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The Rap1 GTPase functions as a regulator of morphogenesis *in vivo*

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The Ras-related Rap GTPases are highly conserved across diverse species but their normal biological function is not well understood. Initial studies in mammalian cells suggested a role for Rap as a Ras antagonist. More recent experiments indicate functions in calcium- and cAMP-mediated signaling and it has been proposed that protein kinase A-mediated phosphorylation activates Rap *in vivo*. We show that *Ras1*-mediated signaling pathways in *Drosophila* are not influenced by *Rap1* levels, suggesting that *Ras1* and *Rap1* function via distinct pathways. Moreover, a mutation that abolishes the putative cAMP-dependent kinase phosphorylation site of *Drosophila* Rap1 can still rescue the *Rap1* mutant phenotype. Our experiments show that *Rap1* is not needed for cell proliferation and cell-fate specification but demonstrate a critical function for *Rap1* in regulating normal morphogenesis in the eye disk, the ovary and the embryo. *Rap1* mutations also disrupt cell migrations and cause abnormalities in cell shape. These findings indicate a role for Rap proteins as regulators of morphogenesis *in vivo*.

Keywords: *Drosophila*/GTPase/morphogenesis/Rap/Ras

Introduction

The *rap* genes, which encode low-molecular-weight GTPases are highly conserved among different organisms and have been characterized in a number of diverse species including *Dictyostelium* (Robbins *et al*., 1991), *Drosophila* (Neuman-Silberberg *et al*., 1984; Hariharan *et al*., 1991) and humans (Pizon *et al*., 1988; Kitayama *et al*., 1989). Rap proteins exhibit ~50% sequence similarity to Ras proteins at the amino acid level. More significantly, the domain in Ras proteins that interacts with effectors is highly conserved in the Rap proteins. This suggests that Rap proteins could potentially interact with Ras effectors and may thus modulate Ras-mediated signaling pathways that have been shown to regulate cell proliferation and differentiation. However, little is known about the normal function of Rap. Unlike *ras* genes, activation of *rap* genes has not been observed in human or animal cancers and so far, no loss-of-function mutations in vertebrate *rap* genes have been described. Thus the function of *rap* remains poorly understood.

Many of the experiments conducted with rap function in mammalian cell lines have highlighted the ability of overexpressed Rap proteins to antagonize Ras-mediated signaling. Indeed, the *Rap1A* gene was also initially identified as one which, when overexpressed, was capable of reverting the phenotype of Ki-ras transformed fibroblasts to a flat morphology (Kitayama *et al*., 1989). Subsequently, a number of studies have shown that either transfected wild-type *rap* or *rap* genes bearing mutations analogous to those that activate *ras* are able to attenuate *ras*-mediated signaling pathways including MAP kinase activation (Cook *et al*., 1993). However, the ability of transfected *rap* genes to interfere with *ras*-mediated signaling may merely reflect a nonproductive and promiscuous interaction of Rap with Ras-pathway components and the two related GTPases may have distinct functions *in vivo*.

Recent studies have linked Rap activation with a variety of second messenger pathways (Vossler *et al*., 1997; Kawasaki *et al*., 1998a; Zwartkruis *et al*., 1998). In PC12 cells, increased cAMP levels result in an increase in the levels of the GTP-bound (active) form of Rap1. Activated Rap1 has been shown to bind to and activate B-raf which in turn phosphorylates and activates MAP kinase, ultimately resulting in activation of the transcription factor Elk-1 (Vossler *et al*., 1997). Since protein kinase A (PKA) has been shown to phosphorylate Rap1 at a unique site (Quilliam *et al*., 1991), it has been argued that the cAMP response results from phosphorylation of Rap1 by PKA (Vossler *et al*., 1997). In blood platelets, Rap1 is activated by a different pathway. Rap1 appears to be activated by a calcium-dependent pathway during platelet aggregation (Franke *et al*., 1997), and recently a Rap-exchange factor that binds calcium and diacylglycerol has been described (Kawaski *et al*., 1998a). A role in the differentiation of PC12 cells and in platelet aggregation suggests a function in the regulation of cell morphology or cell adhesion.

Understanding the normal function of Rap proteins *in vivo* would be aided significantly by the analysis of cells that lack Rap function. In mice, four *rap* genes have been described raising the possibility of overlapping or redundant functions. No ‘knock-out’ experiments have been described so far. In contrast, loss-of-function mutations in *Drosophila Rap1* result in lethality, indicating that the *Rap1* gene provides a nonredundant function *in vivo* (Hariharan *et al*., 1991). The gain-of-function mutation, *Roughened*, which results from a single amino acid substitution in the *Drosophila* Rap1 protein has an easy-to-score rough-eye phenotype. These mutant alleles provide a valuable tool for investigating the role of *Rap1* in a variety of biological processes and for examining the influence of *Rap1* mutations on the functioning of known cellular signaling pathways.

In this study, we have examined the phenotypic consequences of removing *Rap1* during embryogenesis,

