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scribble mutants cooperate with oncogenic Ras or Notch to cause neoplastic overgrowth in *Drosophila*

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Cancer is a multistep process involving cooperation between oncogenic or tumor suppressor mutations and interactions between the tumor and surrounding normal tissue. Here we present the first description of cooperative tumorigenesis in *Drosophila*, by using a system that mimics the development of tumors in mammals. We have used the MARCM system to generate mutant clones of the apical–basal cell polarity tumor suppressor gene, *scribble*, in the context of normal tissue. We show that *scribble* mutant clones in the eye disc exhibit ectopic expression of cyclin E and ectopic cell cycles, but do not overgrow due to increased cell death mediated by the JNK pathway and the surrounding wild-type tissue. In contrast, when oncogenic Ras or Notch is expressed within the *scribble* mutant clones, cell death is prevented and neoplastic tumors develop. This demonstrates, for the first time in *Drosophila*, that activated alleles of Ras and Notch can act as cooperating oncogenes in the development of epithelial tumors, and highlights the importance of epithelial polarity regulators in restraining oncogenes and preventing tumor formation.

Keywords: cell cycle/cell death/cell polarity/cooperative tumorigenesis/*Drosophila*

Introduction

Drosophila has long been recognized as a valuable tool for understanding many aspects of cancer biology, mainly because of the high degree of conservation in signaling pathways between *Drosophila* and mammals, and the ease with which genetic analyses can be carried out in the fly. Studies on a group of three *Drosophila* genes, *discs large* (*dlg*), *lethal giant larvae* (*lgl*) and *scribble* (*scrib*), are now beginning to highlight the critical relationship that exists between loss of epithelial cell polarity and tumor development. Homozygous mutants of *dlg*, *lgl* or *scrib* all exhibit very similar phenotypes. Larvae develop normally; however, as maternal supplies of these proteins are exhausted, cells within the normally monolayered epithelial imaginal discs lose structure and polarity, fail to differentiate and overproliferate to become multilayered amorphous masses of cells that fuse with adjacent tissues (reviewed by Wodarz, 2000). The larvae are unable to initiate pupal development and eventually die as giant overgrown larvae. The three-dimensional and invasive overgrowth exhibited by the mutant tissue, which fails to differentiate properly and lacks morphology, has led to the classification of these three genes as *Drosophila* neoplastic tumor suppressors (Gateff, 1994). Detailed analysis has revealed that *dlg*, *lgl* and *scrib* participate in a common genetic pathway involved in the regulation of both cell polarity and proliferation (Bilder *et al*., 2000). Scrib is a LAP4 protein (16 leucine-rich repeats with four PDZ domains; Bilder and Perrimon, 2000) and Dlg a MAGUK (membrane-associated guanylate kinase) containing three PDZ domains (Woods and Bryant, 1991). In epithelia, both proteins normally localize to a lateral domain of the cell membrane, just basal to the adherens junction, known as the septate junction, a solute barrier thought to be analogous to the tight junction of mammalian cells. Lgl is a cytoplasmic and cortically localized protein with WD40 repeats. In epithelia lacking Scrib, apically localized proteins become redistributed over the baso-lateral surface, and spot adherens junctions remain scattered rather than coalescing into the defined lateral band of the zonula adherens (Bilder and Perrimon, 2000). It is thought that this disruption to cellular architecture perturbs signaling pathways, resulting in a loss of proliferation control.

At present, it is not known if human homologs of Scrib (hScrib or Vartul), Dlg (a family of four proteins; hDlg/SAP97, PSD-93, NE-Dlg/SAP102 and PSD-95/SAP90) and Lgl (Hugl) are tumor suppressors in mammalian cells; however, both hScrib and hDlg are targeted for degradation by the E6 oncoprotein of high-risk human papillomaviruses (Gardiol *et al*., 1999; Nakagawa and Huibregtse, 2000) and, at least in MDCK cells, E6 expression is accompanied by a loss of tight junction integrity (Nakagawa and Huibregtse, 2000). This suggests that the loss of these proteins may have important functional consequences for cell polarity, and hence may contribute to neoplasia progression by the E6 oncoprotein.

In this study, we use an *in vivo Drosophila* model that more closely resembles the clonal nature of mammalian cancer to investigate the role of cell polarity, and Scrib in particular, in tumor development. In mammals, tumorigenesis involves cooperative interactions between tumor suppressor genes and oncogenes (reviewed by Hanahan and Weinberg, 2000), as well as complex interactions between the overproliferating tumor itself and the surrounding stroma (Bissell and Radisky, 2001; Liotta and Kohn, 2001). Models of tumor development in flies must therefore attempt to mimic these added levels of complexity. Using an FLP/*FRT*-mediated clonal analysis, we show that clones of *scrib*$^{-}$ tissue within an otherwise wild-type animal lose polarity and overproliferate; however, the surrounding wild-type tissue ensures that this proliferative advantage is compensated for by Jun N-terminal kinase (JNK) pathway-mediated apoptosis of the mutant tissue. If secondary mutations of activated oncogenes, in particular

