDZ-GEF[ΔNT] fail to associate with Flag-CNK.
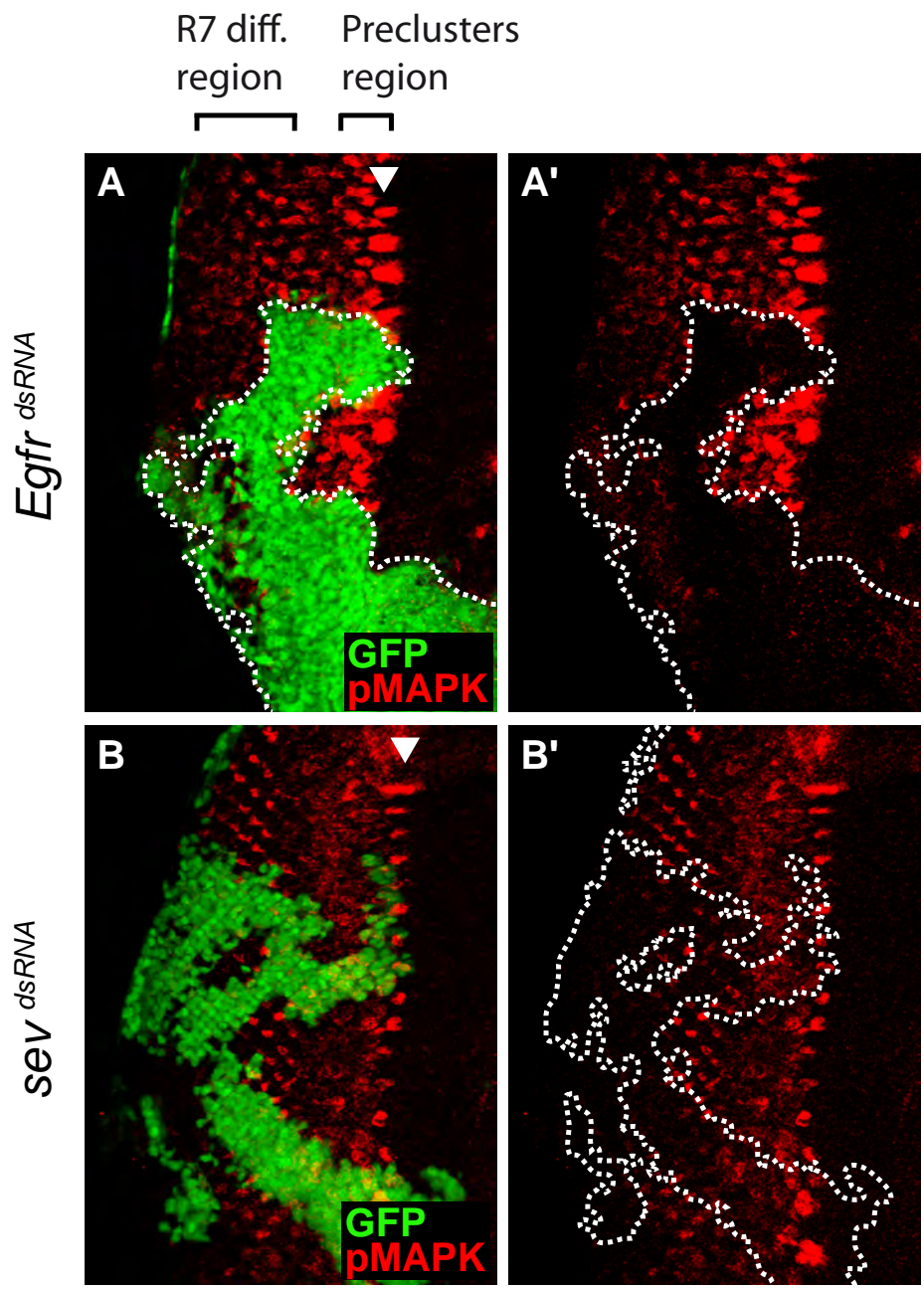
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Figure S5 Comparison of the eye disc areas controlled by EGFR and SEV leading to MAPK activation. (A-A' and B-B') Random clones (GFP positive cells) expressing the indicated dsRNAs were induced using the FLP-out Gal4 system and phosphorylated MAPK (pMAPK) was monitored by immunofluorescence in third instar larval eye discs. Arrowheads refer to the position of the morphogenetic furrow. Anterior is to the right.