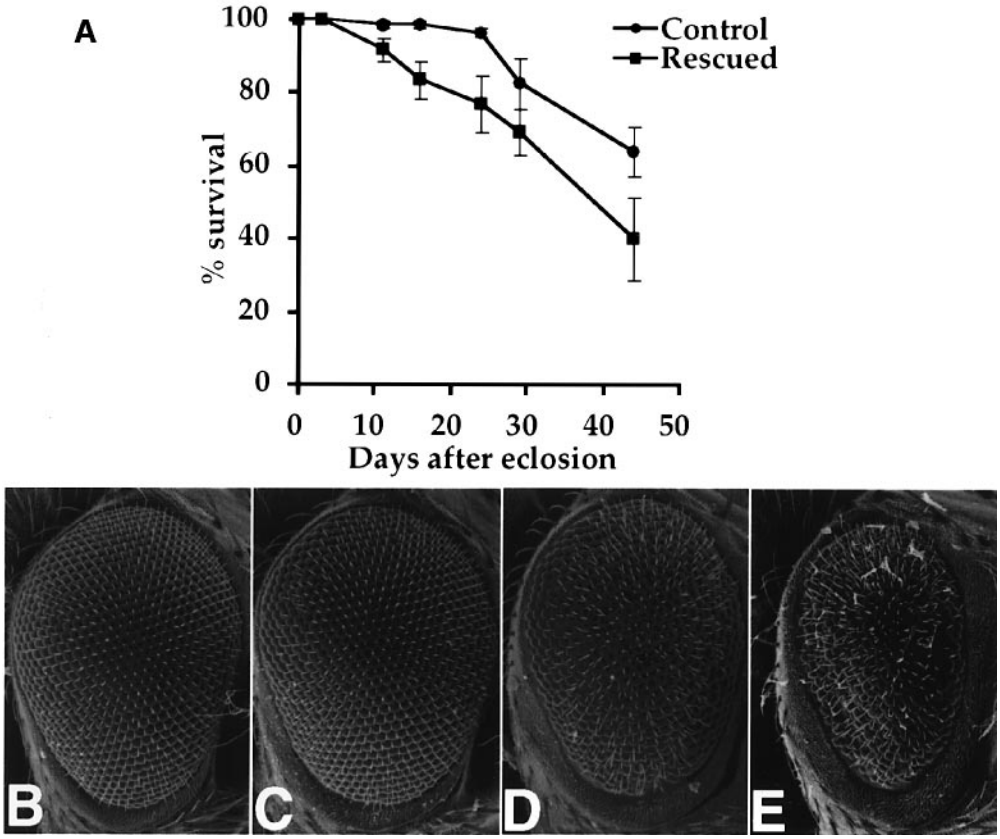

Fig. 1. Rescue of the *Rap1* mutant phenotype by *Rap1* transgenes. (**A**) Percentage of viable adults at varying periods after eclosion. Closed squares indicate numbers of $Rap1^{rv(R)B1}/Df(3L)R^{E}$ flies that had been rescued to the adult stage by induction of a *hsRap1* transgene at 37°C for 30 min. Closed circles indicate numbers of *Rap1*/+ flies that also express the *hsRap1* transgene and had been subject to the identical treatment. (**B**–**E**) Scanning electron micrographs of eyes of adult flies. (B) Wild-type, (C) a $Rap1^{rv(R)B1}/Df(3L)R^{E}$ fly that has been rescued to the adult stage by daily induction of *hsRap1*. (D) The same genotype but has received daily heat shocks only from days 3–7 of development. (E) A $Rap1^{rv(R)B1}/Df(3L)R^{E}$ mutant that was rescued by twice-daily induction of a human *rap1A* gene (*hsrap1A*).

imaginal development and oogenesis. In each of these situations, cell proliferation and cell-fate specification appear to occur relatively normally, whereas morphological aspects of differentiation are severely impaired, particularly in post-mitotic cells. These findings suggest a function for *Rap1* as a regulator of morphogenesis that is distinct from functions attributed to *Ras1*-mediated signaling.

Results

Rap1 is required for imaginal development but not for adult survival

Rap1 homozygotes die at the larval stage because maternally provided *Rap1* is sufficient for normal embryogenesis (see below). Introduction of a genomic DNA fragment that includes the *Rap1* gene fully rescues this lethality. To determine the time during development when *Rap1* is required, we generated lines of transgenic flies which express *Rap1* under the control of the heat-shock promoter. In the absence of heat shock, *Rap1/Rap1* animals die as larvae in cultures maintained either at 18 or 25°C. However, daily 30-min heat shocks generate the expected number of adult flies. These flies (Figure 1C) are indistinguishable in appearance from wild-type flies (Figure 1B). In the eye, each ommatidium contains the normal complement of cells (data not shown). Thus ubiquitous expression of *Rap1* can fully rescue the loss-of-function phenotype. By using a variety of heat-shock regimens, we determined that daily heat shocks from days 3–7 of development were both necessary and sufficient to obtain the normal number of viable adult flies. This indicates a requirement for zygotically expressed *Rap1* during the larval and early pupal phases of development. However, with this minimal heat-shock regimen, many of the flies had rough eyes (Figure 1D).

Unlike the larval and pupal stages which are characterized by cell proliferation and differentiation, adult *Drosophila* are composed mainly of fully differentiated post-mitotic cells. To determine whether *Rap1* has a continued function in such cells, we examined the requirement of *Rap1* for the long-term survival of adult flies (Figure 1A). Flies rescued to the adult stage by the induction of *Drosophila hsRap1* throughout development were maintained at 18°C after eclosion. The rescued *Rap1/Rap1* adults were able to survive for extended periods without any further heat shock (Figure 1A). However, these adults had a slightly higher mortality rate than their *Rap1*/+ siblings. Despite living almost as long as the controls, the rescued females stop laying eggs within 1 or 2 days of cessation of heat shock. Thus the requirement for *Rap1* appears to be either greatly reduced or dispensable in the post-mitotic and fully differentiated cells of the adult fly, but continued expression of *Rap1* is required

during imaginal development and for oogenesis, processes contingent on sustained cell proliferation and differentiation.

Since the human and *Drosophila rap* genes are extremely similar at the amino acid level (88% identity), we also tested whether the human *rap1a* gene can functionally substitute for *Drosophila Rap1* by generating flies that expressed human *rap1a* from the *hsp70* promoter. Rescued adult flies were obtained from cultures that had been given twice-daily heat shocks. However, the frequency of rescue was significantly less than that obtained with *Drosophila Rap1* and the rescued adults had rough eyes (Figure 1E). Thus human *rap1a* can functionally substitute for *Drosophila Rap1*, albeit imperfectly.

Examining for Rap1 function in Ras1 and PKA-mediated signaling pathways

Initial studies in mammalian cell lines led to the proposal that Rap1 functions as an antagonist of Ras-mediated signaling by competing *in vivo* for Ras effectors (Kitayama *et al*., 1989). In view of this proposed antagonism between Ras and Rap, we tested for interactions between the two genes in several ways. First, we tested whether overexpression of Rap1 could interfere with *Ras1*-mediated signaling pathways. Induction of *hsRap1* expression did not interfere with *Ras1*-mediated photoreceptor cell determination either in wild-type flies or even in flies heterozygous for *Ras1* (*Ras1*/+) arguing that *Ras1* and *Rap1* do not function in a delicate antagonistic balance. Secondly, we examined the phenotype of flies expressing reduced levels of *Rap1* for evidence of increased *Ras1*-mediated signaling. By rescuing the *Rap1* mutants using heat shocks only between days 3 and 7 of development, we were able to phenocopy a partial loss of Rap1 function. Rescued adult flies had rough eyes (Figure 1D) characterized by missing photoreceptor and pigment cells. This is almost certainly due to incomplete rescue of the Rap1 loss-of-function phenotype and not due to overexpression of *Rap1* since *Rap1*/+ flies that carried the *hsRap1* transgene and had received the identical heat-shock treatment had eyes of normal appearance. In view of the proposed antagonism between the *Ras*- and *Rap*-signaling pathways, we especially examined these rough eyes for transformation of cone cells to additional R7 cells which is indicative of excessive activity of the *Ras1*-signaling pathway. However, no ommatidia containing additional R7 cells were observed. Thirdly, we investigated the effects of varying *Rap1*-gene dosage on a variety of *Ras1*-mediated signaling pathways. We used components of the Sevenless-signaling pathway including those that are especially sensitive to *Ras1*-gene dosage and tested for effects of varying *Rap1* levels. These included the mutant backgrounds sev^{S11} (Basler *et al*., 1991) and *sev-Ras1Val12* (Fortini *et al*., 1992). In each of the sensitized backgrounds, the retinas of *Rap1*/+ flies were not significantly different to those of +/+ flies. Thus, these three lines of evidence do not support an antagonistic relationship between *Ras1* and *Rap1*-mediated signaling pathways.