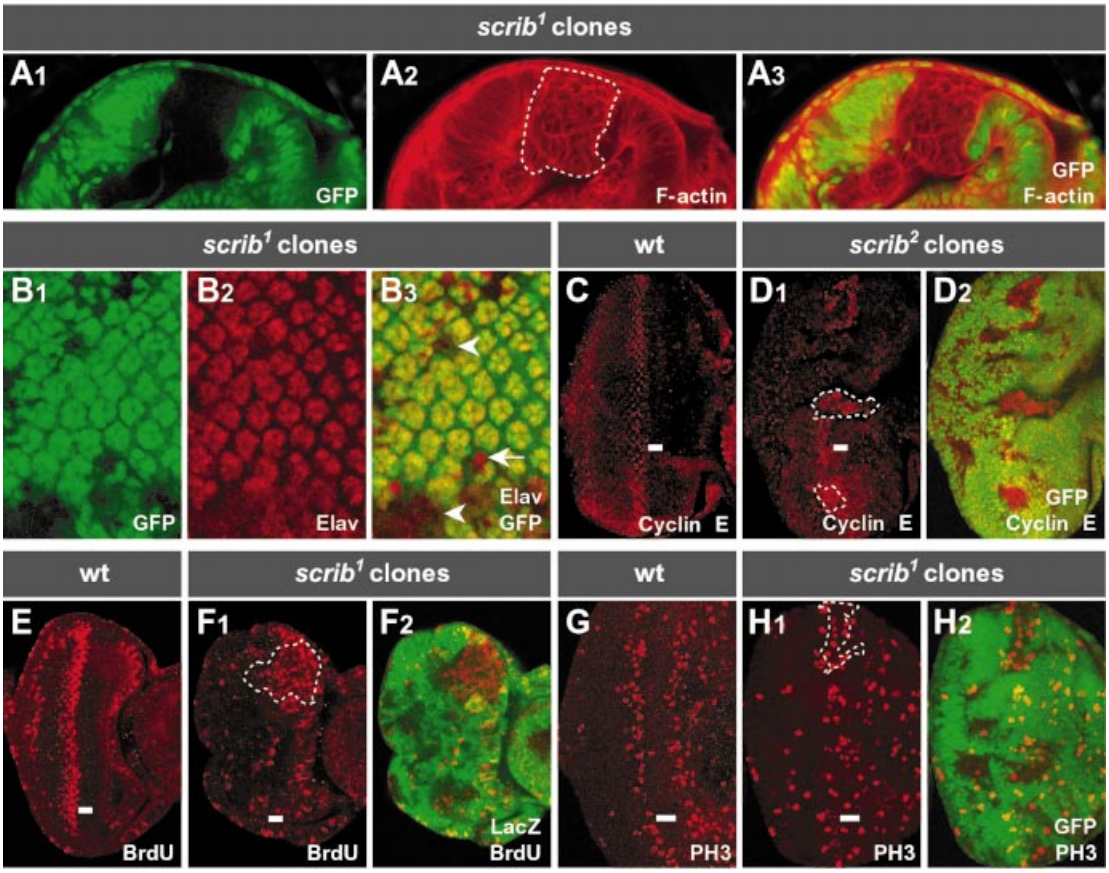


Fig. 1. Somatic eye clones of *scrib* mutant tissue become multilayered and overproliferate. (A) Cross-section and (B–H) planar-sections, anterior to the right, through the monolayered epithelium of third instar larval eye discs. *ey-FLP scrib*^{1} or *scrib*^{2} mutant clones (sometimes outlined by dashed lines) are marked by the absence of GFP, except for BrdU detection (E and F) in which mutant clones are marked by the absence of LacZ staining. Similar results were obtained with both *scrib*^{1} and *scrib*^{2} alleles. In the third instar larval eye disc, the morphogenetic furrow (MF; indicated by a bar), having initiated from the posterior edge of the disc, has progressed half way across, inducing cells to differentiate behind it (posterior). (**A**) Phalloidin staining for F-actin (A2 and 3) shows the columnar epithelium of the wild-type eye disc tissue (GFP-positive), and the multilayered, rounded cells of *scrib*$^{-}$ tissue (GFP-negative). (**B**) Developing photoreceptor cells, marked by Elav staining (B2 and 3), are still able to differentiate in *scrib*$^{-}$ clones (arrow); however, some *scrib*$^{-}$ cells remain undifferentiated (arrowhead). There is also disruption to the regular spacing of ommatidial clusters, which extends into the wild-type tissue. (C–H) In a wild-type eye disc, cyclin E is expressed in a band of cells just posterior to the MF (**C**), which undergo a synchronous S phase (**E**; BrdU incorporation), followed by mitosis (**G**; phospho-histone H3 staining). In *scrib*$^{-}$ clones, and in immediately adjacent wild-type cells, cyclin E levels are elevated, particularly within and anterior to the MF (**D**), resulting in ectopic DNA replication (**F**) and mitoses (**H**).

Ras and Notch, are introduced into the *scrib*$^{-}$ clones, this fate is avoided, resulting in unrestrained tissue overgrowth. The cooperative effect of activated Ras or Notch on *scrib*$^{-}$ tissue cannot be explained solely by the ability of these oncogenes to promote cell survival or cell cycle progression, suggesting that other downstream targets are required for their tumorigenic effects. This study therefore provides the first demonstration of cooperative tumorigenesis in *Drosophila*, and shows how defects in cell polarity could cooperate with oncogenes in the development of cancer.

Results

Somatic clones of scrib mutant tissue in the eye disc lose polarity and overproliferate

The absence of Scrib in homozygous *scrib*$^{-}$ larvae causes a loss of cell polarity and excessive cell proliferation of larval wing imaginal discs and brain lobes, resulting in the formation of neoplastic tumors (Bilder *et al*., 2000). We wished to examine the effects of removing Scrib in somatic clones of tissue within a wild-type tissue context, a situation more akin to the clonal development of mammalian tumors. For this analysis, we used the larval eye imaginal disc, a columnar, monolayered neuroepithelium. During late larval and early pupal stages of development, the morphogenetic furrow (MF) moves across the eye disc (posterior to anterior), inducing cells to differentiate into the photoreceptors and accessory cells that make up the mature ommatidia of the adult eye. Using *FRT*-mediated recombination and FLPase expressed from the *eyeless* (*ey*) promoter (Newsome *et al*., 2000), mitotic clones of *scrib*$^{-}$ tissue were induced early in eye development and examined at the late larval third instar stage. In clones of *scrib*$^{-}$ tissue [*scrib*^{1} and *scrib*^{2}; both null alleles marked by the absence of green fluorescent protein (GFP) in Figure 1, but positively marked by GFP expression in subsequent figures], cells lost their monolayered and columnar shape to become rounded and multilayered (Figure 1A). A marker of photoreceptor differentiation, Elav, showed that many *scrib*$^{-}$ cells, but not all, were defective in their ability to initiate differentiation (Figure 1B). These effects were not strictly cell autonomous, as the regular spacing of ommatidia in the wild-type

tissue immediately surrounding *scrib*$^-$ clones was also often disrupted.

We next determined if cell proliferation control was defective in *scrib*$^-$ clones. Normally, cell proliferation is tightly regulated during third instar larval eye development. Cells in the anterior of the eye disc, which are undifferentiated, cycle asynchronously, until arresting in G_1 within the MF. Subsets of cells initiate differentiation within the MF, and the remainder express a pulse of cyclin E (Figure 1C) and undergo a synchronous S phase (Figure 1E) just posterior to the furrow. Most of these G_2 cells will then also progress into mitosis (Figure 1G), and the resulting pool of cells is recruited into the developing ommatidia. Levels of cyclin E were elevated within *scrib*$^-$ clones (marked by the absence of GFP), as well as in wild-type cells immediately surrounding the clones, and this was most apparent within the zone where cyclin E is usually expressed just posterior to the MF and extended further anteriorly through the MF where cells normally arrest in G_1 (Figure 1D). This was accompanied by ectopic S phases as revealed by bromodeoxyuridine (BrdU) staining (Figure 1F), as well as ectopic mitoses (Figure 1H).

We conclude that during eye development, clones of *scrib*$^-$ tissue lose their regular monolayered columnar shape, become multilayered and rounded, and undergo ectopic cell proliferation. Non-cell-autonomous defects in both photoreceptor differentiation and ectopic cyclin E expression are also observed within wild-type tissue immediately surrounding *scrib*$^-$ clones.

Clones of scrib mutant tissue are eliminated by JNK pathway-mediated apoptosis

Despite increased proliferation of *scrib*$^-$ tissue during third instar larval development, this tissue must eventually be removed by cell death, since by the time the larvae eclose after pupation, little *scrib*$^-$ tissue remains in the adult eye (marked as *w*$^-$ tissue lacking red pigment), and there are signs of necrosis, especially in the middle of the eye (Figure 2B, compared with wild-type eye mosaic in Figure 2A). Indeed, even during third instar larval eye disc development, there is considerable apoptosis (Figure 2D compared with a wild-type disc in Figure 2C), and *scrib*$^-$ tissue (GFP positive), which should account for 50% of the eye antennal disc, is much less represented than wild-type tissue, particularly posterior to the MF where differentiation is occurring (Figure 2F compared with a control mosaic eye disc, Figure 2E).

In both mammals and *Drosophila*, JNK can induce a stress response leading to apoptosis (reviewed by Davis, 2000). We therefore wished to test whether ectopic activation of the JNK pathway in *scrib*$^-$ eye clones could account for the removal of the *scrib*$^-$ tissue. To do this, we used the MARCM system, a method for ectopically and constitutively expressing any GAL4-dependent (*UAS*-) transgene, just within mitotic clones of tissue (marked by *UAS–GFP* expression), and not in the surrounding wild-type tissue (Lee and Lou, 1999; see Materials and methods for a more detailed description of the system). Using this technique, with *ey-FLP* to generate the clones, we observed that the ectopic expression of an activated form of JNKK (Hemipterous), within GFP-expressing clones of otherwise wild-type tissue, resulted in very small clones, consistent with a pro-apoptotic role for the JNK pathway in the eye disc (Figure 2G; Hep^{ACT}). To test if activation of the JNK pathway was responsible for the removal of *scrib*$^-$ tissue, we blocked JNK pathway activity by expressing a dominant-negative version of the *Drosophila* JNK homolog, Basket (Bsk^{DN}), specifically within *scrib*$^-$ eye clones. This resulted in a significant increase in the size of the *scrib*$^-$ clonal tissue (GFP-positive cells, Figure 2H), compared with *scrib*$^-$ control clones (Figure 2F), consistent with the notion that Bsk^{DN} rescues cell death of *scrib*$^-$ tissue. Furthermore, while the expression of Bsk^{DN} in control eye clones resulted in adult flies with only mild eye disorganization (Figure 2I), expression of Bsk^{DN} in *scrib*$^-$ cells resulted in pupal lethality. In contrast, using the MARCM system to downregulate other signaling pathways in *scrib*$^-$ clones, including blocking epidermal growth factor receptor (EGFR)/Ras signaling with a dominant-negative EGFR, Wingless (Wg) signaling with a dominant-negative form of TCF, or Decapentaplegic (Dpp) signaling with ectopic expression of Dad, had the opposite effect, dramatically reducing the viability of the mutant tissue (data not shown). Thus blocking the JNK pathway, specifically, led to increased viability of *scrib*$^-$ tissue. In fact, the effects of downregulating the JNK pathway were even more potent than those achieved by blocking apoptosis with the baculovirus pan-caspase inhibitor, p35. Although expressing p35 in *scrib*$^-$ eye clones also increased the size of the mutant tissue, some flies were capable of eclosing, with eyes showing signs of considerable necrosis (Figure 2J).