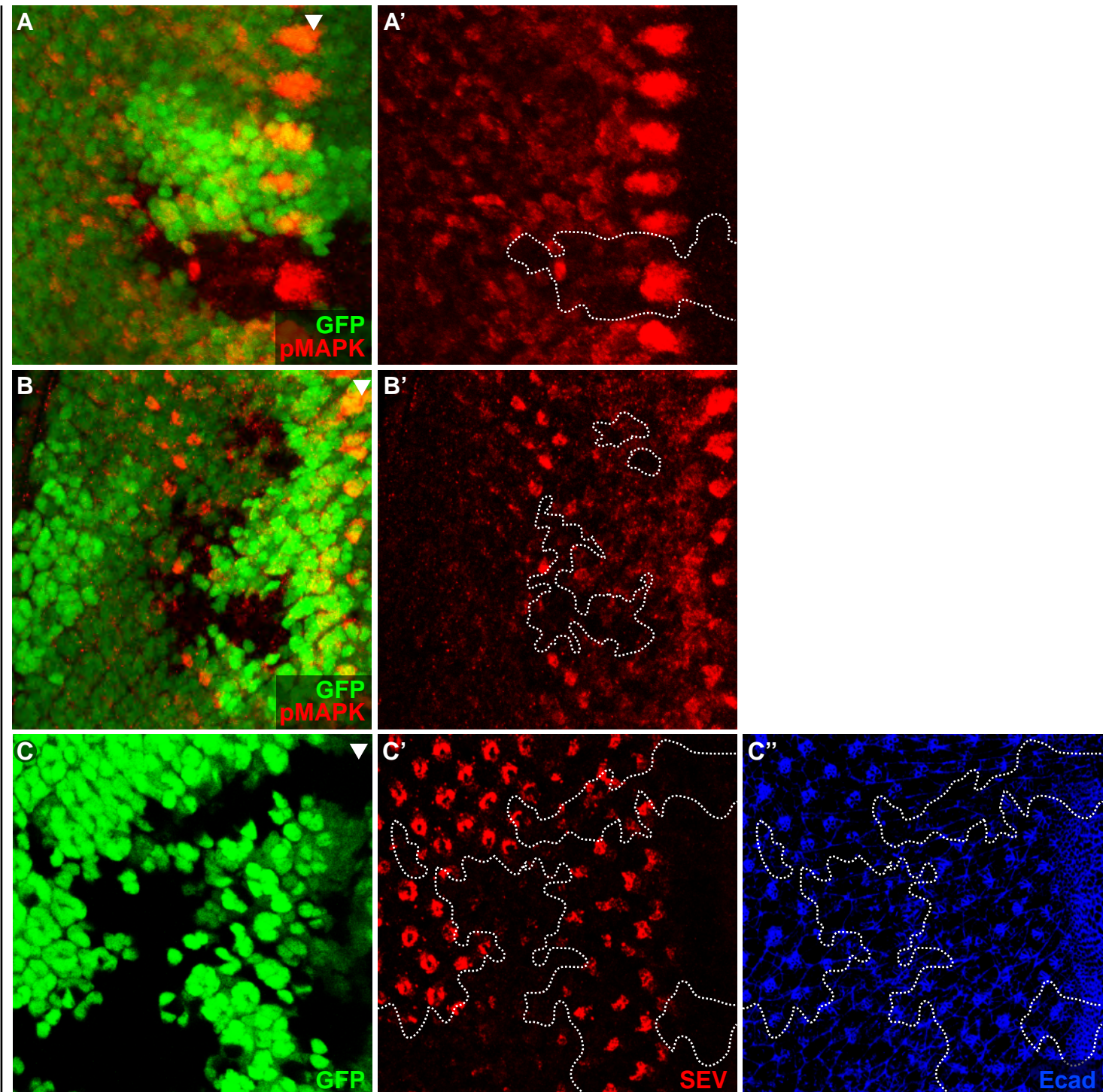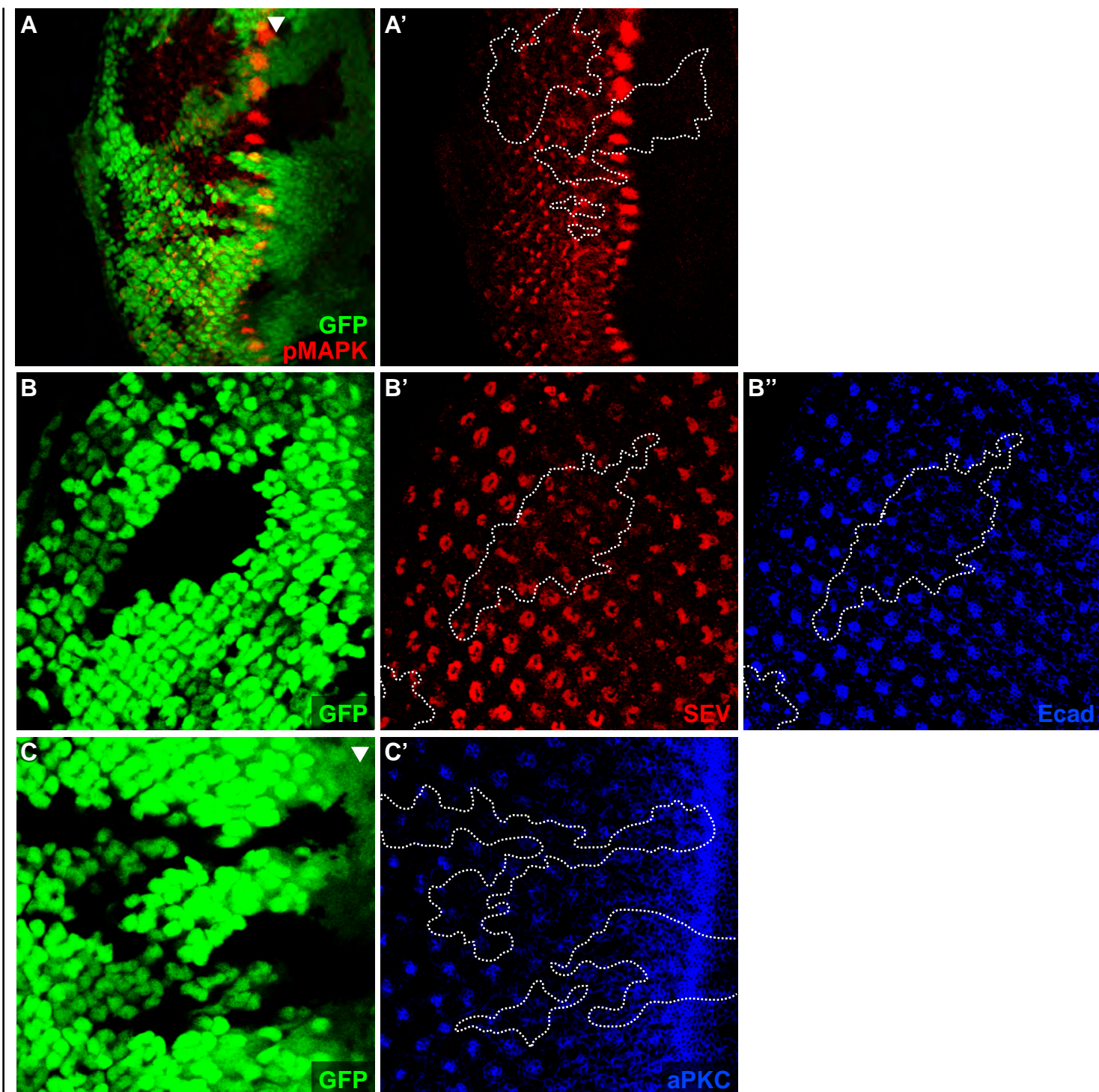
Figure S6 Clonal analysis of *Rap1^CD3^* null allele confirms results obtained with *Rap1* dsRNA in third instar eye discs. (A-A' to C-C'') Random homozygous clones (GFP-negative cells) for the *Rap^CD3^* allele in third instar eye discs were induced using the FLP/FRT system. Eye discs were then immunostained with (A' and B') anti-pMAPK, (C') anti-SEV and (C'') anti-Ecad antibodies. Loss of Rap1 activity does not impede pMAPK levels in cells close to the morphogenetic furrow (A'), but significantly reduces pMAPK levels in posterior areas (B') corresponding to the R7 differentiation region, wherein SEV activity is required. Arrowheads mark the position of the morphogenetic furrow. Anterior is to the right.

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Figure S7 Clonal analysis of *PDZ-GEF*[E-696] null allele confirms results obtained with *PDZ-GEF* dsRNA in third instar eye discs. (A-A' to C-C'') Random homozygous clones (GFP-negative cells) for the *PDZ-GEF*[E-696] allele in third instar eye discs were induced using the FLP/FRT system. Eye discs were then immunostained with (A') anti-pMAPK, (B') anti-SEV, (B'') anti-Ecad, and (C') anti-aPKC antibodies. Loss of PDZ-GEF activity does not impede pMAPK levels in cells close to the morphogenetic furrow (A'), but reduces pMAPK levels in posterior areas corresponding to the R7 differentiation region, wherein SEV activity is required. Arrowheads mark the position of the morphogenetic furrow. Anterior is to the right.