More recently, it has been suggested that human Rap proteins are activated by PKA-mediated phosphorylation (Vossler *et al*., 1997). Human Rap1A has been shown to be phosphorylated by PKA on a unique serine residue

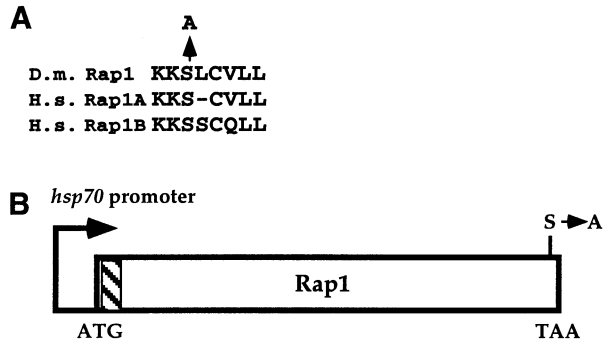

Fig. 2. Mutation in the Rap1 phosphorylation site. (**A**) Alignment of the C-terminal portions of *Drosophila* Rap1 protein (residues 177–184) with human Rap1A (residues 178–184) and Rap1B (residues 177–184). (**B**) Structure of the transgene that encodes the mutant Rap1 S179A protein. The MYC-epitope tag is cross-hatched.

(Ser180) and mutation of this serine to alanine abolishes phosphorylation of Rap1A by PKA (Quilliam *et al*., 1991). This phosphorylation site is conserved in *Drosophila* Rap1 (Figure 2A). Since rescue by *hsRap1* provides a biological assay for Rap1 function, we were able to test the importance of this phosphorylation site *in vivo*. We therefore mutated this serine residue (Ser179) to an alanine residue. Since antisera that detect the *Drosophila* Rap1 protein are not available, we also included a MYC-epitope tag at the N-terminus of Rap1 to facilitate a comparison of the levels of wild-type and mutant proteins *in vivo*. Transformant lines expressing the *Rap1* genes under the control of the *hsp70* promoter were derived (Figure 2B). Expression of the transgene was induced by incubating embryo collections at 37°C for 30 min. Following a recovery period of 2 h, lysates were prepared and analyzed by Western blotting using an antibody directed against the MYC-epitope tag. Comparable levels of the wild-type and mutant protein were expressed following heat shock (data not shown).

Expression of the MYC-tagged wild-type *Rap1* transgene by daily 30-min heat shocks results in complete rescue to the pupal stage (~30% of pupae are Tb^{+}). However, in contrast to untagged versions of the transgene, the majority of the animals die as pharate adults and a minority eclose as viable adults. Thus inclusion of the N-terminal epitope tag impairs Rap1 function to some degree but nevertheless rescues animals, at least to the later stages of pupal life. The MYC-tagged allele bearing the mutation substituting alanine for Ser179 was equally effective in rescuing animals to the pupal stage and resulted in a slightly greater number of rescued adults than the wild-type form of the gene. By using twice-daily heat shocks, we were able to obtain adults with wild-type eye morphology. Retinal sections demonstrated that photoreceptor cell differentiation and formation of the rhabdomere appear to have occurred normally. Thus mutation of the PKA phosphorylation site in *Drosophila* Rap1 does not impair its essential function in viability or its role in photoreceptor cell differentiation.

We also looked for genetic interactions between *Rap1* and mutations that impair the function of the major catalytic subunit of PKA, DC0. First, we altered the gene dosage of *PKA DC0* and examined for alterations in the phenotype of the *Roughened* allele of *Rap1* which has

a rough-eye phenotype. However, no interactions were observed. Secondly, flies overexpressing wild-type *Rap1*, using a *UAS-Rap1* transgene and a *GMR-GAL4* driver (Freeman, 1996), have mildly rough eyes. This phenotype is not modified by removing one copy of *PKA DC0*. Finally, embryos derived from *Rap1* germline clones exhibit morphological abnormalities. The severity and frequency of these abnormalities are increased when these embryos are fertilized by *Rap1* mutant sperm (see below). However, fertilization by *PKA DC0* mutant sperm has no effect on the phenotype. Taken together, our experiments do not provide any evidence that PKA activity is a major regulator of Rap1 function *in vivo*.

Removal of Rap1 disrupts morphological differentiation but not cell proliferation during oogenesis and eye development

Adult *Rap1/Rap1* females rescued by *hsRap1* continue to lay eggs as long as *Rap1* is provided by daily heat shocks. Females stop laying eggs within 3 days of the cessation of the heat shocks. If heat shocks are resumed at this stage, females begin laying eggs once again after a lag of a further 3–4 days. Thus egg production in adult females is absolutely dependent on the continued presence of Rap1 protein.

In wild-type ovarioles, an orderly progression of increasingly more mature stages can be visualized by staining with DAPI and phalloidin (Figure 3A and C) (Cooley and Theurkauf, 1994). In contrast to wild-type ovarioles, those egg chambers examined 5–7 days after the cessation of heat shocks showed significant degeneration. Staining with DAPI revealed many condensed brightly staining nurse cell and follicle-cell nuclei that had undergone fragmentation (Figure 3B). Neither the outlines of the follicle cells nor the actin that separates individual nurse cells was evident by phalloidin staining (Figure 3D). In many egg chambers, the cytoskeletal remnants were condensed into a single spherical mass (Figure 3E). Prior to the complete collapse of the cytoskeleton, discontinuities in the follicle-cell epithelium (Figure 3E) were observed. Thus removal of *Rap1* function results in the degeneration of egg chambers, mainly at the later stages of oogenesis.

Before degeneration, the egg chambers showed significant defects in their organization. In wild-type ovarioles, individual egg chambers are connected by 4–6 interfollicular stalk cells. However, in the ovarioles deprived of *Rap1* for 5–7 days, stalk cells are missing between egg chambers. Moreover, in some of these abnormal egg chambers, the follicle-cell epithelium is no longer a uniform monolayer but appears stratified (not shown). In egg chambers deprived of *Rap1* throughout their development (for 9 days), we observed instances where two egg chambers had fused since they contained twice as many nurse cell nuclei and two oocyte nuclei (Figure 3F). Each oocyte had four ring canals, indicating that the increased number of cells in the egg chamber had not resulted from an additional round of cell division of the cystoblast but rather from the encapsulation of the progeny of two cystoblasts into a single egg chamber. We also observed egg chambers with very few nurse cells (Figure 3G and H) or nurse cell nuclei that were no longer contained within the follicle-cell layer. Thus *Rap1* appears to be necessary for the appropriate interaction between the follicle cells and the cyst which normally results in the enveloping of a 16-cell cyst by follicle cells.

To determine whether these abnormalities reflected a requirement for *Rap1* in the germline or in the soma, we generated mosaic ovaries lacking *Rap1* in each of these compartments. Ovaries derived from females lacking *Rap1* in the germline revealed a number of normal late-stage egg chambers and no signs of degeneration. Furthermore, embryos were derived from these germline clones (see below) arguing that *Rap1* function was not required in the germline for the completion of all stages of oogenesis. These observations suggested a role for *Rap1* in the somatically derived follicle-cell epithelium. We therefore generated clones of mutant follicle cells by FLP/FRT-mediated mitotic recombination. In clones which had been generated at later stages of oogenesis, both the morphology and the proliferative ability of the follicle cells appeared normal. A number of *Rap1/Rap1* mutant follicle-cell clones was observed (data not shown), which were comparable in size to the 'twin spot' derived from the same recombination event, indicating that cells lacking a wild-type copy of *Rap1* are still capable of proliferating for significant periods. However, in clones generated at the early stages of oogenesis, achieved by examining stage 8–10 egg chambers >8 days after the recombination event, degenerative changes were observed. This argues for a role of *Rap1* at an early stage of egg chamber development, possibly in the germarium. Thus Rap1 function seems to be required in the somatically derived follicle cells at a stage when the individual cysts are assembled and surrounded by follicle cells. The degenerative changes might be secondary to abnormal morphogenesis of the individual follicles.

