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Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in *Drosophila* imaginal tissues

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SUMMARY

The *Drosophila Ras1* gene is required for proper cell fate specification throughout development, and the loss-of-function phenotype of *Ras1* suggests an additional role in cell proliferation or survival. A direct role for RAS1 in promoting cell proliferation, however, has not been established. We show that expression of an activated form of RAS1 ($RAS1^{V12}$) during *Drosophila* imaginal disc development is sufficient to drive ectopic cell proliferation and hyperplastic tissue growth. In addition, expression of $RAS1^{V12}$ induces widespread cell death in the imaginal discs, including cells not expressing the transgene, which results in ablation of adult structures. Loss-of-function mutations in the genes encoding RAF, MEK, MAPK and KSR dominantly suppress $RAS1^{V12}$-induced cell proliferation. Furthermore, two RAS effector loop mutations (E37G and Y40C) that block the RAS-RAF interaction, also suppress $RAS1^{V12}$-induced proliferation, consistent with a requirement for the MAPK cascade during the RAS1 mitogenic response. These two RAS effector loop mutants, however, retain some activity and can act synergistically with a MAPK gain-of-function mutation, suggesting that RAS1 may also act through signaling pathway(s) distinct from the MAPK cascade.

Key words: Ras1, Cell proliferation, Cell death, Hyperplasia, *Drosophila*, Imaginal disc

INTRODUCTION

Ras has been implicated in controlling both cell proliferation and differentiation, and its mutational activation is associated with a significant fraction of human tumors (reviewed in Lowy and Willumsen, 1993; Moodie and Wolfman, 1994). RAS activity is tightly controlled by a highly conserved signaling pathway, triggered by growth factor activation of Receptor Tyrosine Kinases (RTK; reviewed in Schlessinger, 1993; van der Geer et al., 1994). In turn, RAS activation leads to the sequential activation of RAF, MEK and MAPK, the highly conserved MAP Kinase cascade (reviewed in Daum et al., 1994).

Our understanding of the role Ras plays during cell differentiation has expanded largely through experiments in genetic systems such as the developing eye of *Drosophila* and vulva of *C. elegans* (review in Wassarman and Therrien, 1997; Sundaram and Han, 1996). *Drosophila*, for example, possess a single homologue of mammalian *N-ras*, *H-ras* and *K-ras* genes called *Ras1* (Simon et al., 1991), which is activated in response to a variety of RTKs throughout development. Among other things, Ras1 is required for proper differentiation of the wing veins and the R7 photoreceptor cells (Diaz-Benjumea and Hafen, 1994; also see reviews Lu et al., 1993; Wassarman and Therrien, 1997). Expression of mutationally activated *Ras1* ($RAS1^{V12}$) under control of the *sevenless* enhancer/promoter (*sev-*$Ras1^{V12}$) bypasses the normal requirement for Sevenless RTK activity and triggers R7 differentiation, and transforms the non-neuronal cone cells into ectopic R7 cells (Fortini et al., 1992). The R7 and cone cell precursors presumably are postmitotic since no mitogenic response to Ras1 signaling is observed. The *sev-*$Ras1^{V12}$ transgene produces a rough eye phenotype, which has been used to isolate mutations in genes that act downstream of Ras1 during R7 differentiation (Karim et al., 1996).

We have a much more limited understanding of how Ras controls cell proliferation and how this differs from its role in differentiation. In the case of mammalian PC12 cells, for example, stimulation with NGF causes a sustained activation of RAS and MAPK and triggers differentiation, whereas stimulation with EGF causes a short-lived activation of RAS and MAPK and triggers proliferation (reviewed in Marshall, 1995). It is unclear, however, whether this phenomenon is specific to PC12 cells or reflects a more general mechanism. Interestingly, Serrano et al. (1997) showed that prolonged expression of mutationally activated *H-ras* (*H-*ras^{V12}) induces senescence in human and rodent primary cell lines, whereas other groups have shown that expression of *H-*ras^{V12} can transform some immortalized mammalian cell lines but not others (reviewed in Lowy and Willumsen, 1993; Moodie and Wolfman, 1994). The varied results obtained using different cell lines illustrates the need for in vivo model systems for

studying Ras-dependent cell proliferation, to better understand how Ras triggers different responses in differing cellular and developmental contexts.

The role of Ras during proliferation in *Drosophila* and *C. elegans* has been even less clear. Recently, Yochem et al. (1997) showed that, in *C. elegans*, *let-60*/*Ras* is not required for cell proliferation or cell survival. Further, *let-60*/Ras plays no apparent role in promoting cell proliferation, since an activating mutation in the endogenous gene produces only differentiation defects (reviewed in Sundaram and Han, 1996). By contrast, strong loss-of-function mutations in *Drosophila Ras1*, *Ksr*, *Raf*, *MEK* and *MAPK* are homozygous lethal and die as third instar larvae completely lacking imaginal discs, which constitute the bulk of the proliferating tissues during larval development (reviewed in Wassarman and Therrien, 1997). Similarly, homozygous mutant clones of *Ras1*, *Ksr*, *Raf*, *MEK* or *MAPK* in an otherwise heterozygous animal fail to survive to the adult stage, which suggests that RAS1 and the MAPK cascade are required for cell proliferation and/or cell survival (Diaz-Benjumea and Hafen, 1994; reviewed in Wassarman and Therrien, 1997). As yet a direct role for Ras1 signaling in promoting cell proliferation has not been established.

Drosophila imaginal discs, which are groups of undifferentiated monolayer epithelial cells that give rise to most of the adult structures, are ideal for studying cell proliferation control (reviewed in Edgar and Lehner, 1996; Bryant, 1996). During larval development imaginal discs proliferate exponentially until just prior to metamorphosis when they reach a stereotypical final size and shape (reviewed in Bryant, 1996). As seen during vertebrate development, imaginal disc cells do not have determined lineages but rather display variable proliferation patterns. Early in imaginal disc development each disc is subdivided into lineage compartments (anterior and posterior) that impose some restriction on the pattern of proliferation and act as units of growth control. Within each compartment, however, the proliferation patterns are heterogeneous and show no lineage dependence. Finally, little cell death is observed in the wing disc implying that disc size is achieved primarily by regulating the rate and pattern of proliferation (review in Edgar and Lehner, 1996; Bryant, 1996); although the data presented by Milan et al. (1997), as well as in this paper, suggest that cell death may provide an additional mechanism of size control.