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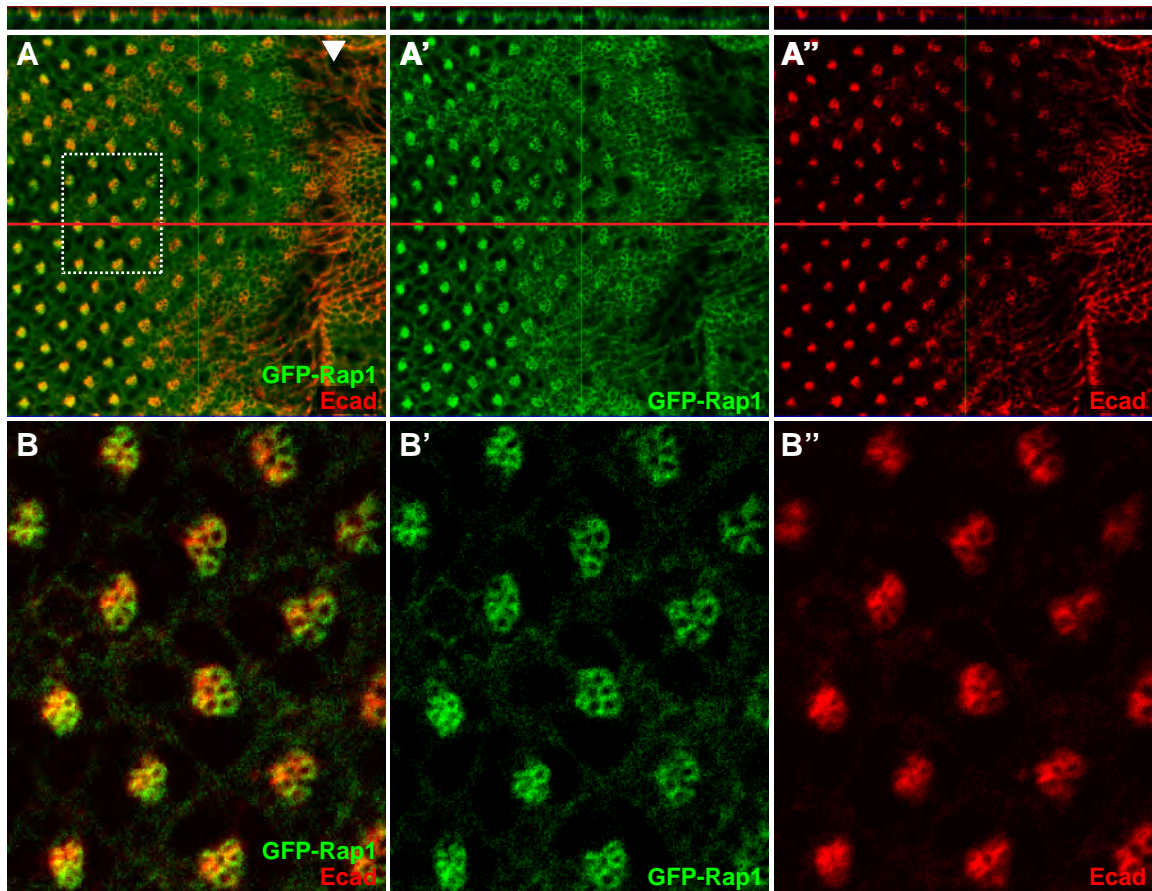
Figure S8 Expression pattern and apical subcellular localization in third instar eye disc of *GFP-Rap1* under the endogenous *Rap1* promoter. (A-A'') GFP-Rap1 accumulates at adherens junctions of developing ommatidia as revealed by co-localization with E-cadherin (Ecad) staining. The red line across each panel corresponds to the position of the optical cross-section shown at the top. (B-B'') Higher magnification of the boxed area shown in (A). Arrowheads denote the position of the morphogenetic furrow. Anterior is to the right.