Mutations in *Notch*, *Delta* and *daughterless* display similar defects during oogenesis and all three have been shown to be required in the follicle cells (Ruohola *et al*., 1991; Xu *et al*., 1992; Bender *et al*., 1993; Cummings and Cronmiller, 1994). Moreover, females that are *trans*-heterozygous for mutations in any two of these three loci display abnormalities in follicle-cell differentiation, suggesting that these three genes function in a common pathway. We found that *Rap1* did not interact dominantly with mutations in *Notch*, *Delta* or *daughterless*. No abnormalities were observed in those ovaries suggesting that *Rap1* may function in a different pathway.

Examination of *Rap1* mutant clones in the eye imaginal disk reinforced the notion that *Rap1* is not required for cell proliferation but rather for terminal differentiation. Clones of *Rap1/Rap1* mutant tissue were generated by inducing FLP/FRT-mediated mitotic recombination during the first larval instar. Imaginal disks dissected during the third larval instar (Figure 4A) and early pupal stages revealed large clones which were comparable in size to the 'twin spot' generated by the same recombination event. However, adult eyes lacked *Rap1/Rap1* tissue but instead displayed scars. Thus the *Rap1/Rap1* tissue did not survive to the adult stage. The scarred regions were mostly composed of tissue that had undergone degeneration and remnants of rhabdomeres were visible. Very occasionally, isolated mutant photoreceptors were apparent (arrows in Figure 4B) which had rhabdomeres displaying abnormal morphology. These observations indicate that mutant precursor cells undergo normal proliferation and that

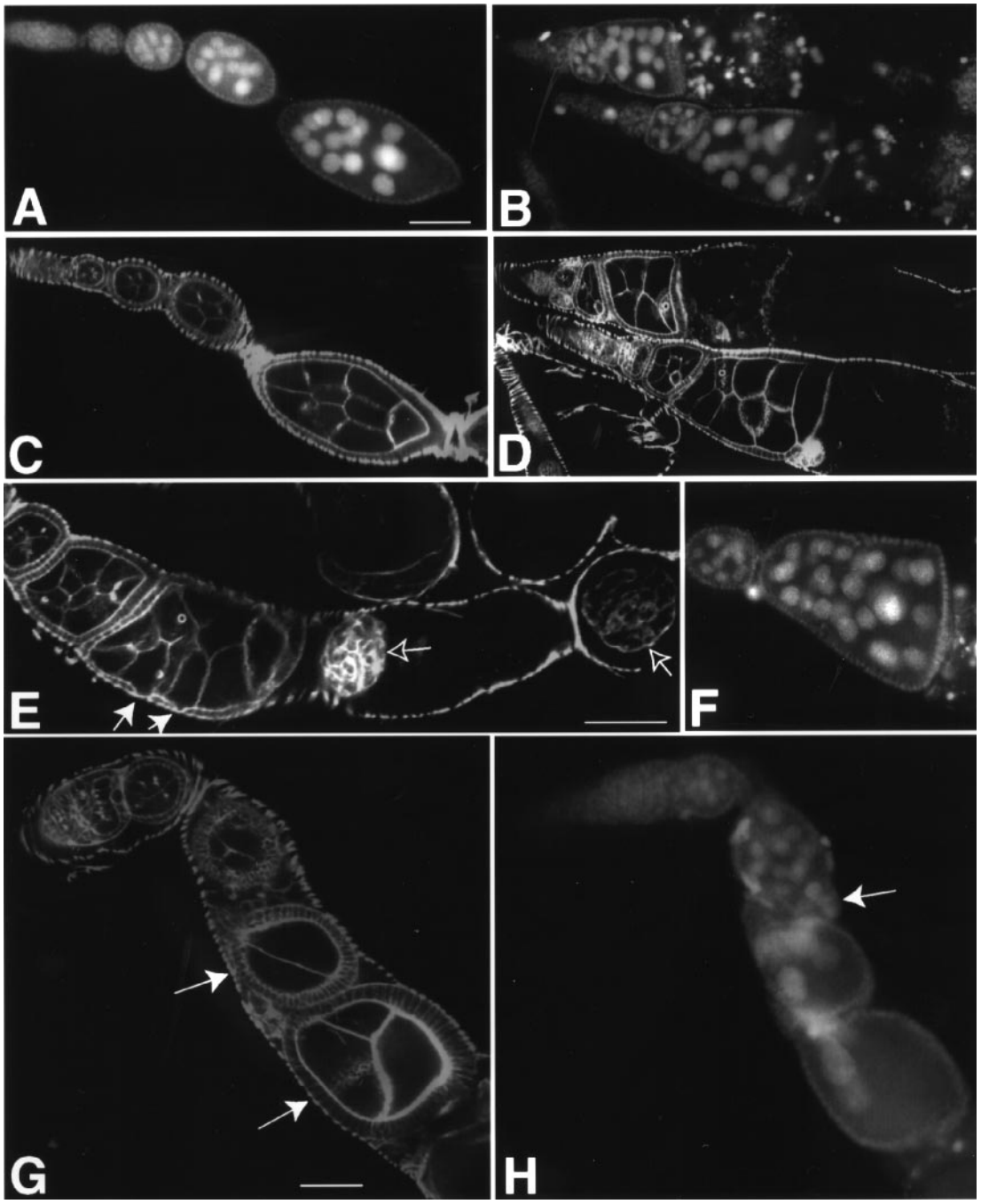

Fig. 3. Defects in oogenesis induced by withdrawal of Rap1. (**A**–**E**) Ovaries from either *hsRap1 Rap1*$^{rv(R)B1}$/+ females (A, C) or *HsRap1 Rap1*$^{rv(R)B1}$/*Df(3L)R*^{E} (mutant) females (B, D, E), 7 days after cessation of heat shocks. (A and B) were stained with DAPI, (C–E) with phalloidin. The open arrows indicate complete degeneration of the cytoskeleton of the egg chamber. The filled arrows indicate discontinuities in the follicle-cell epithelium. (**F**–**H**) Ovaries from mutant females examined 9 days after cessation of heat shocks. (F and H) are stained with DAPI, (G) with phalloidin. The arrows in (G) indicate egg chambers containing greatly reduced numbers of nurse cells. The arrow in (H) indicates a nurse cell nucleus that is outside the follicle-cell epithelium. The scale bar represents 100 μm in (A–F) and 30 μm in (G and H).

photoreceptor cells progress to a relatively late stage of photoreceptor differentiation prior to undergoing degeneration.