We therefore conclude that although the loss of Scrib is associated with ectopic cell proliferation, a JNK-mediated apoptotic response ensures that the mutant tissue does not overgrow.

The surrounding wild-type tissue is important in limiting the overgrowth of scrib mutant clones

The removal of *scrib*$^-$ tissue by JNK-mediated apoptosis could be a purely cell-intrinsic response to a general loss of cell polarity and detachment from the basement membrane, or, alternatively, it could be regulated by the surrounding wild-type tissue context acting to limit the overgrowth of aberrant cells. To distinguish between these two possibilities, we made use of two different genetic techniques to remove the wild-type eye disc tissue surrounding the *scrib*$^-$ clones. First, we expressed the cell death inducer, Hid, from an eye disc driver (*GMR*), specifically in the differentiating wild-type tissue posterior to the MF. Strikingly, removing the wild-type tissue dramatically increased the viability of *scrib*$^-$ clones (GFP-positive cells) posterior to the MF (Figure 3A compared with Figure 2F). This resulted in tissue overgrowth and lethality. In a second, and more extreme experimental option, we removed not just the wild-type tissue posterior to the MF, but nearly all of the wild-type tissue in the eye/antennal disc by using a homozygous cell lethal mutation [*l(3)cl-R3*^{1}; Newsome *et al*., 2000]. Similarly, in this context, the *scrib*$^-$ tissue (GFP-positive) was not eliminated by apoptosis but, instead, overproliferated, losing recognizable structure (Figure 3B, and data not shown), resulting in lethality.

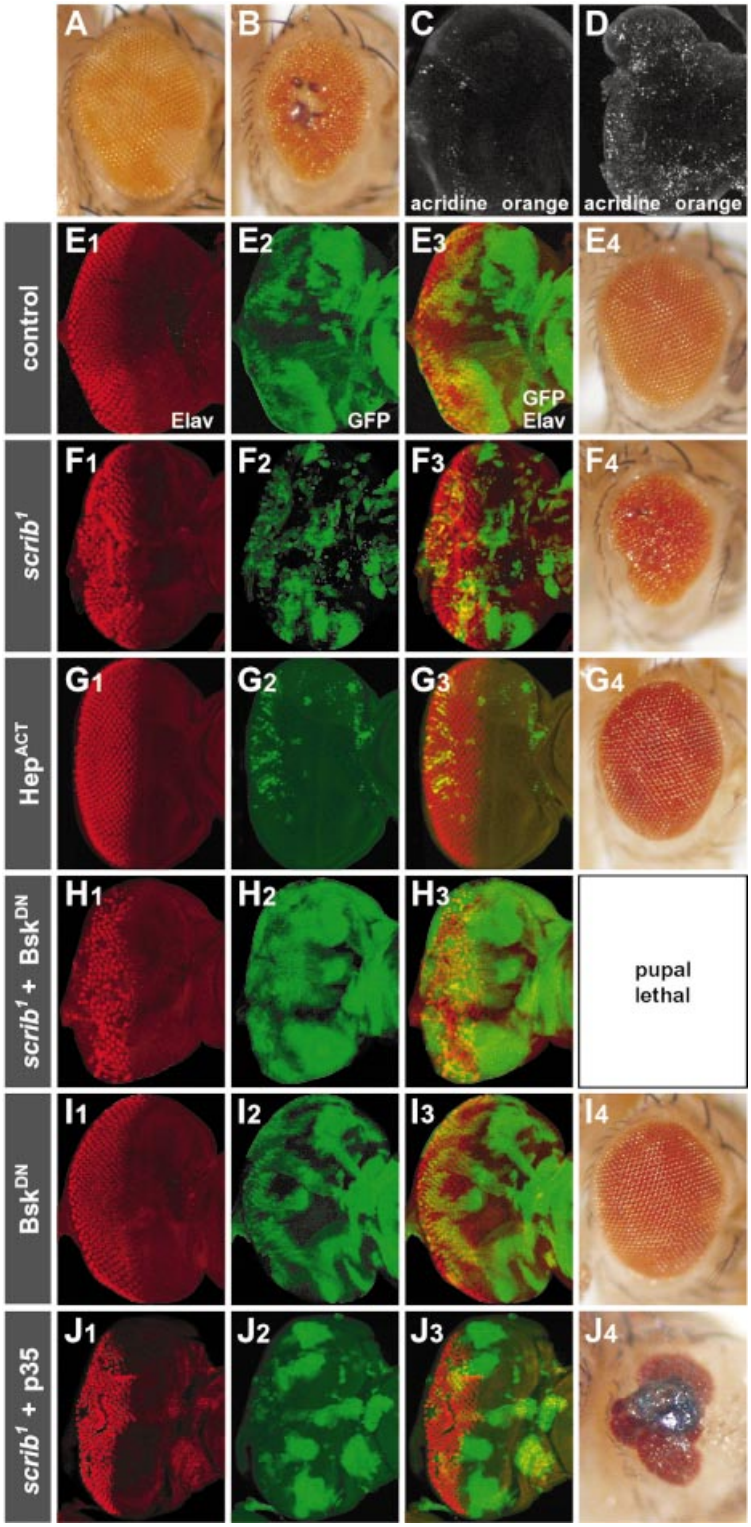
Taken together, these data indicate that the surrounding wild-type tissue is responsible for preventing overgrowth

of the *scrib*$^{-}$ tissue through the induction of JNK-mediated cell death.

Oncogenic forms of Ras and Notch cooperate with scrib mutant clones to result in dramatic overgrowth

Clones of *scrib*$^{-}$ tissue share many characteristics of mammalian tumors, including loss of cell polarity, defective differentiation capacity and ectopic cell proliferation; however, *scrib*$^{-}$ tissue overgrowth is kept in check by a propensity to undergo JNK pathway-mediated apoptosis. We therefore wondered what the consequences would be of using the MARCM system to express secondary oncogenic mutations in the *scrib*$^{-}$ background.

Most striking were the effects of ectopically expressing activated alleles of Ras (Ras^{ACT}) or Notch (N^{ACT}) in *scrib*$^{-}$ clones (Figure 4B and D). Both of these oncogenes were capable of dramatically inducing the overgrowth of the



scrib^{-} tissue (GFP-positive); the wild-type tissue was outcompeted, and the mutant tissue failed to differentiate and continued to grow in three dimensions to many times the normal size of the eye/antennal disc, fusing with the brain lobes and other imaginal tissues. Many larvae failed to pupate, and grew to resemble the giant larvae of homozygous *scrib*$^{-}$ larvae. Importantly, although the ectopic expression of either Ras^{ACT} or N^{ACT} in otherwise wild-type clones of tissue exerted hyperproliferative effects and resulted in pupal lethality, neither gene caused the massive synergistic tissue overgrowth observed in the absence of Scrib (Figure 4A and C).