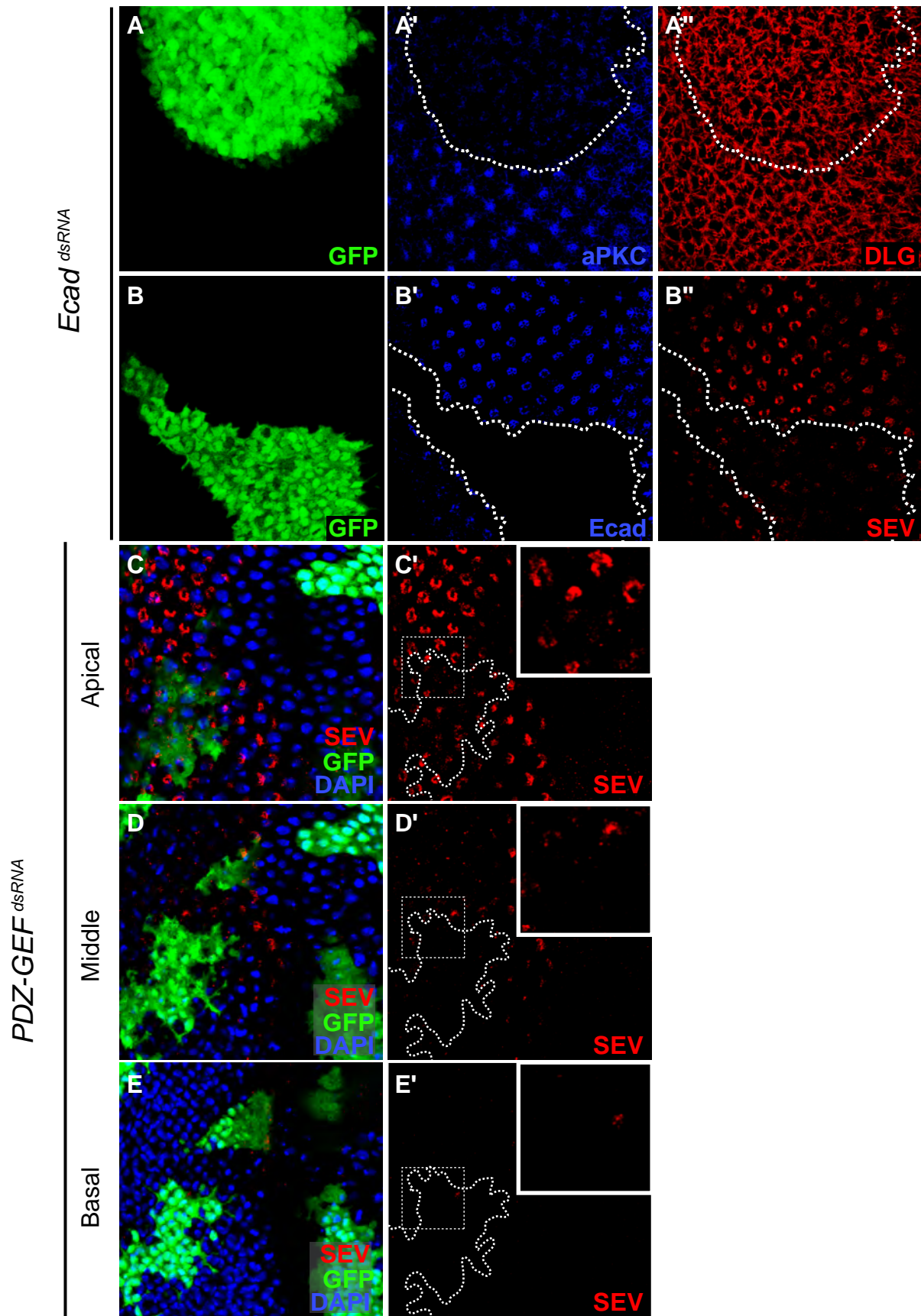
Figure S9 *Ecad* dsRNA phenocopies the loss of Rap1 activity by impairing aPKC and SEV levels in third instar eye discs. (A-A'' to E-E') Random clones (GFP-positive cells) expressing the indicated dsRNAs in third instar larval eye discs were induced using the FLP-out / Gal4 system. Eye discs were then immunostained with (A') anti-aPKC, (A'') anti-DLG, (B') anti-Ecad, and (B'', C'-E') anti-SEV antibodies. The loss of Rap1 signaling, as examplified by depleting PDZ-GEF levels (C-E), reduces SEV levels that normally populate the apical domain and does not lead to a relocalization of SEV to basolateral membranes. The boxed areas in (C'-E') are shown enlarged at the top right corner of each panel. Anterior is to the right.