Withdrawal of Rap1 disrupts embryonic morphogenesis and cellular morphology

If *Rap1* is indeed involved in regulating morphological differentiation of epithelial cells, such defects should become evident in embryos that lack Rap1 function. *Rap1/Rap1* animals die at late larval stages, presumably because maternally supplied Rap1 suffices for normal embryogenesis. By generating germline clones, we were able to remove maternally supplied Rap1. This results in embryonic lethality even when the embryos were derived from germline clones that were fertilized by wild-type sperm; hatching rates of only 2–4% were obtained. This indicates a strong requirement for maternally provided Rap1 for normal embryonic development. In embryos lacking maternally provided Rap1 (hereafter referred to as mutant embryos), early specification of the embryonic

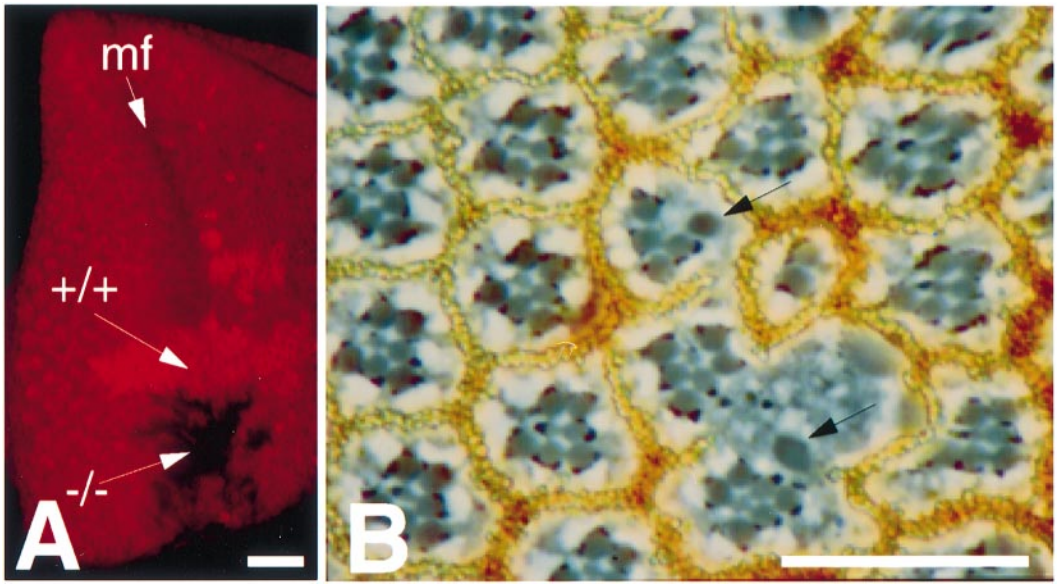

Fig. 4. (**A**) Clones in eye imaginal disk visualized by anti-MYC staining. Anterior to the right. The morphogenetic furrow (mf) is indicated. The $Rap1^{CD3}/Rap1^{CD3}$ mutant clone (–/–) and the wild-type 'twin-spot' (+/+) are shown by arrows. (**B**) A section through a $Rap1^{CD3}/Rap1^{CD3}$ mutant clone in the adult eye. Mutant cells are *white*. Arrows indicate rhabdomeres of mutant (–/–) photoreceptor cells. The scale bars represent 20 μm.

axes occurs normally as assessed by cuticle preparations and by staining with anti-Eve for anteroposterior patterning (Macdonald *et al*., 1986) and by *twist* (Thisse *et al*., 1987) and *zen* (Doyle *et al*., 1989) expression for dorsoventral patterning (not shown). Moreover, expression of *tailless* was normal, indicating that patterning of terminal structures via *torso* and *Ras1* (Duffy and Perrimon, 1994) is not perturbed by removing *Rap1* (Figure 5A and B). Thus another *Ras1*-mediated signaling pathway is not perturbed by alterations in the level of *Rap1*.

In marked contrast, morphogenesis was severely disrupted in mutant embryos undergoing gastrulation. Most commonly (~50% of embryos), there was a failure of the ventral furrow to close (Figure 5C–F), especially in the anterior regions of the embryo leading to a split of the ventral nerve cord in that region (Figure 5G and H). Older embryos (stage 14–15) display defects in head involution (40–50%) (Figure 5I and J) and less frequently (5–10%), defects in dorsal closure (Figure 5K and L) were also apparent. Each of these defects increased in penetrance and severity when eggs were fertilized by *Rap1* mutant sperm.

We examined the formation of the ventral furrow in wild-type and mutant embryos. In wild-type embryos, the ventral furrow is formed at the onset of gastrulation when a stripe of Twist-expressing cells on the ventral region of the embryo undergo apical constriction (Figure 5C). These cells then invaginate and give rise to the mesoderm (Leptin and Grunewald, 1990). This is followed by the apposition in the midline of the two flanking rows of mesectodermal cells (Figure 5E) which leads to closure of the ventral furrow. In mutant embryos, Twist is expressed in the mesodermal precursors (Figure 5D) and they display the apical constriction and membrane blebbing that is characteristic of the invaginating region. Moreover, as in wild-type embryos, the mesectodermal cells express the *sim–lacZ* reporter gene (Nambu *et al*., 1991) normally. However, in many embryos, these cells were not brought together in the midline following ventral furrow formation and remained separated as two distinct rows especially in the anterior regions of the embryo (Figure 5F). Thus, whereas the cell types that participate in ventral furrow formation and closure are specified normally, the morphogenetic events that mediate ventral furrow closure do not occur.

To examine the morphology of the ventral furrow, embryos were sectioned during the stages when the ventral furrow forms and then closes. The shape of the mesodermal cells as well as the other cells in the mutant embryos are extremely abnormal. In wild-type embryos, during stages 7 and 8, the nuclei are located apically in most of the cells and the nuclei move basally in the cells of the ventral furrow which are undergoing apical constriction (Figure 6A). In contrast, in the mutant embryos, nuclei are found at a variety of levels in the dorsal and lateral cells, and many of the nuclei fail to move basally as the ventral furrow forms (Figure 6B).

Mutations in *Rap1* also interfere with normal cell migrations. Internalized mesodermal precursors migrate laterally within the embryo (Beiman *et al*., 1996; Gisselbrecht *et al*., 1996). Their subsequent interaction with the overlying ectoderm leads to the specification of dorsal mesodermal fates. In mutant embryos, mesodermal cell migration is severely perturbed (Figure 6C and D) and dorsal structures such as the heart and dorsal vessel are not formed normally (Figure 5I and J). In contrast, in embryos that proceed to later stages of embryonic development, branching of the tracheal tree is observed suggesting that tracheal cell migrations may be somewhat less sensitive to loss of maternal Rap1. Another dramatic example of cell migration occurs during the formation of the gonad (Williamson and Lehman, 1996) when the germline-derived pole cells migrate from the posterior pole of the embryo through the wall of the posterior midgut to condense with mesodermal tissue to form the gonad (Figure 6E and G). Pole-cell migration is severely perturbed in mutant embryos (Figure 6F and H). Many pole cells fail to migrate through the wall of the midgut and only a small number of pole cells reach the gonad.