In this paper, we demonstrate that ectopic expression of activated *Ras1* (*Ras1^{V12}*) during imaginal disc development is sufficient to drive cell proliferation and causes hyperplastic growth of imaginal discs. Further, *Ras1^{V12}* induces hyperplasia of both the eye imaginal disc and the adult eye when expressed under the control of an *eyeless* enhancer, which is an early eye-specific enhancer element. In addition, activated Ras1 induces non-autonomous cell death in the imaginal discs and, at high expression levels, can lead to the complete ablation of the adult eye. We also show that the MAPK cascade is required for RAS1 mitogenic response since loss-of-function mutations in genes encoding RAF, MEK, MAPK and KSR dominantly suppress hyperplastic growth, as do mutations in the RAS1 effector loop that disrupt the RAS-RAF interaction. Two of these RAS1 effector loop mutants can, however, act synergistically with a gain-of-function mutation in *MAPK*, which raises the possibility that alternate pathway(s) downstream of RAS1 may also be required.

MATERIALS AND METHODS

Fly stocks

Flies carrying *UAS-Ras1^{wt}*, *UAS-Ras1^{V12}*, *UAS-Ras1$^{V12\ S35}$*, *UAS-Ras1$^{V12\ G37}$*, *UAS-Ras1$^{V12\ C40}$* and *eyEh-Ras1^{V12}* were generated by P-element transformation. Flies carrying the *dpp-GAL4* transgene (lines 40C.6 and 57A) are described in Staehling-Hampton et al. (1994). The *wg-lacZ* and *hh-lacZ* enhancer trap lines are described in Kassis et al. (1992) and Lee et al. (1992), respectively. *Sevenmaker* mutant flies are described in Brunner et al. (1994). The strains *wg-lacZ; dpp-GAL4(40C.6)/Tl(2;3) SM6-TM6B* and *dpp-GAL4(57A); hh-lacZ/Tl(2;3) SM6-TM6B* were kindly provided by Jessica Treisman. The *UAS-P21* lines and the *UAS-P35* lines were generous gifts from Iswar Hariharan and Bruce Hay, respectively.

Construction of the UAS-Ras1 and P[eyEh-Ras1^{V12}]

The Ras1^{wt} and Ras1^{V12} genomic fragments were cut out of Bluescript vector (Fortini et al., 1992) as *Kpn*I (blunt-ended)/*Not*I fragments and ligated into the pUAST vector at *Eco*RI (blunt-ended)/*Not*I. The Ras1 effector loop mutants were generated by PCR-mediated in vitro mutagenesis and confirmed by sequencing. Each effector loop mutant fragment (*Eco*RI/*Bgl*II) was combined in a three-way ligation with a *Bgl*II/*Not*I fragment encoding the remainder of the Ras1 sequences and *Eco*RI/*Not*I digested pUAST vector.

The 3.4 kb *Eco*RI genomic enhancer fragment from the *eyeless* gene (a generous gift from U. Walldorf and W. Gerhing) was blunt-end ligated into the *Xho*I site upstream of the hsp70 promoter of P-CaSpeR-heat shock (Thummel et al., 1988). The Ras1^{V12} genomic fragment was cut out of pBS vector (Fortini et al., 1992) as *Cla*I (blunt-ended)/*Not*I and ligated into the P[eyEh] vector at *Hpa*I/*Not*I.

Histology

UAS-lacZ, *hh-lacZ* and *wg-lacZ* expression patterns in wing imaginal discs were detected by X-gal staining as described by Brand and Perrimon (1993). BrdU labeling was done following a modified protocol described by Truman and Bate (1988). Third instar wing discs were dissected in phosphate buffer and incubated in a 60 μg/ml solution of BrdU in phosphate buffer for 30 minutes at 25°C. Acridine orange staining of third instar larval wing discs was done as described in Spreij (1971). Flies were prepared for scanning electron microscopy as described in Kimmel et al. (1990).

RESULTS

Expression of Ras1^{V12} induces ectopic cell proliferation and hyperplastic growth of imaginal discs

To determine whether Ras1 activation is sufficient to drive cell proliferation, we used the GAL4-UAS system (Brand and Perrimon, 1993) to express in imaginal discs both the wild-type form of Ras1 (*UAS-Ras1^{wt}*) and a mutationally activated form of Ras1, in which valine is substituted for glycine at position 12 (*UAS-Ras1^{V12}*). In these studies, transgenic flies carrying either *UAS-Ras1^{wt}* or *UAS-Ras1^{V12}* (see Materials and Methods) were crossed to a fly strain in which *GAL4* was expressed under the control of the *decapentaplegic* (*dpp*) disc enhancer fragment (*dpp-GAL4*; Staehling-Hampton et al., 1994). *dpp-GAL4* was chosen because it drives expression of *GAL4* in all of the imaginal discs throughout development, and in only a limited portion of each disc resembling the pattern of endogenous *dpp* expression, as shown by *UAS-lacZ* (Fig. 1A).

The *UAS-lacZ*-staining pattern in *UAS-Ras1^{wt}/UAS-lacZ/dpp-GAL4* wing discs (Fig. 1A) is indistinguishable from

discs carrying only *UAS-lacZ* and *dpp-GAL4* (data not shown). In *UAS-Ras1^{V12}/UAS-lacZ/dpp-GAL4* wing discs *lacZ* staining is distorted and more diffuse (Fig. 1B), and the discs are often slightly larger than normal.

Two enhancer trap lines, *hedgehog* (*hh-lacZ*) and *wingless* (*wg-lacZ*), were used to examine the effects that ectopic expression of *Ras1^{wt}* and *Ras1^{V12}* have on wing disc morphology and patterned gene expression. *UAS-Ras1^{wt}/dpp-GAL4* has no effect on either *hh-lacZ* or *wg-lacZ*-staining patterns (Fig. 1C and E). On the contrary, *UAS-Ras1^{V12}/dpp-Gal4* causes severe disruptions in the normal folding pattern on the anterior side of the A/P border, while the folding pattern in the posterior compartment appears to be relatively unaffected (Fig. 1D). Noteworthy are the appearance of new folds not normally present in wild-type discs, particularly along the A/P border. *UAS-Ras1^{V12}* disrupts *wg-lacZ* expression on both the dorsal and ventral edges of the wing pouch at the A/P border where *dpp-GAL4* drives its expression (see arrowheads in Fig. 1F). Along the wing margin, *wg-lacZ* staining is unbroken, although it is somewhat distorted and more diffuse in the region of *UAS-Ras1^{V12}* expression. Disruption of *wg-lacZ* staining does not appear to be due to loss of expression but rather appears to result from displacement of the *wg*-expressing cells, such that they now abut the A/P border (see arrowheads in Fig. 1F). Alterations of both the folding pattern and *wg-lacZ* expression appear to be caused by ectopic tissue growth induced by *Ras1^{V12}*.