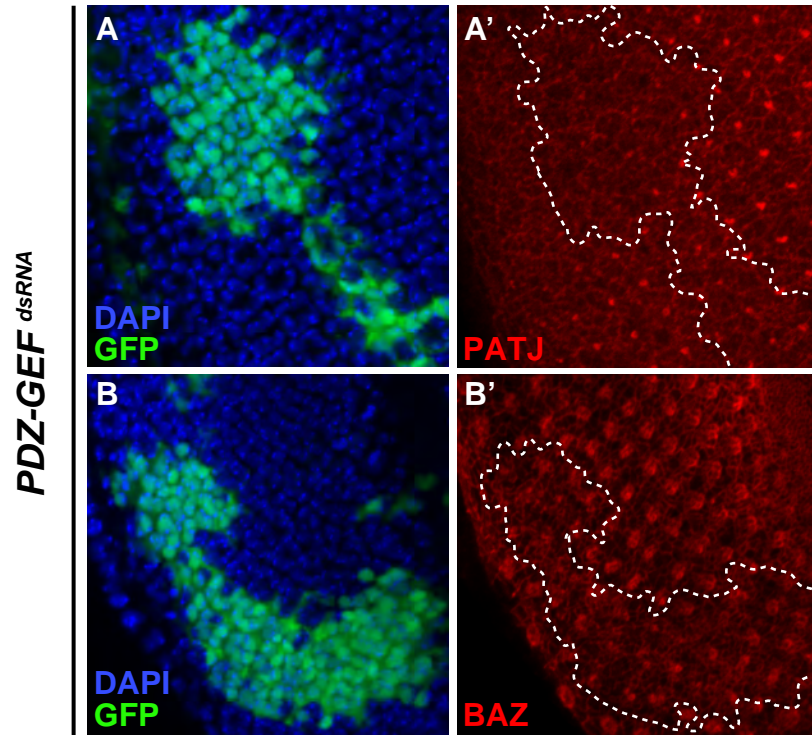
Figure S10 Knockdown of PDZ-GEF during eye development impairs apical localization of PATJ and Bazooka. Random clones (GFP-positive cells) expressing *PDZ-GEF* dsRNA were induced using the FLP-out / Gal4 system. Eye discs were then immunostained with (A') anti-PATJ or (B') anti-Bazooka. Anterior is to the right.
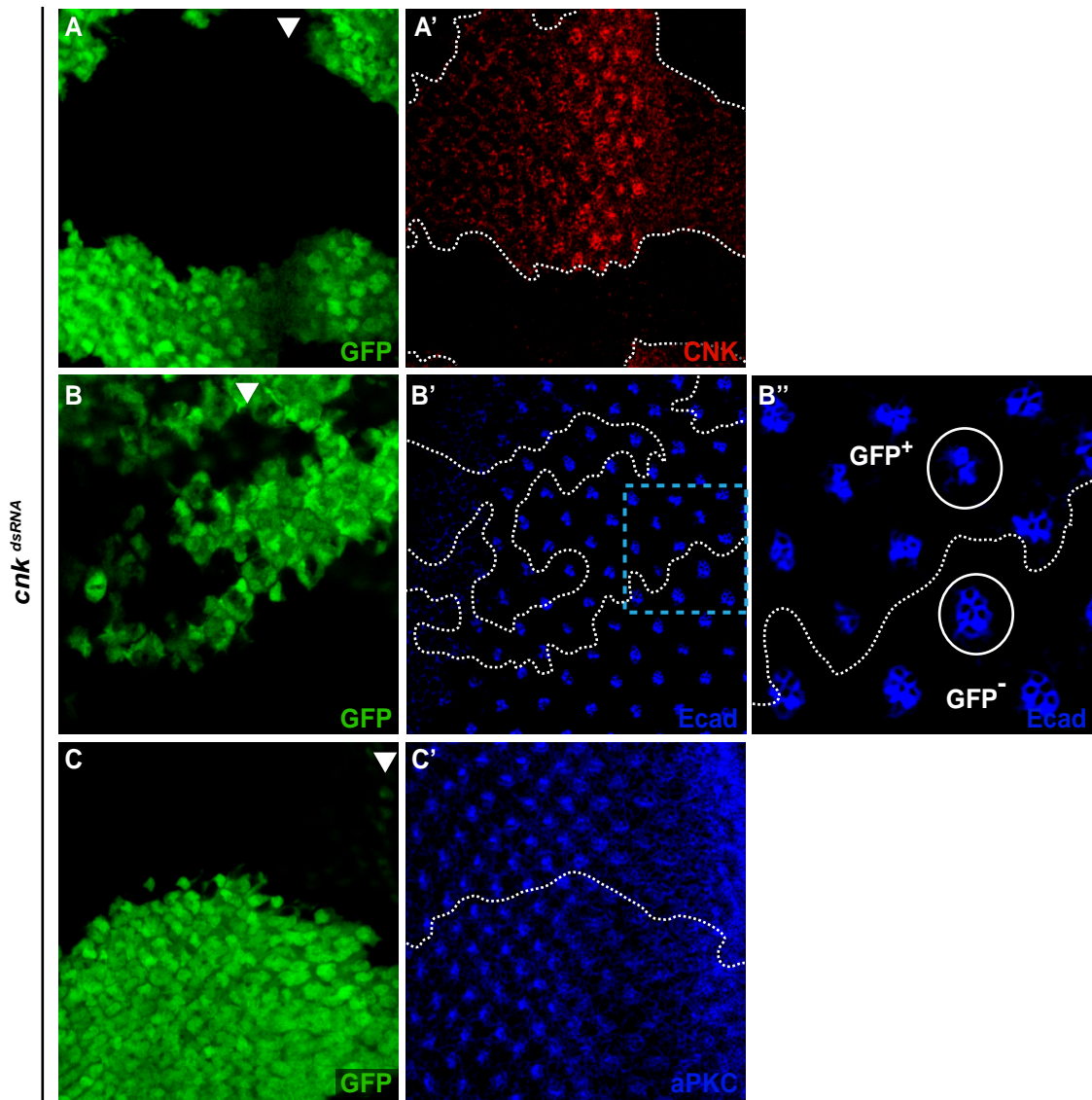
Figure S11 Knockdown of CNK in third instar larval eye discs has no impact on the level of adherens junction or apical domain markers per individual ommatidial cell. (A-A' to C-C') Random clones (GFP-positive cells) expressing *cnk* dsRNA in third instar eye discs were induced using the FLP-out / Gal4 system. Eye discs were then immunostained with (A') anti-CNK, (B' and B'') anti-Ecad; or (C') anti-aPKC antibodies. B'' corresponds to the boxed area shown in B'. Because CNK depletion impedes cell differentiation, less cells join ommatidial clusters