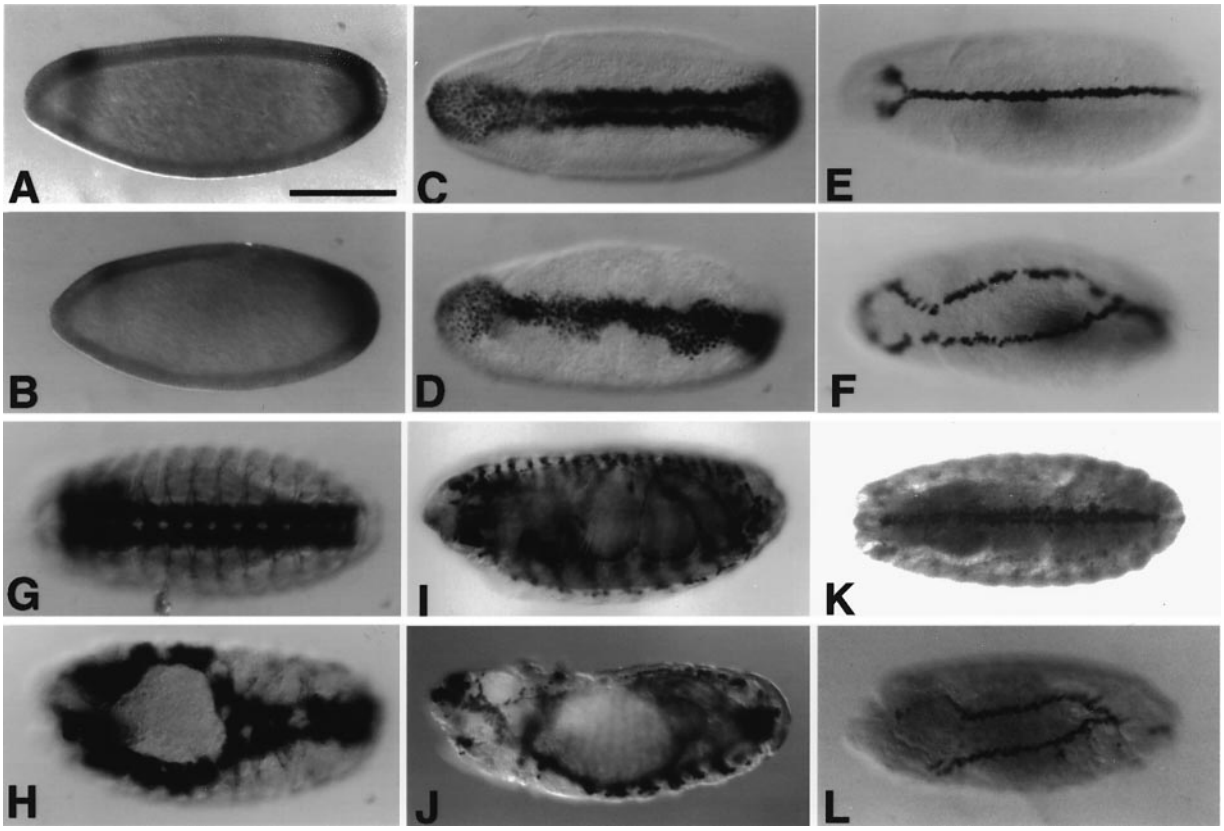

Fig. 5. Abnormal morphogenesis in embryos lacking Rap1. Wild-type embryos (A, C, E, G, I, K) and embryos derived from $Rap1^{CD3}/Rap1^{CD3}$ germline clones (B, D, F, H, J, L) fertilized by wild-type sperm. (**A**, **B**) Blastoderm embryos, *tailless* RNA was detected by *in situ* hybridization; (**C**, **D**) ventral views of embryos that have initiated gastrulation stained with anti-Twist antibody; (**E**, **F**) embryos undergoing germ-band elongation stained with anti-β-galactosidase to detect expression of a paternally provided *sim–lacZ* reporter in mesectodermal cells; (**G**, **H**, **I**, **J**) embryos that have completed germ-band retraction and are at a stage when the wild-type embryos have completed dorsal closure. (G, H) Ventral views stained with mAb BP102; (I, J) lateral views stained with anti-D-mef2; (**K**, **L**) dorsal views of embryos stained with anti-β-galactosidase to detect expression of a paternally provided *puc–lacZ* reporter in cells of the leading edge. Anterior is to the left in all panels. The scale bar represents 100 μm.

Thus, a lack of *Rap1* function disrupts the migration of at least two different cell types.

Defects in the shape and organization of epithelial cells as well as in cell migrations is suggestive of abnormalities in the organization of the cytoskeleton. We therefore examined the apical profile cells in blastoderm embryos and in gastrulating embryos with an antibody directed against Neurotactin (BP106) (Hortsch *et al*., 1990). Consistent with the defects observed in the cross sections (Figure 6B and D), the cell outlines were more irregular than in wild-type embryos. However, staining with antibodies to beta heavy-spectrin (Thomas and Kiehart, 1994) and with phalloidin to visualize filamentous actin did not reveal any obvious abnormalities in the intensity of staining or abnormalities in the cellular distribution of these proteins. Thus the defects in cell shape must result from more subtle defects in cytoskeletal organization.

Discussion

Interaction of Rap1 with signaling via Ras1 and PKA

Initial studies of Rap function in mammalian cells argued for a mechanism where Rap interacted with Ras effectors in a nonproductive manner and hence antagonized the function of Ras. Our results suggest that *Drosophila* Ras and Rap proteins do not have an analogous antagonistic relationship. First, we observe no diminution in *Ras1*-mediated signaling when *Rap1* is expressed at increased levels, even when the gene dosage of *Ras1* is reduced. Secondly, phenotypes observed when *Rap1* function is compromised do not mimic those observed in situations of increased *Ras1* activity. Finally, certain mutant backgrounds that are extremely sensitive to changes in *Ras1* gene dosage are not particularly sensitive to alterations in *Rap1* levels.

The gain-of-function allele of *Rap1*, *Roughened*, has a dominant eye phenotype which is consistent with antagonism of *Ras1*-mediated photoreceptor cell determination. The *Roughened* mutation results in a single amino acid substitution in Rap1. The analogous mutation in mammalian Ras results in global structural changes and an increase in the levels of the active GTP-bound form (Quilliam *et al*., 1995). However, since the genetic tests we have conducted do not reveal any effect of the levels of the wild-type form of *Rap1* on the *Ras1* pathway, it seems possible that the *Roughened* phenotype may reflect properties of the mutant protein which are not shared by the wild-type form.

More recently, there is accumulating evidence that Rap proteins in mammalian cells are activated by increases in intracellular levels of cAMP (Vossler *et al*., 1997). In