The consequences of ectopically activating the major growth and patterning pathways of Hedgehog (Hh), Dpp and Wg in *scrib*$^{-}$ clones (GFP-positive) were very different from the effects of Ras^{ACT} and N^{ACT}. An oncogenic, activated form of β-catenin (Arm^{ACT}) which mimics ectopic Wg pathway signaling effectively blocked photoreceptor differentiation in control clones and maintained the tissue in a proliferative state (Figure 4E), but in *scrib*$^{-}$ clones, although photoreceptor differentiation remained blocked by the expression of Arm^{ACT}, the *scrib*$^{-}$ tissue did not exhibit unrestrained overgrowth, and occasionally adult flies eclosed, albeit with severely disorganized eyes (Figure 4F). Activation of either Dpp (Tkv^{ACT}; Figure 4G and H) or Hh (Ci-155; Figure 4I and J) signaling also caused patterning defects, but even ectopic Hh signaling in *scrib*$^{-}$ clones, which resulted in pupal lethality, did not induce the strong cooperative overgrowth effect with *scrib*$^{-}$ observed with either Ras^{ACT} or N^{ACT}. Furthermore, while the ectopic activation of Wg, Dpp or Hh signaling pathways in *scrib*$^{-}$ clones generally increased the size of the clonal tissue, this was not with the same consistency, nor extent, as that induced by blocking the JNK pathway.

In summary, these data demonstrate that extensive tumor overgrowth can be induced in *Drosophila* by cooperative interactions between the loss of the tumor suppressor, *scrib*, and, specifically, the oncogenic forms of Ras or Notch.

The effects of activated Ras on scrib mutant tissue overgrowth are not solely a consequence of blocking apoptosis and enhancing cell cycle progression

We chose to focus on the effects of Ras^{ACT} on *scrib*$^{-}$ tissue in more detail to determine which effectors of Ras might be responsible for the synergistic overgrowth observed in combination with the absence of Scrib. In mammals, oncogenic Ras is thought to exert its effect through a number of different effectors including Raf and the canonical mitogen-activated protein kinase (MAPK) pathway, the growth regulator phosphatidylinositol 3-kinase (PI3 kinase) and cell architecture regulators such as Ral and Rho family members (reviewed by Shields *et al*., 2000). However, in the *Drosophila* eye disc, ectopic expression of PI3 kinase in *scrib*$^{-}$ clones did not mimic the effects of activated Ras (Figure 4L), nor could activated alleles of the cytoskeletal regulators, Ral, Rho, Rac and Cdc42 (data not shown). Thus it seemed likely that downstream targets of the MAPK pathway would be responsible for the effects of Ras^{ACT}. Indeed, expression of a gain-of-function allele of Raf (Raf^{ACT}) in *scrib*$^{-}$ clones (GFP-positive) completely phenocopied the overgrowth effects of Ras^{ACT} in *scrib*$^{-}$ tissue (Figure 5A and B).

Ras and the MAPK pathway have been implicated in a number of different developmental processes in the *Drosophila* eye disc, including differentiation (Halfar *et al*., 2001; Yang and Baker, 2001), protection from cell death (Bergmann *et al*., 1998, 2002; Kurada and White, 1998; Sawamoto *et al*., 1998), and increased cell proliferation (Karim and Rubin, 1998). We had already demonstrated that blocking apoptosis in *scrib*$^{-}$ clones enhanced overgrowth of the mutant tissue (Figure 2) but, as this was not to the same extent as Ras^{ACT}, we considered it likely that downstream effectors of Ras involved in cell proliferation would also be important. Indeed, one of the downstream effectors of Ras^{ACT} is cyclin E, since the ectopic expression of Ras^{ACT} in otherwise wild-type eye clones (GFP-positive) increases cyclin E protein levels (Figure 5C). Although *scrib*$^{-}$ clones also ectopically express cyclin E, Ras^{ACT} may increase the levels of cyclin E further and thereby enhance the overgrowth of *scrib*$^{-}$ tissue.

To test the importance of cyclin E in the Ras^{ACT}-induced overgrowth of *scrib*$^{-}$ tissue, we blocked cyclin E activity by overexpressing the *Drosophila* p21 cyclin E–Cdk inhibitor homolog, Dacapo (Dap), in Ras^{ACT}-expressing *scrib*$^{-}$ eye clones, and this effectively rescued the tumorigenic overgrowth phenotype (Figure 5D and F). Similarly, we also tested the involvement of another important cell cycle regulator, E2F1, in Ras^{ACT}-induced overgrowth. Attenuating E2F1 activity in Ras^{ACT}-expressing *scrib*$^{-}$ clones, by using an *e2f1* null allele (*e2f1*^{91}; Duronio *et al*., 1995; Royzman *et al*., 1997), similarly rescued the overgrowth effects (Figure 5H).

Since increased proliferation mediated through cyclin E and E2F1 was therefore clearly important in mediating the

Fig. 2. Clones of *scrib* mutant eye tissue are eliminated by JNK pathway-mediated apoptosis. (A and B) By using a *w*$^{+}$ gene to mark wild-type tissue in the adult eye, an *ey-FLP* control eye incorporates an equal amount of *w*$^{+}$ and *w*$^{-}$ tissue (**A**). An *ey-FLP scrib*^{1} mosaic eye incorporates little *scrib*$^{-}$ tissue (*w*$^{-}$) in the adult, the eyes are reduced in size and necrotic spots are often observed in the centre of the eye (**B**). (C and D) Acridine orange staining of third instar larval eye discs reveals few apoptotic cells in a wild-type eye/antennal disc (**C**), but in an *ey-FLP scrib*^{1} mosaic eye/antennal disc (**D**) many apoptotic cells are detected. (E–J) The MARCM system was used to generate *ey-FLP* clones expressing different transgenes, with GFP as a positive clonal marker, in either wild-type control clones (**E**, **G** and **I**) or *scrib*^{1} clones (**F**, **H** and **J**). Developing photoreceptor cells in third instar larval eye discs are shown by Elav staining. In wild-type clones, only expressing GFP as a clonal marker, clonal tissue makes up ~50% of the tissue (E2 and 3) and the adult eyes are normal (E4). In *scrib*^{1} clones, mutant tissue is significantly less represented than wild-type tissue (F2 and 3), and the adult eyes are often reduced in size and disorganized (F4). Ectopic activation of the JNK pathway by expressing an activated allele of JNKK (Hep^{ACT}) in control wild-type clones (G1–3) nearly eliminates all clonal tissue, resulting in slightly roughened adult eyes (G4). Blocking the JNK pathway, by expressing a dominant-negative form of JNK (Bsk^{DN}), in *scrib*^{1} clones (H1–3) causes a pronounced expansion of the *scrib*$^{-}$ tissue in the eye disc (H2; compared with *scrib*^{1} clones, F2), and complete pupal lethality. Expression of Bsk^{DN} in control clones (I1–3) results in adult flies with only slight eye disorganization (I4). The expression of the pan-caspase inhibitor, p35, in *scrib*^{1} mutant clones (J1–3) results in an increase in the size of *scrib*$^{-}$ clonal tissue compared with clones of *scrib*^{1} alone (F2), and the eyes of the resulting adult flies are more disorganized and necrotic (J4) compared with *scrib*^{1} clones alone (F4).

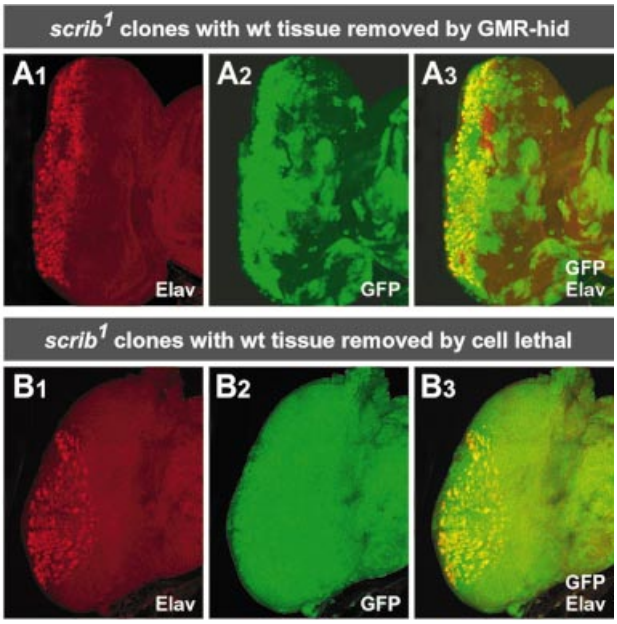


Fig. 3. The surrounding wild-type tissue limits the overgrowth of the *scrib* mutant tissue. (**A**) *scrib*^{1} mosaic eye discs were generated, and the remaining wild-type tissue posterior to the MF was eliminated by expressing the cell death inducer, Hid, from the *GMR* promoter. The *scrib*^{1} clonal tissue, marked by the expression of GFP, is not eliminated posterior to the MF. Elav staining marks developing photoreceptor cells. (**B**) *scrib*^{1} mosaic eye discs were generated, and the surrounding wild-type tissue eliminated by the presence of a homozygous cell-lethal mutation. The *scrib*^{1} clonal tissue, marked by the expression of GFP, overgrows in three dimensions, with limited differentiation as judged by Elav staining of developing photoreceptor cells.