The effects of *UAS-Ras1^{V12}* expression are temperature-sensitive and dose-sensitive. For example, *UAS-Ras1^{V12}/dpp-GAL4* animals raised at 18°C rather than 25°C show less severe disruptions of *wg-lacZ* staining (see arrowheads in Fig. 1G) and instead of dying as early pupae die as young adults with severe developmental defects (see Fig. 2B and D). Two copies of *UAS-Ras1^{V12}* at 25°C cause more severe disruption of *wg-lacZ* staining, including the staining along the wing margin (Fig. 1H). Also, the anterior compartment of the wing disc is dramatically overgrown, while the posterior compartment is more compacted (Fig. 1H, see asterisk). Similar Ras1^{V12}-dependent overgrowth was generated in the posterior compartment when an *engrailed-GAL4* line was used (data not shown). Under each of these conditions, the discs appear to remain as monolayer epithelia (unpublished observations).

To determine whether *Ras1^{V12}* induces cell proliferation, third instar wing discs were labeled with BrdU, which is incorporated by cells during S-phase. *UAS-Ras1^{wt}/dpp-GAL4* wing discs have a uniform pattern of cell division, which is indistinguishable from wild-type (Fig. 1I; see also Schubiger and Palka, 1987; Milan et al., 1996). In contrast, the *UAS-Ras1^{V12}/dpp-GAL4* wing discs have a dramatic increase in BrdU incorporation in a broad band along the A/P border, corresponding to the domain of *dpp-GAL4* expression (see brackets in Fig. 1J). No increase in BrdU incorporation is seen in the posterior compartment or the regions just anterior to this band of high BrdU incorporation. Thus, ectopic expression of *Ras1^{V12}* under control of *dpp-*

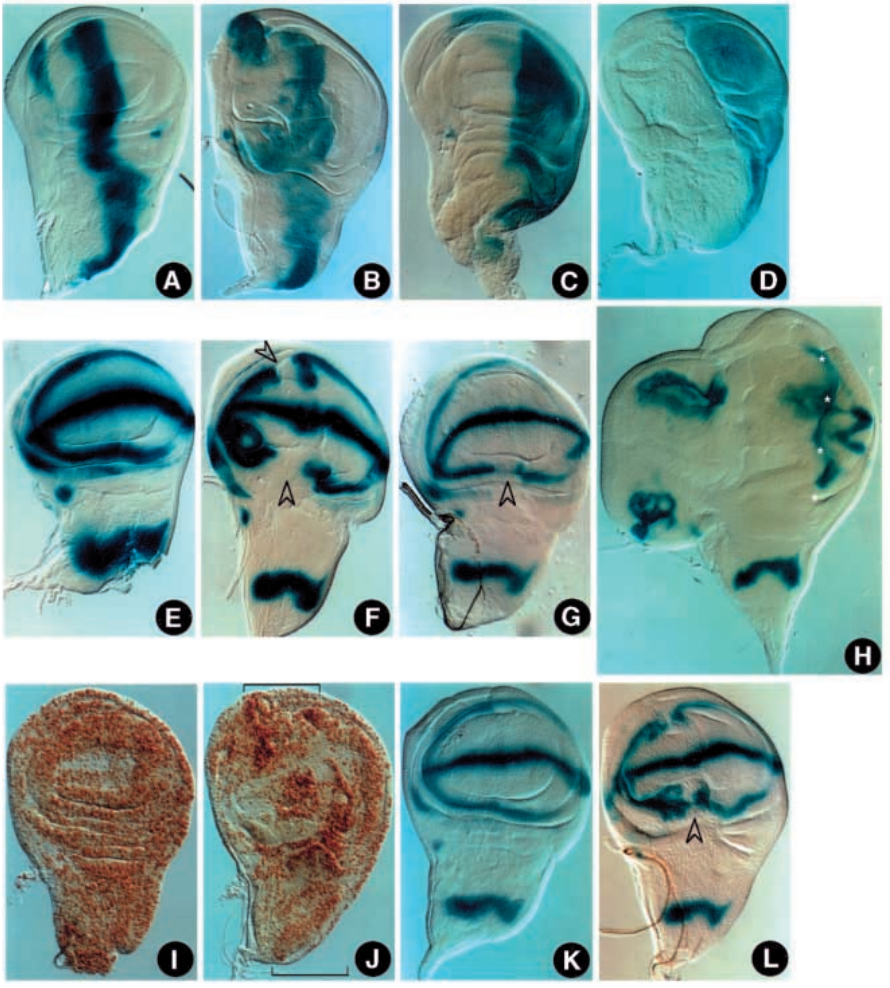

Fig. 1. Expression of activated Ras1 induces ectopic cell proliferation and hyperplastic growth in imaginal discs. Third instar wing discs were stained for β-galactosidase activity (blue; A-H,K,L), and with anti-BrdU antibody (brown; I,J). All discs are shown with anterior to the left. All tissues were isolated from animals raised at 25°C, except for the one shown in G, which was isolated from an animal raised at 18°C. In wing imaginal discs, *dpp-GAL4* expression, and therefore expression of *UAS-Ras1^{wt}* and *UAS-Ras1^{V12}*, occurs in a stripe along the anterior-posterior compartment border (A/P border) just on the anterior side, as visualized by *UAS-lacZ* staining (A,B). *hh-lacZ* is expressed exclusively in the posterior compartment (C,D), whereas *wg-lacZ* is expressed along the wing margin and surrounding the wing pouch (E-H,K,L). The asterisks (H) show the approximate location of the A/P border. The genotypes are as follows: (A) *UAS-Ras1^{wt}/+; dpp-GAL4 /UAS-lacZ*; (B) *UAS-Ras1^{V12}/+; dpp-GAL4 /UAS-lacZ*; (C) *UAS-Ras1^{wt}/dpp-GAL4 /+; hh-lacZ/+*; (D) *UAS-Ras1^{V12}/dpp-GAL4; hh-lacZ/+*; (E,I)*UAS-Ras1^{wt}/wg-lacZ; dpp-GAL4 /+*; (F,G,J)*UAS-Ras1^{V12}/wg-lacZ; dpp-GAL4 /+*; (H) *UAS-Ras1^{V12}/wg-lacZ; dpp-GAL4 /UAS-Ras1^{V12}*; (K) *wg-lacZ/+; dpp-GAL4/UAS-P21*; (L) *UAS-Ras1^{V12}/wg-lacZ; dpp-GAL4 /UAS-P21*.

GAL4 induces cell proliferation, which leads to hyperplastic growth that disrupts both the normal folding and patterned gene expression in the anterior compartment.