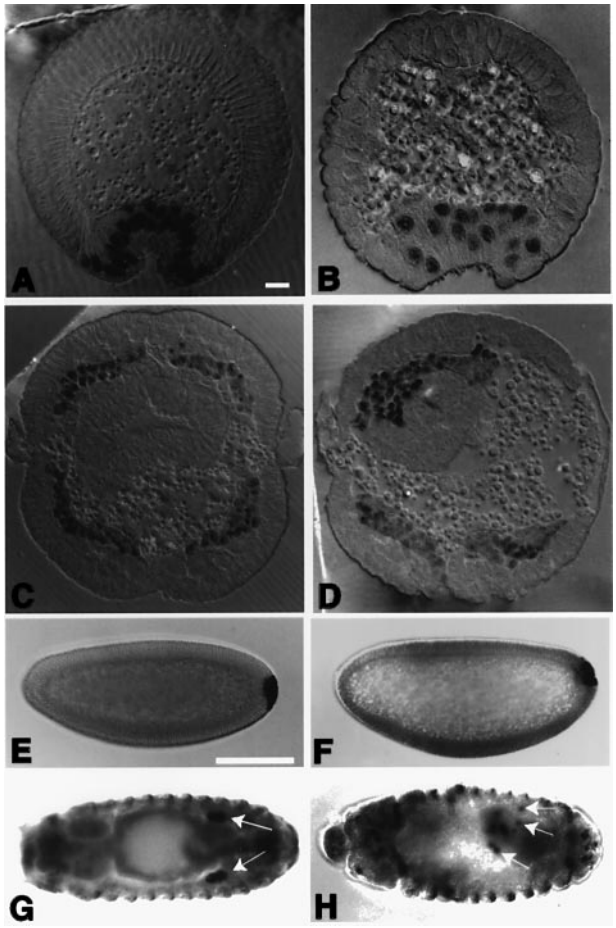

Fig. 6. Abnormal migration of mesodermal precursors and pole cells in mutant embryos. Wild-type embryos (**A**, **C**) or embryos derived from *Rap1/Rap1* germline clones (**B**, **D**) have been stained with anti-Twist and sectioned at 50% egg length. Embryos in (A) and (B) were staged as late stage 7/early stage 8. Some mutant embryos have fewer Twist-staining nuclei. Embryos in (C) and (D) were stage 10. (**E**, **F**, **G**, **H**) Embryos stained with anti-Vasa antibody, (E and F) are lateral views of blastoderm embryos and (G and H) are focused at the level of the gonads in embryos that have completed germ-band retraction. Anterior is to the left in (E–H). The scale bar represents 10 μm in (A–D) and 100 μm in (E–H).

PC12 cells, Rap appears to activate MAP kinase via B-Raf. The precise mode by which alterations in cAMP levels lead to changes in the levels of GTP-bound Rap has not yet been elucidated. It has been proposed that activation of Rap requires phosphorylation of Rap by PKA. We have mutated the conserved PKA phosphorylation site in *Drosophila* Rap1 and demonstrated that the construct expressing the mutant Rap1 protein is able to function *in vivo* as well as an analogous construct expressing wild-type Rap1. Thus phosphorylation of this conserved site is not essential for Rap1 function *in vivo*. Moreover, we have been unable to detect mobility shifts of epitope-tagged Rap1 proteins (data not shown) even in flies that express a constitutively active PKA (Jiang and Struhl, 1995). This raises the possibility that cAMP could activate Rap1 by a PKA-independent pathway. Such a mechanism may operate via cAMP-activated Rap1 guanine-nucleotide exchange factors which have recently been discovered by two laboratories (de Rooij *et al*., 1998; Kawasaki *et al*., 1998b). Alternatively, redundant mechanisms may operate *in vivo* to mediate the activation of Rap1 by PKA.

Role of Rap1 in morphogenesis

We have shown that *Rap1* function is required during embryogenesis, imaginal development and oogenesis, and seems unnecessary or is minimally required for fully differentiated mitotically quiescent cells in adult *Drosophila*. These observations are consistent with a role in some aspect of development such as cell proliferation, cell-fate specification or terminal differentiation rather than in functions essential for cell survival. In each situation where *Rap1* function has been removed, cell proliferation and the appropriate specification of a wide variety of cell types occurs normally. Cells of the embryonic epidermis appear to have undergone the normal number of cycles as assessed by staining with the anti-phos-H3 antibody (de Nooij *et al*., 1996) which stains mitotic cells. In the eye imaginal disk and in the follicle cells of the egg chamber we were able to show that clones of mutant tissue were comparable in size with the twin spot of wild-type tissue that had been generated from the same recombination event. Thus *Rap1* mutant cells appear to proliferate normally.

An important role for *Rap1* in cell-fate specification seems unlikely since most cell types are specified normally in *Rap1* mutants. In the instances where some cell types were under-represented in mutant embryos, for example dorsal mesodermal fates, these could be accounted for by abnormalities in morphogenesis or cell migration where precursor cells are not exposed to the appropriate inductive signals. In the eye imaginal disk, ELAV-staining nuclei are observed in the developing ommatidial clusters (data not shown) indicating that neuronal fates are specified in mutant clones. This contrasts with mutations in the related *Ras1* gene which block neuronal cell fate specification in the eye imaginal disk.

Our experiments demonstrate that morphological aspects of differentiation are seriously impaired in the absence of *Rap1* function. During embryonic development, closure of the ventral furrow and head involution fail to occur frequently. Moreover, cell morphology is grossly abnormal in gastrulating embryos, with cells displaying aberrant shapes and nuclear positions. Cell migrations are also significantly perturbed. At later developmental stages, photoreceptor cells and follicle cells degenerate at a time when they normally undergo significant morphological changes. These findings point towards a role for *Rap1* in either directing or facilitating morphological aspects of differentiation. Significantly, not all developmental processes that require changes in cell morphology are disrupted in *Rap1* mutants. Germ-band extension occurs normally in most instances as does axon elongation since the longitudinal connectives of the central nervous system (CNS) are formed in mutant embryos.

A conserved role for Rap1 in regulating morphogenesis

Recent studies in mammalian tissue culture cells support the notion that Rap is a regulator of cell morphology. Expression of a dominant-negative form of rap1B, is able to block the neuronal differentiation of PC12 cells in response to nerve growth factor (NGF) (York *et al*., 1998). NGF induces an early phase of MAP kinase activation via Ras and Raf and a subsequent sustained phase of MAP kinase activation which is mediated via Rap and

B-Raf. Sustained activity of MAP kinase appears to be necessary for the differentiation response, since stimulation of PC12 cells by the EGF receptor results solely in the early phase of MAP kinase activation and results in cell proliferation. The observation that mutations in *Drosophila* Rap1 fail to disrupt cell proliferation yet have profound effects on morphological differentiation are consistent with this observation. Indeed at least two *Drosophila* receptor tyrosine kinases (RTKs), those encoded by the *breathless* and *heartless* loci, regulate morphogenesis by regulating the migration and the morphology of post-mitotic cells. The migration of mesodermal precursors which normally occurs in response to Heartless-mediated signaling is perturbed in *Rap1* mutant embryos. Thus Rap1 might have a conserved role in mediating RTK signaling during morphogenesis. If, as in PC12 cells, such signals are mediated via B-Raf, the elucidation of that pathway in *Drosophila* awaits the characterization of the *Drosophila* B-Raf ortholog.

In mammalian cells Rap is activated by signals other than those emanating from RTK activation. For instance, Rap1B is activated to high levels during platelet aggregation apparently in response to an increase in intracellular calcium (Franke *et al*., 1997). Platelet aggregation is contingent on reorganization of the cytoskeleton and on alterations in cell–cell and cell–substrate adhesion. All these observations argue for a role for Rap1 in mediating certain morphological changes, possibly in response to several different intracellular signaling pathways.

In *Saccharomyces cerevisiae*, the *Rap1*-like gene *BUD1* has a crucial role in bud site selection (Chant, 1996). *Bud1* mutants grow and divide normally but choose their bud sites randomly in contrast to wild-type yeast which follow a unipolar or bipolar budding pattern. Once the bud choice is made, *Bud1* mutants are able to polarize their cytoskeleton appropriately towards the site of bud outgrowth. Overexpression of human Rap in wild-type yeast has been shown to phenocopy the *Bud1* mutation arguing that Rap1 can interact with the Bud1 pathway (McCabe *et al*., 1992). It is unclear as to what cellular processes are analogous to bud-site selection in the cells of a multicellular eukaryote. *Rap1* mutations do not seem to abolish asymmetric divisions in *Drosophila* since neuroblast division occurs and the cells of the peripheral nervous system (PNS) are specified appropriately. However, the abnormal cell shapes and nuclear positions observed in gastrulating embryos may reflect abnormalities in some of the same processes that affect choice of the budding site in yeast.