tumorigenic effects of Ras^{ACT} in *scrib*$^{-}$ clones, we next tested to see whether we could recapitulate the synergistic overgrowth effects of Ras^{ACT} in *scrib*$^{-}$ clones by promoting cell proliferation with the ectopic expression of cyclin E or E2F1 while also blocking apoptosis. However, neither the co-expression of cyclin E with the apoptosis inhibitor p35, nor that of E2F1/DP with p35 could mimic the effects of Ras^{ACT} on *scrib*$^{-}$ (GFP-positive) tissue (Figure 5I and J). Although pupal lethality resulted in both instances, analysis of larval eye discs revealed that the aggressive overgrowth and lack of differentiation induced by Ras^{ACT} in *scrib*$^{-}$ clones did not occur (Figure 5I and J). As blocking JNK pathway activity had more pronounced effects on the viability of the *scrib*$^{-}$ mutant tissue than the use of p35, we also expressed cyclin E, or E2F1/DP, in Bsk^{DN}-expressing *scrib*$^{-}$ clones, but this also failed to mimic the dramatic overgrowth effects of Ras^{ACT} (data not shown).

These results suggest that although protection from apoptosis and enhanced cell cycle progression are important factors in mediating Ras^{ACT}-induced overgrowth of *scrib*$^{-}$ tissue, other effectors of Ras^{ACT} signaling are also likely to be important in mediating the cooperative overgrowth. The general tissue disruption induced by the ectopic expression of Ras^{ACT} in wild-type clones, when the proliferative effects were minimized by either the expression of Dacapo (Figure 5E) or the absence of E2F1 (Figure 5G), is also suggestive of additional effectors other than those involved in cell proliferation and protection from apoptosis.

Discussion

In this study on the *Drosophila* tumor suppressor, *scribble*, we have adopted a clonal approach, more closely resembling the clonal nature of mammalian cancer, to analyze the effects of removing Scrib function on tumor formation. Our analysis indicates that *Drosophila scrib*$^{-}$ tumors: (i) lose tissue architecture, including apical–basal cell polarity; (ii) fail to differentiate properly; (iii) exert non-cell-autonomous effects upon the surrounding wild-type tissue; (iv) upregulate cyclin E and undergo excessive cell proliferation; (v) are restrained from overgrowing by the surrounding wild-type tissue via a JNK-dependent apoptotic response; and (vi) show strong cooperation with oncogenic alleles of Ras and Notch to produce large amorphous tumors. These conclusions are summarized in a model for tumor development in *Drosophila* in Figure 6. We suggest that the role of epithelial cell polarity regulators in restraining oncogenes is likely to be of general significance in mammalian tumorigenesis.

Upregulation of cyclin E and overproliferation of scrib mutant clones

scrib^{-} clones ectopically express cyclin E and undergo ectopic S phases and mitoses. Since cyclin E is rate limiting for cell cycle progression in the developing eye (Richardson *et al*., 1995), it is likely that upregulation of cyclin E in *scrib*$^{-}$ clones is critical for the ectopic cell proliferation. Indeed we originally isolated alleles of *scrib* and *lgl* as dominant suppressors of a hypomorphic *cyclin E* allele, *DmcycE*^{JP} (A.M.Brumby, J.Secombe, J.Horsfield, M.Coombe, N.Amin, D.Coates, R.Saint and H.E.Richardson, in preparation), suggesting that these cell polarity genes normally play a critical role in limiting cyclin E expression. We currently are investigating which signaling pathways are altered in *scrib, dlg* or *lgl* mutants that could be responsible for *cyclin E* upregulation. A recent study in human lung epithelial cells showed that disrupting cell polarity allowed mixing of the heregulin-α ligand and the erbB2-4 receptor, which are normally physically separated, resulting in activation of the pathway and cell proliferation (Vermeer *et al*., 2003). Further studies are required to determine whether the ectopic expression of cyclin E observed in the absence of Scrib is simply a consequence of the tissue disorganization induced by disrupting cell polarity, or if Scrib has a direct role in limiting cell proliferation independent of cell polarity. Interestingly, the rounding up of cells in the absence of Scrib appeared to be predominantly a cell-autonomous effect, yet clearly non-cell-autonomous defects were also apparent, including the upregulation of cyclin E. This would suggest that altered cell–cell interactions between wild-type and mutant cells can also alter signaling pathways within wild-type cells, and that the loss of apical–basal polarity and collapse of the columnar epithelium is not intrinsically responsible for the deregulated expression of cyclin E. A deeper understanding of the relationship between epithelial cell polarization and cell proliferation is clearly important for understanding the development of cancer, since a loss of cell polarity often accompanies tumor progression and metastasis.