and as a result reduces the overall area that normally stains positive for Ecad in each ommatidium. Circles in B'' highlight typical examples of the size difference between ommatidial clusters expressing (GFP+) or not (GFP–) *cnk* dsRNAs. However, Ecad or aPKC levels per cell in differentiating *cnk* dsRNA-expressing cells do not appear to be reduced compared to control cells. Arrowheads denote the position of the morphogenetic furrow. Anterior is to the right.
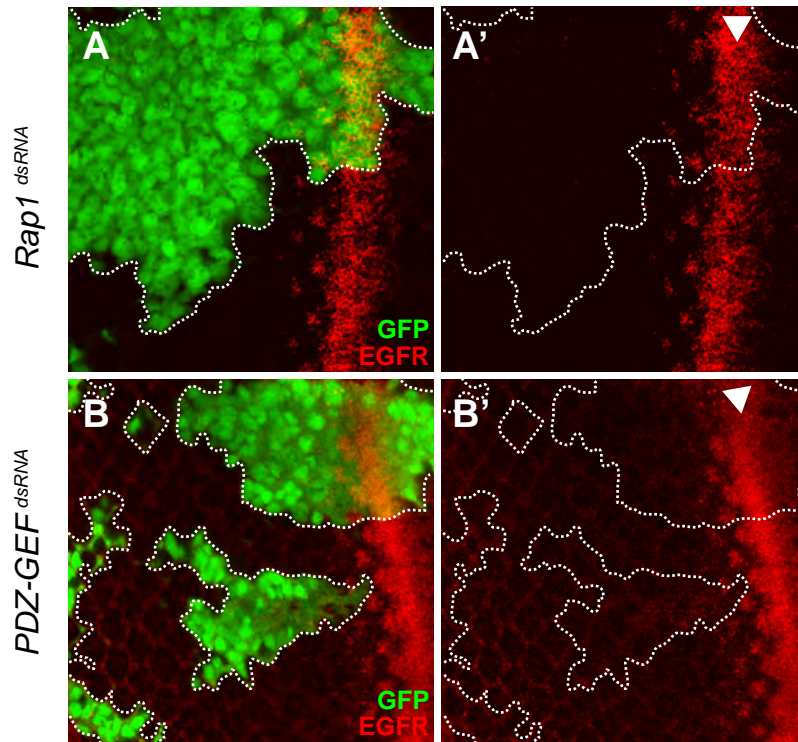
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Figure S12 Reduction of Rap1 activity during eye development does not alter Egfr expression pattern. (A-A') Rap1 dsRNA or (B-B') PDZ-GEF dsRNA were clonally expressed (marked by GFP) in the third instar eye discs using the FLP-out Gal4 system. Discs were then stained with an anti-Egfr antibody. The arrowhead refers to the position of the morphogenetic furrow. Anterior is to the right.