Human cyclin-dependent kinase inhibitor, P21$^{CIP1/WAF1}$ suppresses Ras1^{V12}-dependent cell proliferation

We attempted to suppress Ras1^{V12}-induced cell proliferation by co-expressing the human cyclin-dependent kinase inhibitor, P21$^{CIP1/WAF1}$ (*UAS-P21*; Harper et al., 1993; El-Deiry et al., 1993). Ectopic expression of human P21 has been successfully used to block the second mitotic wave in the developing eye imaginal disc (de Nooij and Hariharan, 1995). *UAS-P21/dpp-GAL4* wing discs are morphologically normal in size and shape (Fig. 1K). At 25°C, co-expression of *UAS-P21* appears to suppress most, but not all, of the *UAS-Ras1^{V12}*-induced cell proliferation and hyperplastic growth seen in wing discs (see arrowhead in Fig. 1L). Under these conditions, however, one copy of *UAS-P21* is not sufficient to suppress the lethality but does delay the lethal phase, similar to what is seen in *UAS-Ras1^{V12}/dpp-GAL4* flies raised at 18°C (data not shown).

Flies carrying two copies of *UAS-P21* and *dpp-GAL4* raised at 18°C are morphologically normal, except for loss of the anterior cross vein and part of the L3 wing vein (see arrowhead in Fig. 2E,G). In addition, cells in the L3-L4 intervein region are larger than normal as visualized by the more widely spaced wing hairs (see arrowhead in Fig. 2E,G). At 18°C, co-expression of two copies of *UAS-P21* with *UAS-Ras1^{V12}* results in the complete suppression of lethality associated with activated Ras1 expression. Morphologically, the wing is restored to its normal shape and size, although *UAS-P21* only partially suppresses the extra wing vein material along L3 (see arrowhead in Fig. 2F,H). Interestingly, expression of *UAS-Ras1^{V12}* completely suppresses the larger cell size caused by *UAS-P21* expression (Fig. 2H).

Ras1^{V12} induces non-autonomous cell death

As noted earlier, *UAS-Ras1^{V12}/dpp-GAL4* animals raised at 18°C die as young adults with deletions of certain structures, particularly those arising from cells adjacent to the domain where activated *Ras1* is expressed, e.g., the tarsal segments of the leg (Fig. 2B,D). To address whether these deletions result from *UAS-Ras1^{V12}*-induced cell death, we stained third instar wing imaginal discs with acridine orange. Very little, if any, cell death is observed in either wild-type (data not shown) or *UAS-Ras1^{wt}/dpp-GAL4* wing discs (Fig. 2I). Wing discs isolated from animals carrying one or two copies of *UAS-Ras1^{V12}*, however, have a marked increase in cell death (Fig. 2J,K) that includes but is not limited to the region where *Ras1^{V12}* is expressed. This suggests that ectopic expression of *Ras1^{V12}* induces cell death non-autonomously. To confirm this non-autonomy, the baculovirus cell death inhibitor *P35* and *Ras1^{V12}* were co-expressed (*UAS-P35*; B. Hay, personal communication; also see Hay et al., 1994). Cell death is greatly reduced in the region where *dpp-GAL4* drives co-expression of *UAS-Ras1^{V12}* and *UAS-P35* but is still considerable in other regions of the disc, especially in its anterior portion (see brackets in Fig. 2L). When raised at 18°C, *UAS-Ras1^{V12}/UAS-P35/dpp-GAL4* animals still die as young adults with deletions of structures (data not shown),

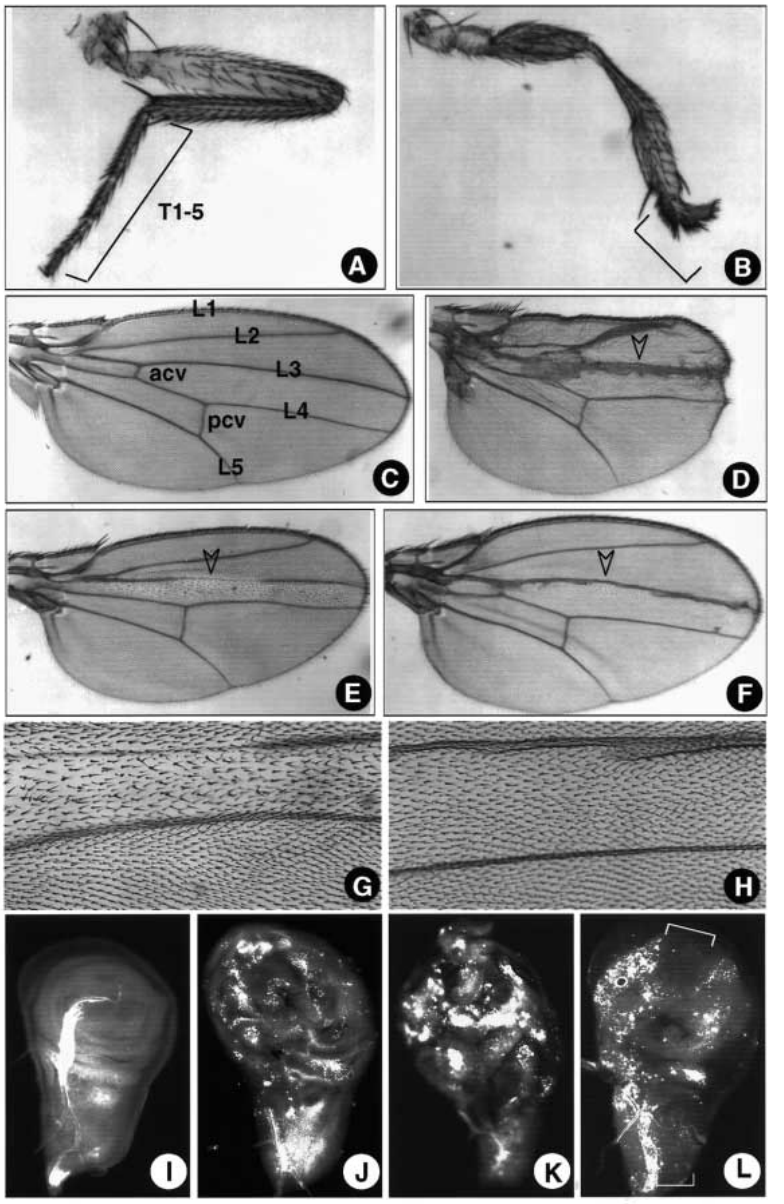

Fig. 2. Activated Ras1 induces non-autonomous cell death in imaginal discs. Adult legs (A,B) and wings (C-H) are shown with anterior up. The longitudinal wing veins (L1-L5), as well as the anterior (acv) and posterior cross veins (pcv) are labeled (C). Acridine orange staining of third instar wing discs reveals dying cells (I-L). Note, the brightly stained region in I is a piece of trachea. *UAS-Ras1^{V12}/dpp-GAL4* animals raised at 18°C die as young adults with mild rough eyes (data not shown), severely malformed legs with missing or fused tarsal segments (see brackets in B) and smaller wings with broad L3 wing vein (see arrowhead in D). The genotypes are as follows: (A,C,I) *UAS-Ras1^{wt}/+; dpp-GAL4 /+*; (B,D,J) *UAS-Ras1^{V12}/+; dpp-GAL4/+*; (E,G) *UAS-P21/+; +/+; dpp-GAL4/UAS-P21*; (F,H) *UAS-P21/+; UAS-Ras1^{V12}/+; dpp-GAL4/UAS-P21*; (K) *UAS-Ras1^{V12}/+; dpp-GAL4/UAS-Ras1^{V12}*; (L) *UAS-Ras1^{V12}/+; dpp-GAL4/UAS-P35*.

presumably as a consequence of this non-autonomous cell death.