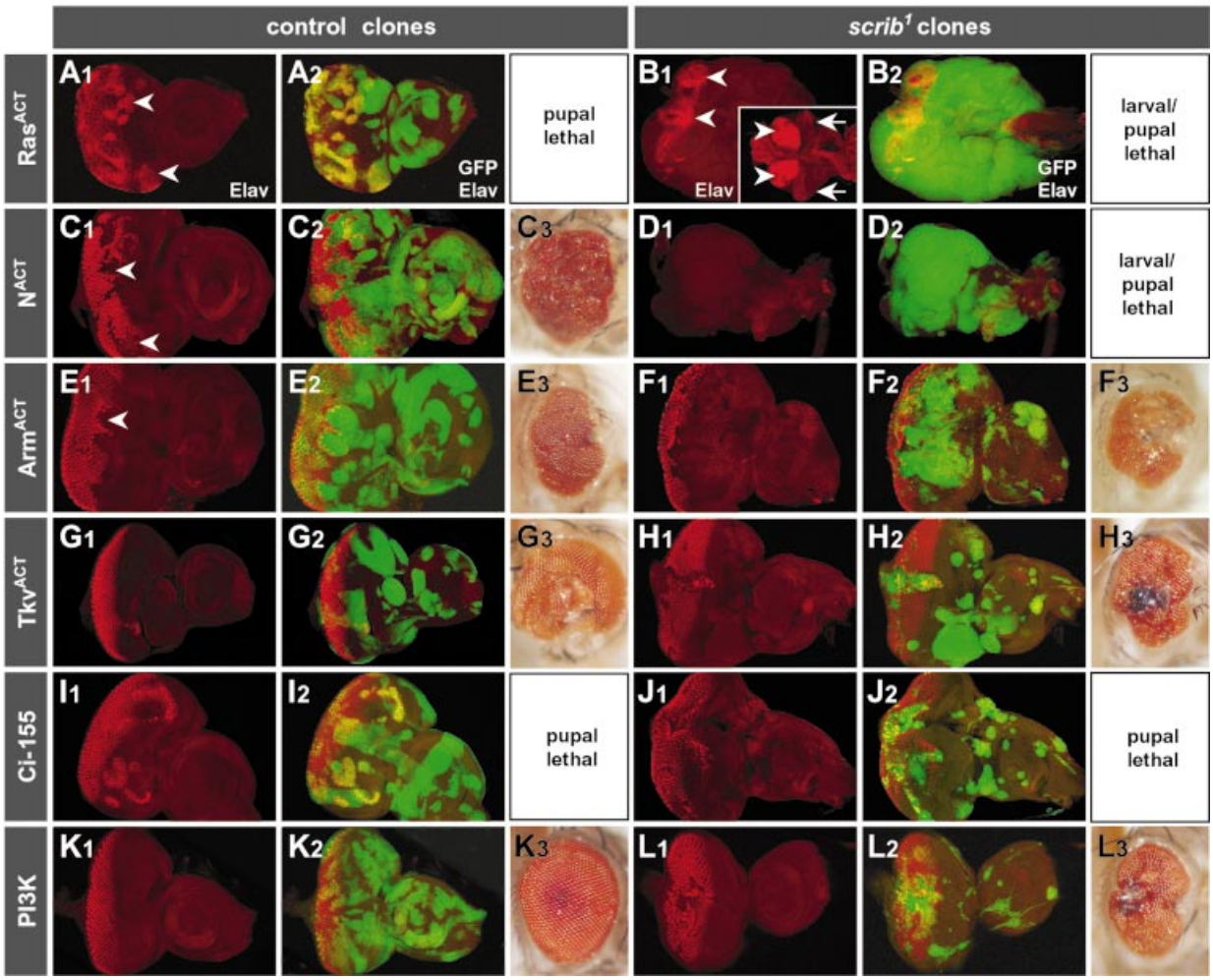


Fig. 4. Ectopic activation of Ras and Notch signaling pathways, but not Wg, Dpp, Hh or PI3 kinase pathways, in *scrib* mutant eye clones induces massive cooperative overgrowth. (A–L) The MARCM system was used ectopically to activate the EGFR/Ras pathway (Ras^{ACT}; **A** and **B**); Notch pathway (N^{ACT}; **C** and **D**); Wg pathway (Arm^{ACT}; **E** and **F**); Dpp pathway (Tkv^{ACT}; **G** and **H**); Hh pathway (Ci-155; **I** and **J**); or PI3 kinase pathway (PI3K; **K** and **L**) in either wild-type control eye clones (A, C, E, G, I and K) or *scrib*^{1} clones (B, D, F, H, J and L). All clones are marked by the expression of GFP. Developing photoreceptors are shown by Elav staining (A1–L1; merged with GFP in A2–L2). The resulting adult eyes (A3–L3), or pharate adults dissected from the pupal case (C3), are shown where applicable. Expression of Ras^{ACT} in control clones (A) induces precocious photoreceptor differentiation anterior to the MF (arrowhead). In *scrib*^{1} clones (B), Ras^{ACT} fails to initiate differentiation, and instead, massive three-dimensional tissue overgrowth is induced, resulting in fusion of the eye/antennal discs to each other as well as the brain lobes (arrowheads). The insert (B1) shows wild-type brain lobes (arrowheads) and eye antennal discs (arrows) at the same magnification (magnification is half that of the other panels in this figure) for comparison. Expression of N^{ACT} in control clones (C) interferes with photoreceptor differentiation (arrowheads), and adult flies die before eclosure with overgrown eyes. In *scrib*$^{-}$ clones (D), N^{ACT} induces a similar degree of three-dimensional overgrowth, and lack of differentiation, as Ras^{ACT} (a planar section is shown, but overgrowth is in three dimensions). Ectopic activation of Wg pathway signaling effectively blocks photoreceptor differentiation in control clones (E; arrowhead), and this remains the case in *scrib*^{1} clones (F). Ectopic activation of Dpp or Hh signaling induces patterning defects in both control clones (G and I), as well as *scrib*^{1} clones (H and J). Ectopic PI3 kinase signaling in *scrib*^{1} clones (L) has little effect.

Compensatory JNK-mediated elimination of scrib mutant tissue

Overproliferation of *scrib*$^{-}$ clones in the eye disc is compensated for by JNK-mediated apoptosis. Blocking JNK pathway activity in *scrib*$^{-}$ eye clones greatly increases the proportion of clonal tissue, and results in lethality to the host. As downregulating JNK pathway activity in otherwise wild-type clones of tissue did not induce increased cell proliferation (A.M.Brumby and H.E.Richardson, unpublished data), we suggest that JNK pathway activity in *scrib*$^{-}$ clones induces apoptosis. This is consistent with previous reports on the pro-apoptotic effects of the JNK pathway in the *Drosophila* eye (Takatsu *et al*., 2000) and our own observations (Figure 2G). Recent studies in mammals would also suggest that activation of the JNK pathway can limit the growth of tumors *in situ*, possibly by increasing apoptosis (Kennedy *et al*., 2003).

How JNK-mediated apoptosis is induced in *scrib*$^{-}$ clones is not known. While Scrib could play a direct role in repressing JNK pathway activity, it is also possible that the JNK pathway is activated indirectly, in response to other cellular defects. In the wing disc, removal of cells by JNK-mediated apoptosis is linked to discontinuities in a cell's response to morphogen gradients, most notably the antero-posterior patterning regulator, Dpp, in a process probably related to cell competition, with the purpose of eliminating aberrant or slow growing cells (Adachi-Yamada *et al*., 1999; Adachi-Yamada and O'Connor,