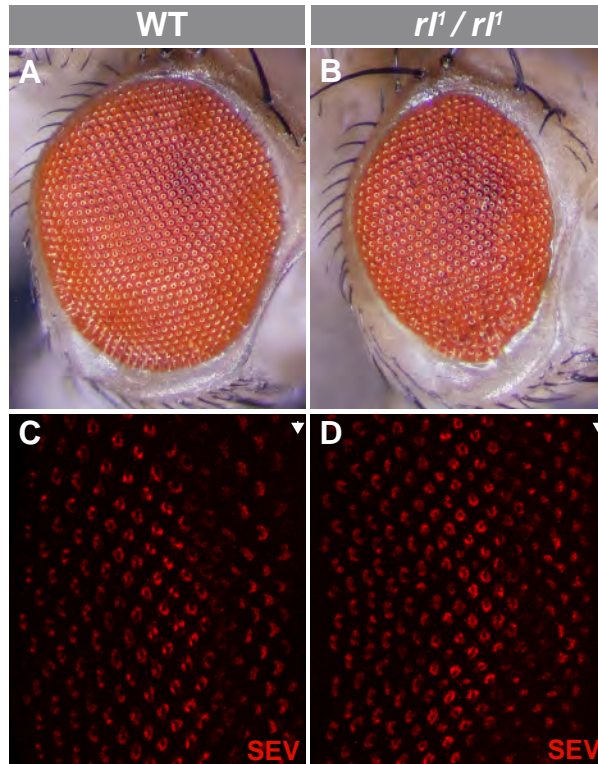
Figure S13 Reduction of MAPK activity does not alter SEV levels. Adult fly retinae (A-B) or third instar eye discs stained using an anti-SEV antibody (C-D) are shown. Compared to WT (A-C), the homozygous *rolled^1* (*rl^1*) mutation (B-D) causes eye roughness owing to the loss ommatidial cells, but does not modify SEV levels or expression pattern. Arrowheads mark the position of the morphogenetic furrow. Anterior is to the right.
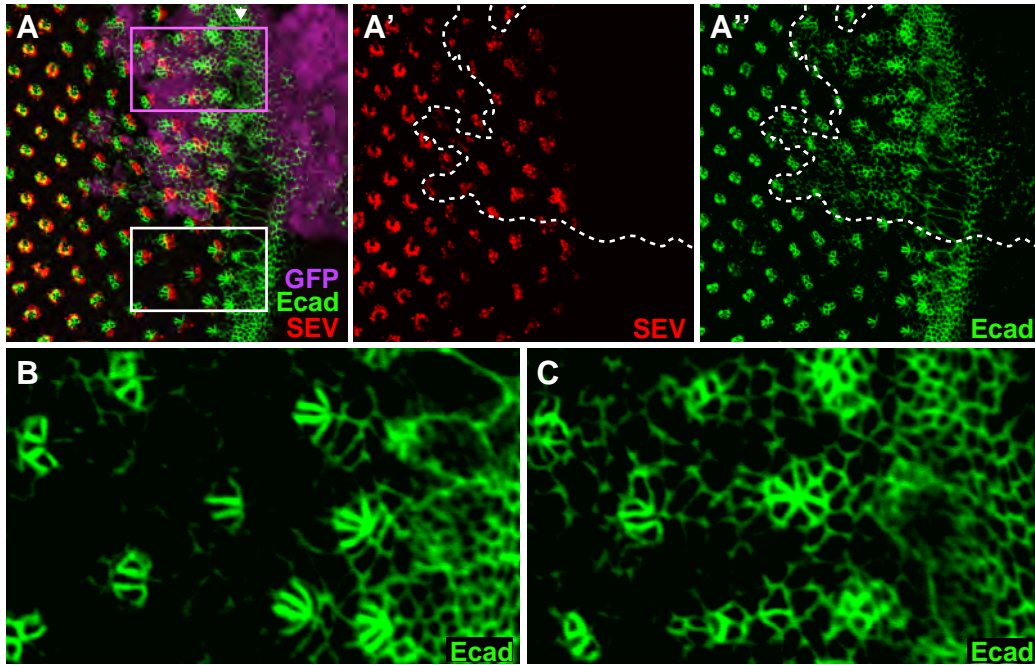
Figure S14 Rap1[V12] does not alter SEV expression, but perturbs ommatidial cluster assembly. (A-A'') Random clones (GFP-positive cells) expressing Rap1[V12] in third instar eye discs were induced by heat shock using the FLP-out/Gal4 system. Tissues were then immunostained with anti-SEV and anti-Ecad antibodies. Dotted line in (A'-A'') marks the Rap1[V12]-expressing clonal area. The white and magenta boxes in (A) encompass control and Rap1[V12]-expressing areas that are respectively shown enlarged in (B) and (C). Arrowheads denote the position of the morphogenetic furrow. Anterior is to the right.

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