The MAP kinase cascade is required for Ras1^{V12}-dependent cell proliferation

The RAF serine/threonine kinase, which is the best characterized Ras effector, is recruited to the plasma membrane upon Ras activation via a direct protein-protein interaction between the effector loop of RAS and the Ras-binding domain within the N-terminal domain of RAF (reviewed in Morrison and Cutler, 1997; Katz and McCormick, 1997). A dominant-negative *Raf* allele, *Raf$^{XS\text{-}2541}$*, was tested for its ability to suppress as a heterozygote (i.e., dominantly) the Ras1^{V12}-induced proliferation. At 25°C, *Raf$^{XS\text{-}2541}$/+* dominantly suppresses most of the hyperplastic growth seen in *UAS-Ras1^{V12}/dpp-GAL4* wing discs (see arrowheads in Fig. 3B). Similarly, loss-of-function mutations in the genes encoding the *Drosophila* homologues of MEK, MAPK and KSR dominantly suppress most of the Ras1^{V12}-induced proliferation (data not shown), consistent with the MAPK cascade playing a critical downstream role during cell proliferation. Conversely, a dominant gain-of-function mutation in *Drosophila* MAPK, *Sevenmaker* (*Sem*, *rolled^{Sem}*, *MAPK^{Sem}*; Brunner et al., 1994), dominantly enhances activated Ras1 overproliferation, resulting in a much larger disc with severe disruptions of morphology (Fig. 3C).

Although *Raf$^{XS\text{-}2541}$/+* suppresses most of the overproliferation seen at 25°C, it only partly suppresses the cell death and lethality associated with *UAS-Ras1^{V12}/dpp-GAL4*. The surviving adults have severe developmental defects, similar to *UAS-Ras1^{V12}/dpp-GAL4* animals raised at 18°C (data not shown). The residual hyperplastic growth seen in *Raf$^{XS\text{-}2541}$/UAS-Ras1^{V12}/dpp-GAL4* animals may reflect residual RAF activity present in *Raf$^{XS\text{-}2541}$/+* heterozygotes. Since animals that are hemizygous or homozygous mutant for a null allele of *Raf* (*Raf$^{l1\text{-}29}$*) die as third instar larvae lacking imaginal discs (Nishida et al., 1988), we tested to see whether expression of activated *Ras1* can bypass a requirement for *Raf*. Indeed, no imaginal disc proliferation is seen in hemizygous *Raf$^{l1\text{-}29}$* males in which *UAS-Ras1^{V12}* is expressed under control of *dpp-GAL4* (data not shown), consistent with a requirement for *Raf* and the MAPK cascade.

RAS1 may also act through signaling pathway(s) distinct from the MAPK cascade

Although *Raf* is required for Ras1-dependent proliferation, it may not be sufficient for mediating all Ras1 signaling. In mammalian cells, RAS not only interacts with RAF but can also interact with effectors distinct from RAF, some of which act independently or in parallel to RAF (reviewed in Katz and McCormick, 1997). Three different Ras effector loop mutants (T35S, E37G, Y40C) that block or impair the ability of RAS to bind RAF have been used to distinguish between RAF and alternate effector pathways downstream of mammalian RAS (White et al., 1995; Khosravai-Far et al., 1996; Joneson et al., 1996). These effector loop mutants can act synergistically with each other to transform mammalian cells, which suggests that they differentially affect parallel downstream pathways (White et al., 1995; Joneson et al., 1996; Khosravai-Far et al., 1996).

Based on these observations and the conservation of RAS1 effector loop sequence, we generated three different effector loop mutants (T35S, E37G and Y40C) in the activated form of *Drosophila Ras1* (*Ras1$^{V12\ S35}$*, *Ras1$^{V12\ G37}$* and *Ras1$^{V12\ C40}$*). Unlike mammalian cells, in which H-RAS$^{V12\ S35}$ is attenuated in its ability to transform cells, *UAS-Ras1$^{V12\ S35}$/dppGAL4* produces approximately the same amount of hyperplastic growth in third instar wing disc as *UAS-Ras1^{V12}* (see arrowhead in Fig. 3D). In contrast, both *UAS-Ras1$^{V12\ G37}$* and *UAS-Ras1$^{V12\ C40}$* mutants display little, if any, hyperplastic growth in wing discs (Fig. 3E,F, respectively) In both cases, animals survive to adulthood but have minor differentiation defects, such as mild rough eyes (data not shown) and extra wing vein material in the case of *UAS-Ras1$^{V12\ G37}$/dpp-GAL4* but not in *UAS-Ras1$^{V12\ C40}$/dpp-GAL4* (see arrowheads in Fig. 4B,C). *UAS-Ras1$^{V12\ G37}$* and *UAS-Ras1$^{V12\ C40}$* do not act synergistically and produce no ectopic proliferation in the imaginal discs and the differentiation defects seen in the adults appear to be additive rather than synergistic (data not shown). These results confirm that activation of the MAPK cascade is an essential downstream event during RAS1-induced proliferation.

The mild differentiation defects associated with both RAS1$^{V12\ G37}$ and RAS1$^{V12\ C40}$ indicate that these mutant proteins retain some signaling activity, which might result from either a residual capacity to bind and activate RAF, or from signal transmission via a Raf-independent pathway. Under either scenario, one might expect these effector loop mutants to act synergistically with *MAPK^{Sem}*, the MAPK gain-of-function mutation. *MAPK^{Sem}* flies display no apparent proliferation defect on their own. The adults have moderately rough eyes and extra wing vein material (Brunner et al., 1994), although extra vein material is only occasionally seen along the L3 wing vein (see arrowhead in Fig. 4D). In fact, *UAS-Ras1$^{V12\ G37}$/dpp-GAL4* and *MAPK^{Sem}* do act synergistically to produce a small amount of hyperplastic growth in third instar wing discs (see arrowhead in Fig. 3G), but only a fraction of that seen with *UAS-Ras1^{V12}/dpp-GAL4* at 25°C. These animals die as pharate adults at 25°C and have deletions of tarsal segments and extra wing vein material along L3, similar to *UAS-Ras1^{V12}/dpp-GAL4* animals raised at 18°C (data not shown). No apparent ectopic proliferation is seen in imaginal discs of animals carrying *MAPK^{Sem}* and *UAS-Ras1$^{V12\ C40}$/dpp-GAL4* (data not shown). The adults, however, often have wing blisters and extra vein material along the L3 vein not normally seen in either *MAPK^{Sem}* or *UAS-Ras1$^{V12\ C40}$/dpp-GAL4* flies alone (see arrowhead in Fig. 4E). If the synergistic activity between these effector loop mutants and *MAPK^{Sem}* is a consequence of residual RAS-RAF interaction, then it should be dramatically suppressed by the dominant-negative *Raf* allele. In fact, the extra wing vein material and blisters seen in *MAPK^{Sem}/UAS-Ras1$^{V12\ C40}$/dpp-GAL4* flies are not dramatically suppressed by *Raf$^{XS\text{-}2541}$/+* (see arrowhead in Fig. 4F), consistent with RAS1 acting via a pathway distinct from the MAPK cascade.