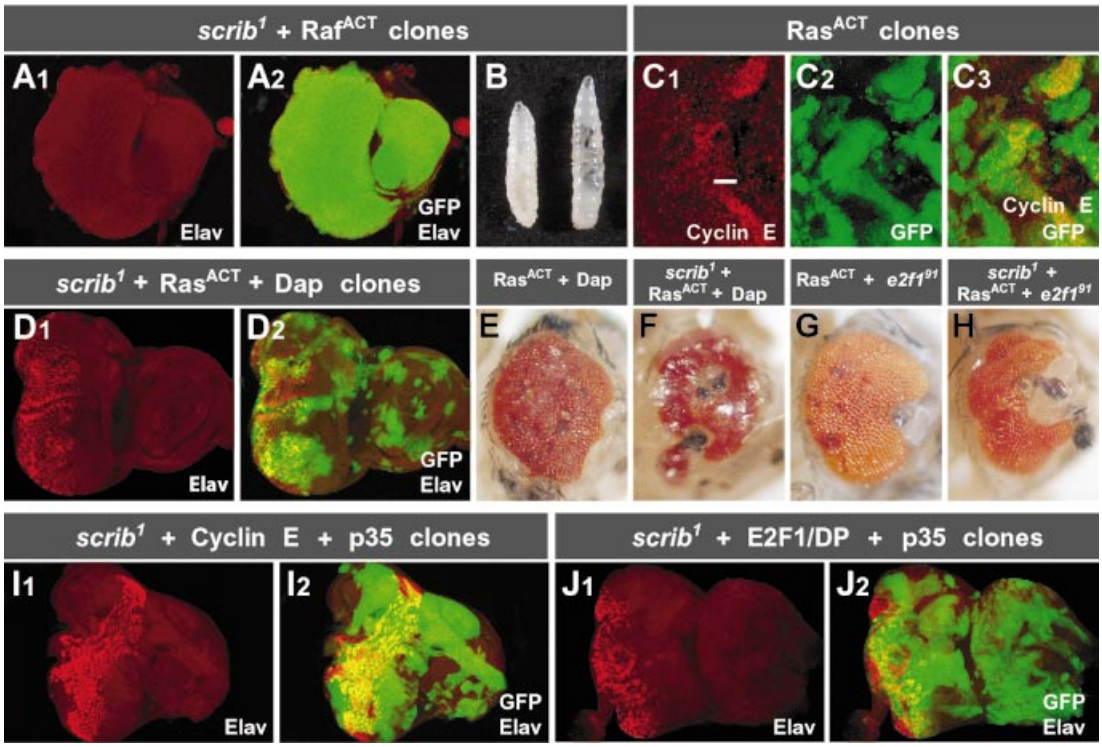


Fig. 5. The effects of Ras^{ACT} in *scrib* mutant eye clones are mediated through the MAPK cascade, but cannot be mimicked by enhancing cell cycle progression and preventing apoptosis. (**A** and **B**) Expression of Raf^{ACT} in *scrib^{1}* eye clones mimics the effects of Ras^{ACT}. Elav staining shows a block to photoreceptor differentiation in third instar larval eye discs (A1), and the *scrib^{-}* tissue, marked by the expression of GFP, greatly overproliferates (A2). As a consequence, many larvae fail to pupate and overgrow (B, wild-type larvae on the left) like homozygous *scrib* mutants. (**C**) Expressing Ras^{ACT} in control wild-type eye clones, marked by GFP expression (C2 and 3), induces cyclin E (C1 and 3), and is pupal lethal. The position of the MF is indicated by a bar. (**D–F**) Blocking cyclin E activity by expressing Dacapo (Dap) in Ras^{ACT}-expressing control clones can rescue the pupal lethality associated with the constitutive expression of Ras^{ACT}, although the resulting adult eyes are severely disorganized (E). Expressing Dap in Ras^{ACT}-expressing *scrib^{1}* clones can also rescue the overproliferation phenotype, although Elav staining (D) shows that Ras^{ACT} is still not effective at inducing photoreceptor differentiation of *scrib^{-}* tissue, and the developing adult flies fail to eclose and have severely disorganized eyes (F). (**G** and **H**) Mutating E2F1 (*e2f1^{91}*) in Ras^{ACT}-expressing control eye clones rescues the pupal lethality associated with the constitutive expression of Ras^{ACT} in wild-type clones (G), as does mutating E2F1 (*e2f1^{91}*) in Ras^{ACT}-expressing *scrib^{1}* mutant clones (H). (I and J) Co-expression of cyclin E with the apoptosis inhibitor, p35, in *scrib^{1}* eye clones (**I**) results in overgrowth but fails to reproduce the effects of Ras^{ACT} in *scrib^{1}* clones. Co-expression of E2F1, DP and p35 in *scrib^{1}* eye clones (**J**) results in overgrowth; however, the overgrowth is still not as aggressive as that produced by Ras^{ACT} or Raf^{ACT} in *scrib^{1}* clones.

2002; Moreno *et al*., 2002a). Although this form of compensatory JNK-mediated apoptosis has not yet been demonstrated within the eye disc, the observation that the surrounding wild-type tissue context plays an important role in limiting the overgrowth of *scrib^{-}* tissue argues against a simple cell-intrinsic apoptotic response of *scrib^{-}* cells to a loss of cell polarity, and is more consistent with an integrative response mediated by both the tumor cells and the surrounding wild-type cells, as exemplified by cell competition. Whether this is dependent on a failure of *scrib^{-}* cells to transduce Dpp signaling is not known; however, other interesting possibilities also warrant further investigation. Notable is the recent identification of a tumor necrosis factor-induced apoptotic signaling pathway involving the JNK pathway (Igaki *et al*., 2002; Moreno *et al*., 2002b). We are also mindful of the involvement of the JNK pathway in orchestrating cell shape changes during the morphogenetic movements of dorsal and thorax closure (reviewed by Kockel *et al*., 2001) and wound healing (Ramet *et al*., 2002). We note that clones of *scrib^{-}* tissue expressing Bsk^{DN} (JNK^{DN}) appeared morphologically different from those expressing the apoptosis inhibitor p35; most notably, the clones were generally larger and less rounded than those expressing p35 (Figure 2H, and data not shown). This would imply either that p35 is not as effective as Bsk^{DN} in preventing cell death, or that there are other effectors of the JNK pathway that are important in the inhibition of *scrib^{-}* tumor overgrowth. We currently are investigating the possibility that JNK activation could play a role in eliminating *scrib^{-}* tissue from the epithelium in a process reminiscent of wound healing.

It has been reported previously that *dlg* and *lgl* mutant clones also show poor viability (Woods and Bryant, 1991; Agrawal *et al*., 1995), suggesting that JNK-mediated apoptosis could be a common response to the loss of cell polarity and overproliferation induced by the absence of these tumor suppressors. Indeed, while other regulators of epithelial cell polarity, such as Crumbs and E-cadherin, apparently do not act as tumor suppressors in *Drosophila*, the effects of these mutations on cell proliferation when cell death is blocked warrant further examination. Interestingly, in mammalian systems, the polarized nature of epithelia is also important in protecting cells from an

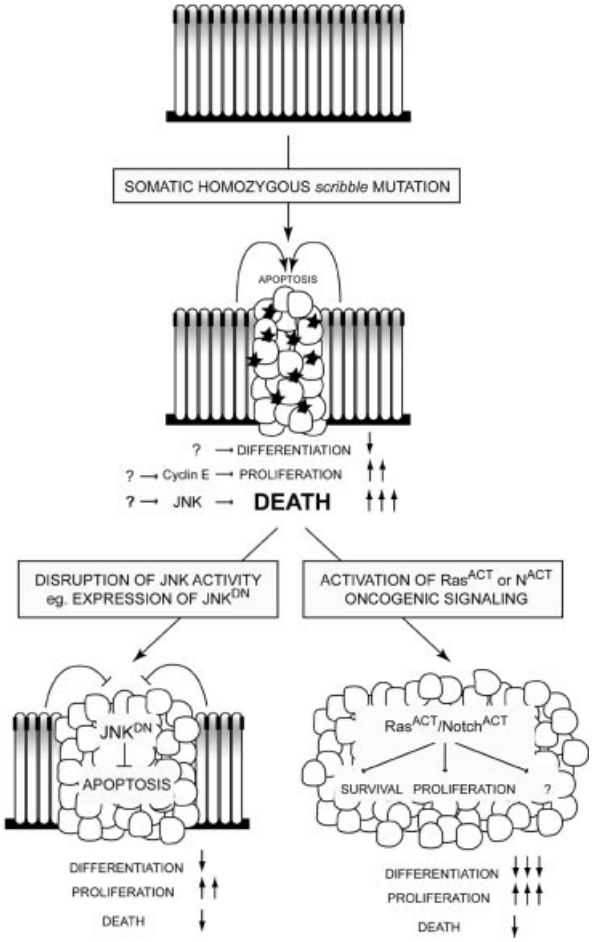


Fig. 6. A model for cooperative tumorigenesis in *Drosophila*. A wild-type larval eye disc is a monolayered columnar epithelium, in which cell proliferation is tightly regulated. Cell architecture is maintained by the formation of adherens junctions (black boxes), the apical localization of Scribble (gray shading) and adhesion to the basement membrane. Mutation of *scrib* results in loss of apical–basal polarity, leading to multilayering and rounding up of cells. *scrib*$^-$ tissue also shows impaired differentiation, and ectopic cyclin E expression (by an unknown mechanism) leads to ectopic cell proliferation. Unrestrained overgrowth and tumor formation of *scrib*$^-$ cells is held in check by compensatory JNK-mediated apoptosis (black stars), dependent upon the presence of surrounding wild-type cells. Secondary mutations are required to avoid this apoptotic fate. If JNK activity is blocked within *scrib*$^-$ cells, by expressing a dominant-negative form of JNK, apoptosis is prevented, resulting in tissue overgrowth and lethality. Even more aggressive overgrowth results from the addition of activating oncogenic alleles of Ras or Notch. In addition to promoting cell survival, these oncogenes must also promote tumor cell proliferation; however, we propose that other downstream effectors of these oncogenes are likely also to be important, since we could not mimic the cooperative overgrowth effects of Ras^{ACT} or N^{ACT} on *scrib*$^-$ tissue by simply blocking apoptosis and enhancing cell proliferation.

apoptotic response, and this acts as a brake on tumor development when polarity is disrupted (reviewed by Jacks and Weinberg, 2002).

Cooperative tumorigenesis induced by oncogenic Ras and Notch

In *Drosophila*, activated Ras exerts its oncogenic effects through Raf and the MAPK pathway (Figure 5A and B). Downstream targets of MAPK in the eye disc previously have been shown to promote differentiation, cell survival and cell proliferation. Our work also demonstrates that Ras can increase cyclin E protein levels in the eye disc. In combination with *scrib*$^-$, the differentiation output of Ras^{ACT} signaling appears to be attenuated, and the proliferative and anti-apoptotic responses prevail.