In *S.cerevisiae*, a complex of proteins including Bud1p, functions upstream of the Cdc42p GTPase (Chant and Stowers, 1995). This raises the possibility that *Drosophila Rap1* may function in a similar signaling pathway. We were unable to observe any genetic interactions between *Rap1* and mutant alleles of *Cdc42* (Fehon *et al*., 1997), *Rho1* (Strutt *et al*., 1997) and the gene encoding the Rho1 exchange factor *DRhoGEF2* (Barrett *et al*., 1997). However, the absence of such interactions does not exclude the possibility that those genes function in a pathway *in vivo*.

The demonstration of a role for *Rap1* in regulating morphogenesis provides a starting point for the examination of Rap function in a variety of organisms. By analogy with other Ras family GTPases, it is possible that Rap functions via multiple effector pathways. These pathways will possibly be elucidated by a combination of genetic and biochemical approaches.

Materials and methods

Drosophila stocks

Two loss-of-function alleles, $rv(R)^{B1}$ and $rv(R)^{B3}$ were generated by reverting the dominant gain-of-function mutation *Roughened* (Hariharan *et al*., 1991). These alleles will subsequently be referred to as $Rap1^{rv(R)B1}$ and $Rap1^{rv(R)B3}$. Both alleles have premature stop codons in the N-terminal part of the protein and are possibly null alleles. An additional allele, $Rap1^{CD3}$, was kindly provided by Tim Sliter; it has a 4.5 kb deletion that deletes the entire coding region of the *Rap1* gene. All three alleles behave as genetic nulls and their phenotypes are indistinguishable. The *hsRap1* transgene was generated by cloning a PCR-amplified genomic fragment of *Rap1* that includes the entire coding region into the pCaSpeR-hs vector. In addition, tagged versions of *Rap1* were generated by PCR mutagenesis. PCR was used to insert a MYC-epitope tag in the *Rap1* gene and to add *Eco*RI and *Xba*I sites to the 5′ and 3′ ends of the *Rap1* gene. The resulting protein had a 12 amino acid tag (EEQKLISEEDLL) between amino acids 2 and 3 of Rap1. The S179A mutant was generated by altering codon 179 TCC (Ser) to GCC (Ala). The altered *Rap1* genes were cloned into pCaSpeR-hs cleaved with *Eco*RI and *Xba*I. Multiple transgenic lines expressing each construct were generated by injecting the constructs into w^{1118} embryos. The genomic DNA rescue construct was generated by cloning a 4.5 kb *Eco*RI fragment into pCaSpeR and the UAS-Rap1 construct was generated by cloning into pUAST cleaved with *Eco*RI and *Xba*I. To express human *rap1A* in *Drosophila*, a human *rap1A* cDNA was cloned into into pCaSpeR-hs.

In each case, the transgene was recombined onto a chromosome harboring the $Rap1^{rv(R)B1}$ mutation. Flies carrying this chromosome were crossed to flies heterozygous for the deletion $Df(3L)R^{E}$ which removes the *Rap1* gene. Each chromosome was maintained over the balancer *TM6B Tb Hu*. Rescued pupae were therefore Tb^{+} and rescued adults were Hu^{+}. Expression of the tagged *Rap1* transgenes was assessed by Western blotting. An overnight collection of embryos was heat shocked at 37°C for 30 min. Following recovery for 2 h at 25°C, embryos were dechorionated, homogenized in loading buffer and subjected to SDS–PAGE. Protein blots were probed with the anti-MYC monoclonal antibody (mAb) 9E10 at a dilution of 1:4.

Fly stocks used for interaction crosses included sev^{S11} (Basler *et al*., 1991), *sev-Ras Val12* (Fortini *et al*., 1992), DCO^{B3} (Lane and Kalderon, 1993), hsp70-PKA* (Jiang and Struhl, 1995), $RhoA^{72O}$ (Strutt *et al*., 1997), $DRhoGEF2^{4.1}$ (Barrett *et al*., 1997), $cdc42^{1}$ (Fehon *et al*., 1997), *Notch*, *Delta* and *daughterless* (da^{1} and da^{IIB3i}) (Brown *et al*., 1996). The *puc–lacZ* and *sim–lacZ* reporter fusions have been described previously (Nambu *et al*., 1991; Ring and Martinez-Arias, 1993).

Generation of somatic clones

Rap1 clones were generated using the null alleles $Rap1^{CD3}$, $Rap1^{rv(R)B1}$ and $Rap1^{rv(R)B3}$ as described previously (Xu and Rubin, 1993). Mosaic patches were generated in the eye imaginal disks of larvae and pupae as well as in follicle cells using the FLP/FRT-mediated mitotic recombination. Males of the genotype *Rap1 P[ry*$^{+}$*; hs-neo FRT]80B /TM6B* were mated to *hsFLP1; P[mini-w*$^{+}$*; hs NM]67B, P[ry*$^{+}$*; hs-neo FRT]80B*. Mitotic recombination was induced by heat shocks in first instar larvae for imaginal disk clones and in 2-day-old adult females for follicle-cell clones.

Generation of germline clones

Germline clones were produced using the FLP/DFS system (Chou and Perrimon, 1996). *Rap1* $FRT^{3L\text{-}2A}$*/TM6B* females were mated with *y w P[ry*$^{+}$*; FLP]*^{22}*; P[ovo*^{D1}*]*^{3L} $FRT^{3L\text{-}2A}$ males. Mitotic recombination was induced in larvae on days 4, 5 and 6 AEL for 1 h at 37°C. Female progeny of this cross were mated either to males which were *Rap1/TM3 ftz-lacZ* or to wild-type males. In some experiments, the wild-type males carried *lacZ* reporter genes (*puc–lacZ* or *sim–lacZ*).

Immunohistochemistry

Ovaries were fixed for 15 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), 0.3% Triton X-100, and stained with DAPI (1 mg/ml) and rhodamine phalloidin (1 mg/ml) overnight at 4°C, and

then washed (3× 5 min) in PBS and mounted in 90% glycerol, 1×PBS, 0.5% *n*-propyl gallate. Antibody staining of embryos was performed using standard techniques (Patel, 1994). Eye imaginal disks were prepared for microscopy as described previously (Xu and Rubin, 1993). Images were collected using a Leica confocal microscope and processed using Adobe Photoshop. Scanning electron micrographs were prepared as described previously (Hariharan *et al*., 1991). The following rabbit polyclonal antibodies were used: anti-β-galactosidase (Capell), anti-Twist (provided by S. Roth), D-mef2 (provided by H.Nguyen). The mAbs BP102 and anti-MYC (9E 10) (Evan *et al*., 1985) were obtained from the Developmental Studies Hybridoma Bank and the anti-Vasa antibody from R.Lehmann.

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