Expression of Ras1^{V12} under control of an *eyeless*-enhancer construct causes hyperplastic growth of both the eye imaginal disc and the adult eye

To facilitate future genetic screens, we wished to generate a stable transgenic fly strain in which Ras1^{V12}-induced hyperproliferation does not lead to lethality. Expression of activated Ras1 exclusively in the eye imaginal disc throughout development would be ideal, since the eye is dispensable for

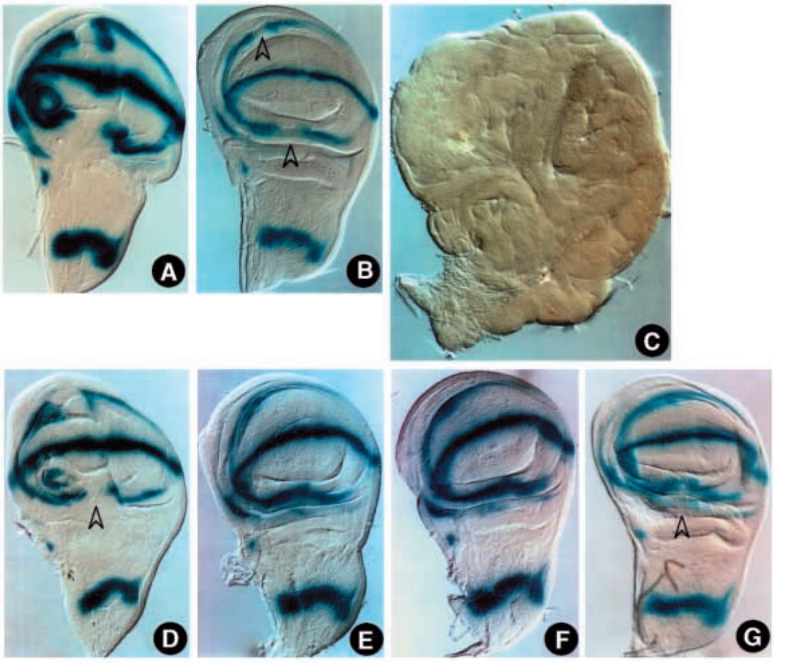

Fig. 3. The Raf/MAP kinase cascade is necessary but may not be sufficient for RAS^{V12}-induced proliferation. Third instar wing discs (anterior is to the left) were stained for β-galactosidase activity to reveal the *wg-lacZ* expression pattern (blue; A,B,D-G; the wing disc shown in C does not carry the *wg-lacZ* enhancer trap line). All wing discs are displayed at the same magnification. The genotypes are as follows: (A) *UAS-Ras1^{V12}/wg-lacZ; dpp-GAL4 /+*; (B) *Raf$^{XS-2541}$/+; UAS-Ras1^{V12}/wg-lacZ; dpp-GAL4 /+*; (C) *UAS-Ras1^{V12}/MAPK^{Sem}; dpp-GAL4 /+*; (D) *UAS-Ras1$^{V12\ S35}$/wg-lacZ; dpp-GAL4 /+*; (E) *UAS-Ras1$^{V12\ G37}$/+; wg-lacZ/+; dpp-GAL4/+*; (F) *UAS-Ras1$^{V12\ C40}$/+; wg-lacZ/+; dpp-GAL4 /+*; (G) *UAS-Ras1$^{V12\ G37}$/+; MAPK^{Sem}/wg-lacZ; dpp-GAL4/+*.

viability and fertility and, unlike the wing, is easily scored in adults that fail to eclose. To achieve this, we used a 3.4 kb genomic enhancer fragment from the *eyeless* (*ey*) gene that has been shown to confer expression of a lacZ reporter throughout eye development (Walsdorf and Gehring, personal communications). Eye imaginal discs from *eyEh-Ras1^{V12}* third instar larvae raised at 18°C are larger than normal (Fig. 5G), consistent with ectopic cell proliferation and hyperplastic growth, but the adult eyes are rough and slightly smaller than normal (Fig. 5B). When raised at 25°C, the eye imaginal discs from *eyEh-Ras1^{V12}* animals are dramatically larger than wild-type (Fig. 5H). These animals survive as adults and often have larger-than-normal eyes, with protruding outgrowths of eye tissue and cuticle emanating from the posterior margin of the eye (Fig. 5C). Occasionally, rather than having larger eyes, ventral portions or all of one eye may be missing (data not shown). When raised at 29°C, *eyEh-Ras1^{V12}* larvae have no eye-antennal discs (data not shown). These animals die as pharate adults and completely lack all head structures derived from the eye-antennal disc. Only the proboscis, which develops from the labial disc, is seen protruding from the thorax where the head is normally located (see arrowhead in Fig. 5D).

We crossed mutants that suppressed or enhanced $Ras1^{V12}$-induced differentiation effects (Karim et al., 1996) to *eyEh-Ras1^{V12}* at 18°C, 25°C and 29°C. Loss-of-function mutations in *Raf*, *MEK*, *MAPK* and *Ksr* dominantly suppress both the lethality and disc overgrowth seen at all three temperatures, although the mutations suppress these phenotypes to varying degrees and none completely at 29°C. For example, *Raf$^{XS-2541}$* dominantly suppresses *eyEh-Ras1^{V12}* at 29°C such that most animals have head structures and some survive to adulthood with a limited amount of hyperplasia (Fig. 5E). In addition, several enhancers isolated in the *sev-Ras1^{V12}* screen also enhance *eyEh-Ras1^{V12}* such that the animals die at 18°C. These are usually missing most or all head structures, similar to *eyEh-Ras1^{V12}* at 29°C (data not shown).

DISCUSSION

In this paper, we demonstrate that expression of mutationally activated Ras1 ($Ras1^{V12}$) induces ectopic cell proliferation, hyperplastic growth and non-autonomous cell death during imaginal disc development.

When *dpp-GAL4* is used to drive *UAS-Ras1^{V12}* expression in the wing disc, ectopic cell proliferation occurs in a stripe

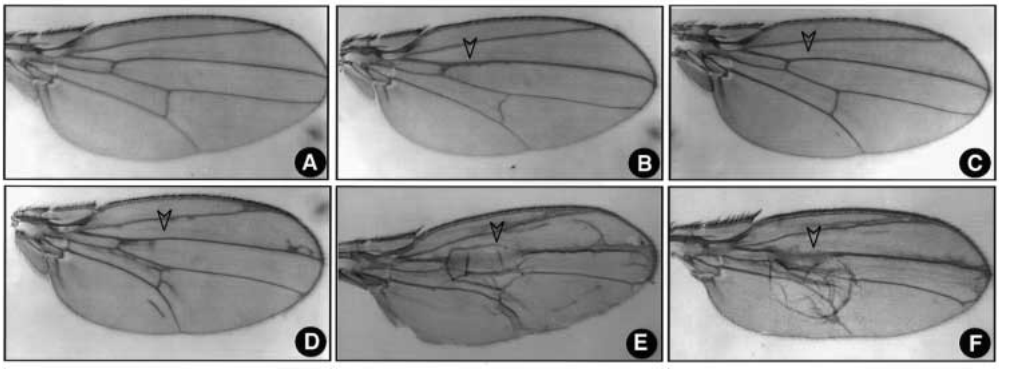

Fig. 4. The E37G and Y40C Ras effector loop mutants block $RAS1^{V12}$-induced proliferation but retain some residual activity indicative of an additional downstream pathway parallel to RAF. Adult wings (A-F) are shown with anterior up. The genotypes are as follows: (A) wild type; (B) *UAS-Ras1$^{V12\ G37}$/+; dpp-GAL4/+;* (C) *UAS-Ras1$^{V12\ C40}$/+; dpp-GAL4 /+*; (D) *MAPK^{Sem}/+*; (E) *UAS-Ras1$^{V12\ C40}$/+; MAPK^{Sem}/+; dpp-GAL4/+*; (F) *UAS-Ras1$^{V12\ C40}$/Raf$^{XS-2541}$; MAPK^{Sem}/+; dpp-GAL4/+*.

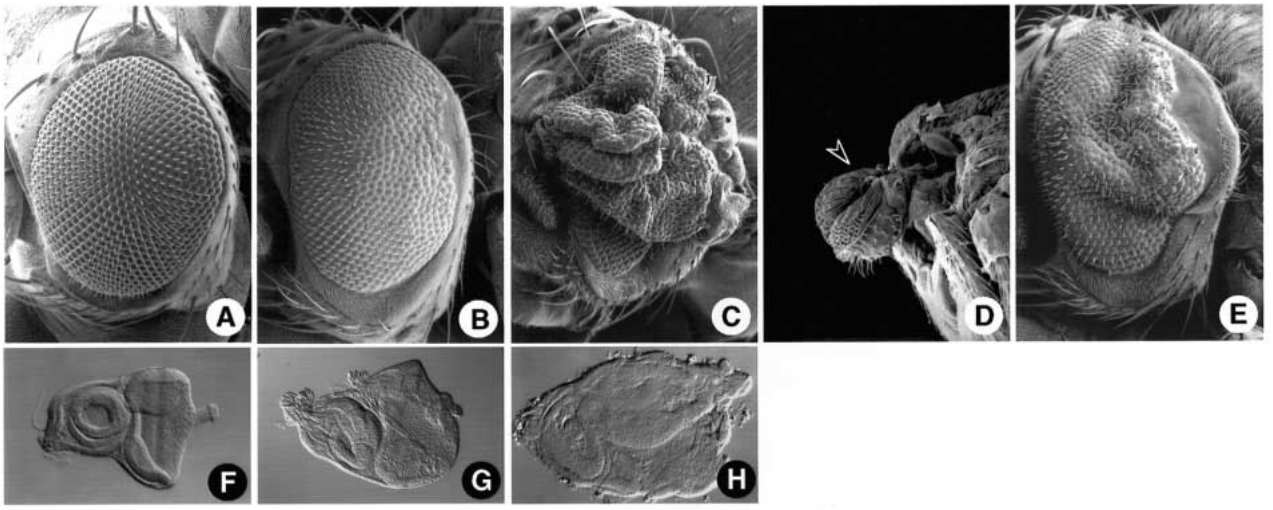

Fig. 5. Expression of *Ras^{V12}* under the control of an eyeless enhancer element (*eyEh-Ras1^{V12}*) triggers hyperplastic growth in the eye imaginal disc and the adult eye. Scanning electron micrographs of adult eyes (A-E) and eye-antennal imaginal discs (F-H) are shown with anterior to the left. The animal in panel D has no head, only the proboscisis present (arrowhead). The genotypes are as follows: (A,F) wild type; (B,G) *eyEh-Ras1^{V12}* raised at 18°C; (C,H) *eyEh-Ras1^{V12}* raised at 25°C; (D) *eyEh-Ras1^{V12}* raised at 29°C; (E) *Raf$^{XS\text{-}2541}$*/+; *eyEh-Ras1^{V12}* raised at 29°C.

along the anterior side of the A/P border. Dramatic expansion of the disc profile occurs with high levels of activated Ras1 activity (e.g., with two copies of *UAS-Ras1^{V12}*, or in combination with the *MAPK^{Sem}*), yet the growth appears to be hyperplastic rather than neoplastic since the disc remains a monolayer epithelium and is not invasive (data not shown).

Two types of overgrowth phenotypes are observed in *Drosophila* imaginal discs, hyperplastic and neoplastic. Neoplastic growth of imaginal discs can be characterized by the loss of its monolayer epithelial character and its inability to differentiate. In addition, neoplastic growths are invasive when transplanted into normal hosts. Hyperplastic growths retain their monolayer epithelial structure and their ability to differentiate, but are not invasive (reviewed in Gateff, 1994; Bryant et al., 1993). Hyperplastic growths can be further subdivided into two classes; those that result as a consequence of a pattern duplication (e.g., the overgrowth that results from ectopic *dpp* expression; reviewed in Edgar and Lehner, 1996), and those that display no pattern duplication but instead result from a general loss of growth control (e.g., as seen in *warts* or *fat* loss-of-function mutants; reviewed in Bryant et al., 1993). RAS1^{V12}-induced hyperplastic growth displays no obvious pattern duplication in the discs or adult structures, but instead displays larger than normal tissues (Fig. 5C,H).

These results demonstrate that Ras1 plays an important role in the control of imaginal disc cell proliferation; however, it is unclear how Ras1 signaling is integrated with signaling pathways that control pattern formation.

Is non-autonomous cell death a manifestation of cell competition?

Ras1^{V12} animals that survive to adulthood often have deletions of adult structures (e.g., deletions of tarsal segments in *UAS-Ras1^{V12}/dpp-GAL4* animals and head structures in *eyEh-Ras1^{V12}* animals). In each case, the structures most commonly deleted include those that arise from cells that lie adjacent to the Ras1^{V12}-expressing cells within the imaginal disc. Presumably the loss of wild-type cells results from induction of non-autonomous cell death. It is, however, unclear whether *UAS-Ras1^{V12}*-expressing cells trigger the non-autonomous cell death by sending a lateral signal, or whether this occurs indirectly as a consequence of excess proliferation in the disc. Consistent with this latter possibility, co-expression of two copies of P21, which should block cell proliferation but not affect a lateral signal, suppresses the deletion of structures in the adult (Fig. 2F, data not shown). Complementary to our results, which indicate that ectopic proliferation leads to the induction of non-autonomous cell death, Milan and Garcia-Bellido (1997) have shown that experimentally induced cell death in one region of the disc triggers non-autonomous cell death, as well as cell proliferation.

One interpretation is that non-autonomous cell death is the manifestation of cell competition. The notion of cell competition was first proposed when it was observed that clones of *Minute*/+ mutant cells, which have a cell-autonomous slower rate of division, could not be recovered when generated in non-*Minute* (+/+) animals, even though heterozygous mutants are not otherwise cell lethal (Morata and Ripoll, 1977). Additionally, when homozygous wild-type clonal tissue was generated in *Minute*/+ mutant animals, they grew to be very large at the expense of *Minute* mutant cells, hence, the term cell competition. In the *Minute* experiments, cell competition is restricted by compartment boundaries, and mosaic compartments are always of normal size and shape. This is not true in the Ras1^{V12} experiments since the anterior compartment of the wing disc is usually bigger in *UAS-Ras1^{V12}/dpp-GAL4* animals. In this case, *Ras1^{V12}*-expressing cells might ‘compete’ with neighboring non-expressing cells and induce non-autonomous cell death. This cell death, however, is insufficient to completely compensate for the increased proliferation, so the discs are not normal size or shape. Increased cell death may represent a mechanism to compensate for excessive proliferation and regulate the overall disc size. Consequently, the final outcome of *Ras1^{V12}* expression on adult structures

appears to be a balance between the amount of proliferation versus cell death.

The MAPK cascade is necessary for Ras-induced proliferation

Our results raise important questions about the nature of Ras1 signaling during cell proliferation and how it differs during cell differentiation. Understanding Ras signaling during oncogenesis requires a better understanding of its downstream effector(s). Two lines of evidence demonstrate that the MAPK cascade is necessary for Ras1-dependent proliferation: First, loss-of-function mutations in *Raf*, *MEK*, *MAPK* and *Ksr* genes strongly suppress of $RAS1^{V12}$-induced hyperplasia. Second, Ras effector loop mutants (E37G and Y40C) that disrupt the RAS-RAF interaction completely suppress $RAS1^{V12}$-induced proliferation.

Even though the RAS1 effector loop and the Ras-binding domain of *Drosophila* RAF are conserved between flies and mammals, the RAS1 effector loop mutants reveal some differences between RAS1 and mammalian RAS. For example, the T35S effector loop mutant, which impairs but does not block H-RAS-RAF interaction in the yeast two-hybrid system and blocks mammalian cell transformation, does not significantly alter the ability of activated RAS1 to drive cell proliferation. H-$RAS^{V12\ G37}$ and H-$RAS^{V12\ C40}$ are impaired in their abilities to stimulate the MAPK cascade (White et al., 1995; Khosravai-Far et al., 1996), but both retain a limited ability to transform certain mammalian cell lines, suggestive of Raf-independent pathways, and together they can act synergistically to more effectively cause transformation. *UAS-Ras1$^{V12\ G37}$* and *UAS-Ras1$^{V12\ C40}$* do not produce ectopic cell proliferation on their own, but do produce mild differentiation defects indicative of some residual activity. Furthermore, $RAS1^{V12\ G37}$ and $RAS1^{V12\ C40}$ do not have complementary activities, since co-expression produces additive rather than synergistic phenotypes, although both can act synergistically with *$MAPK^{Sem}$*, a gain-of-function allele. This synergistic activity might result from $MAPK^{Sem}$ augmenting a low level activity generated by a residual capacity of $RAS1^{V12\ G37}$ or $RAS1^{V12\ C40}$ to bind and activate RAF. Alternatively, $RAS1^{V12\ G37}$ and $RAS1^{V12\ C40}$ might retain signaling activity via a pathway parallel to RAF, and this is augmented by $MAPK^{Sem}$ gain-of-function activity. The former does not seem likely since *$Raf^{XS-2541}$/+* does not dominantly suppress the extra wing vein material in either *UAS-Ras1$^{V12\ G37}$/dpp-GAL4* (data not shown) or *Sem/UAS-Ras1$^{V12\ C40}$ /dppGAL4* flies (Fig. 4F). These results are instead consistent with an alternate signaling pathway(s) functioning downstream of RAS1 but parallel to the MAPK cascade.

Ras signal transduction during cell proliferation

Mutationally activated H-RAS is implicated in the development of some human cancers, which is born out by its ability to transform certain mammalian cell lines. Paradoxically, H-RAS^{V12} triggers senescence in human and rodent primary cell lines (Serrano et al., 1997). These different responses presumably reflect the differing mutational histories of these cell lines, which can lead to confusing or contradictory conclusions regarding Ras function. The high degree of conservation of Ras signaling pathways between *Drosophila* and humans demonstrates that flies provide a good in vivo system for understanding Ras-dependent proliferation. Furthermore, both fly and mammalian cells require Ras for proliferation, and activated Ras is sufficient to induce proliferation. This does not appear to be true of all metazoan organisms since, in *C. elegans*, Ras does not appear to play a role in proliferation (Yochem et al., 1997). $RAS1^{V12}$-induced proliferation generated with *UAS-Ras1^{V12}* and *eyEh-Ras1^{V12}* combined with the genetic and molecular tools available in flies provide a powerful in vivo model system for addressing the role of Ras during cell proliferation, both in general and in a developmental context. Key points that this system can be used to address include the following. How does Ras activation lead to differentiation versus a mitogenic response? What signaling pathways in addition to the MAPK cascade contribute to transmission of the proliferation signal? What are the critical links between Ras signaling and the cell cycle machinery? What constrains the $Ras1^{V12}$-dependent proliferation so that these cells maintain the monolayer?

The ability to address these questions within a developmental context should reveal how Ras signaling is integrated with the cell cycle machinery and with other signaling pathways during development and oncogenesis.

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