Activated Notch also cooperates with *scrib*$^-$, resulting in neoplastic overgrowth, and although no anti-apoptotic role for Notch signaling in the eye has been described previously, N^{ACT} exerts hyperproliferative effects in flies (Go *et al*., 1998; Baonza and Garcia-Bellido, 2000), and Notch signaling is required for proliferation of eye disc cells (Cho and Choi, 1998; Dominguez and de Celis, 1998). Although we do not know if N^{ACT} induces the same critical downstream targets as Ras^{ACT} to cause overgrowth of *scrib*$^-$ tissue, removing *ras* function in *scrib*$^-$ cells overexpressing N^{ACT} rescues the overgrowth phenotype, suggesting that the effects of N^{ACT} are at least partially dependent on Ras (A.M.Brumby and H.E.Richardson, unpublished data).

Initially it seemed likely that the cooperative effects of Ras^{ACT} or N^{ACT} on *scrib*$^-$ tissue could be explained by the ability of these oncogenes to promote cell proliferation while blocking apoptosis. However, the expression of neither cyclin E nor E2F1/DP, in combination with the apoptosis inhibitor p35 (or with the inhibitor of JNK pathway activity, Bsk^{DN}), was capable of phenocopying the effect of Ras^{ACT} or N^{ACT} in *scrib*$^-$ clones. We therefore suggest that other downstream effectors, apart from anti-apoptotic and cell cycle regulators, must be important in mediating the oncogenic effects of Ras^{ACT} or N^{ACT}. In fact, in *Drosophila*, Ras has also been shown to be a potent inducer of cellular growth (Prober and Edgar, 2000), while cyclin E and E2F1 mainly promote cell cycle progression (Neufeld *et al*., 1998). Whether N^{ACT} also promotes cell growth in *Drosophila* has not been examined in detail. If growth promotion targets downstream of Ras^{ACT} or N^{ACT} are critical in promoting the overgrowth of *scrib*$^-$ tumors, these are likely to be independent of the PI3 kinase pathway since ectopic PI3 kinase signaling in *scrib*$^-$ clones did not induce synergistic overgrowth, and Raf^{ACT} was able to induce overgrowth as equally extensive as Ras^{ACT}.

Finally, we note that in mammalian systems, evidence exists for a role for Ras signaling in modulating cell junction complexes and enhancing epithelial to mesenchymal transitions (e.g. Chen *et al*., 2000; Janda *et al*., 2002), and in *Drosophila* also, constitutive Ras^{ACT} signaling in clones alters cell affinities and changes the levels of E-cadherin and β-catenin (Prober and Edgar, 2002). Whether Ras^{ACT} or N^{ACT} signaling destabilizes adherens junctions in *Drosophila* and this potentiates *scrib*$^-$ neoplastic overgrowth or whether alterations in the structure of the adherens junction resulting from the absence of Scrib alters a cells response to constitutive activation of these oncogenes are important future questions.

Concluding remarks

In this study, we have described a novel multi-hit model of tumorigenesis in *Drosophila* (Figure 6). Furthermore, although it has been suspected that disruptions to cell polarity could potentiate tumor progression and metastasis, our work in *Drosophila* demonstrates for the first time how the oncogenic effects of activated Ras and Notch are

unleashed in the absence of epithelial polarity regulators. We predict that in mammals also, defects in apical–basal polarity could cooperate with oncogenes during neoplastic development. Our approach in *Drosophila* can now be used to screen for novel oncogenes that, when specifically overexpressed in *scrib*$^{-}$ clones, are capable of inducing cooperative tumorigenesis, and can also be extended to identify cooperative interactions between other tumor suppressors and oncogenes within a whole animal context.

Materials and methods

Fly stocks

Mutants used were *scrib*^{1}, *scrib*^{2} and *e2f1*^{91}. For the generation of eye mosaics, we used *w ey-FLP1*, *FRT82B*, *FRT82B P[Ubi-nlsGFP]*, *FRT82B P[mini-w] P[arm-lacZ]*, *FRT82B scrib*^{1}, *FRT82B scrib*^{2}, *FRT82B P[Ubi-nlsGFP] scrib*^{1}, *FRT82B e2f1*^{91} and *FRT82B e2f1*^{91} *scrib*^{1}.

For the generation of eye mosaics with the wild-type tissue removed, the stocks employed were *ey-GAL4, UAS-FLP1;;FRT82B GMR-hid* and *ey-FLP2;;FRT82B l(3)cl-R3*^{1}, and for the generation of MARCM eye mosaics, *w ey-FLP1,UAS-mCD8-GFP;;tub-GAL4 FRT82B tub-GAL80*.

Transgenes used for expression of: Ras^{ACT}, *UAS-Ras1*^{V12}; Raf^{ACT}, *UAS-Draf*^{GOF}; EGFR^{DN}, *UAS-Egfr*^{DN}; N^{ACT}, *UAS-N*^{intra}; Arm^{ACT}, *UAS-arm*^{S10}; dTCF^{DN}, *UAS-dTCFΔN3*; Tkv^{ACT}, *UAS-tkv*^{Q253D}; Dad, *UAS-dad*; Ci-155, *UAS-ci*; PI3K, *UAS-Dp110*; PI3K^{DN}, *UAS-Dp110*^{D945A}; Hep^{ACT}, *UAS-hep*^{ACT}; Bsk^{DN}, *UAS-bsk*^{DN}; cyclin E, *UAS-DmcycEII*; p35, *UAS-p35*; E2F1/DP, *UAS-dE2F1,UAS-dDP*; Dap, *UAS-dap*; Ral^{ACT}, *UAS-Ral*^{V20}; Rac^{ACT}, *UAS-Drac1*^{V12}; Rho^{ACT}, *UAS-Rho1*^{V12}; Cdc42^{ACT}, *UAS-Dcdc42*^{V12}.

Generation of mitotic eye clones

Mitotic eye clones, marked by the absence of GFP or LacZ, were generated by crossing *ey-FLP1;;FRT82B P[Ubi-nlsGFP]* flies or *ey-FLP1;;FRT82B P[mini-w] P[arm-lacZ]* flies to *FRT82B scrib*^{1} or *FRT82B scrib*^{2} stocks.

To generate *scrib*^{1} eye clones with the surrounding wild-type tissue removed, *ey-GAL4, UAS-FLP1;;FRT82B GMR-hid* or *ey-FLP2;;FRT82B l(3)cl-R3*^{1} flies were crossed to *FRT82B P[Ubi-nlsGFP] scrib*^{1} flies. *scrib*^{1} mutant tissue was marked by the expression of GFP.

To express different transgenes within mutant clones, the MARCM system was used (Lee and Lou, 1999). With the MARCM system, both the transcriptional activator GAL4 and the repressor GAL80 are ubiquitously expressed from *tubulin* promoters, but upon *FLP*-mediated recombination, a *FRT* site flanking *tub-GAL80* ensures the loss of GAL80 in clones, and subsequent derepression of GAL4-dependent transcription, including a *UAS-mCD8-GFP* transgene as a positive clonal marker, specifically within the mutant clonal tissue. *ey-FLP1,UAS-mCD8-GFP;;tub-GAL4 FRT82B tub-GAL80* flies were crossed to flies carrying the *UAS*-transgene with either *FRT82B* as a control, or *FRT82B scrib*^{1} to express the transgene in *scrib*^{1} clones. To express Ras^{ACT} in *e2f1*^{91} mutant clones, *FRT82B e2f1*^{91} or *FRT82B e2f1*^{91} *scrib*^{1} stocks were used. If the *UAS*-transgene was on the third chromosome, recombinants were generated carrying the *UAS* transgene with either *FRT82B* or *FRT82B scrib*^{1}.

Immunohistochemistry

For the analysis of eye/antennal discs, larvae were picked at the third instar stage and tissues were fixed in 4% formaldehyde for 20 min. Antibodies used were mouse anti-Elav (Developmental Studies Hybridoma Bank, 1:5), rat anti-cyclin E (1:1000), rabbit anti-phosphohistone H3 (Santa Cruz, 1:400), mouse anti-BrdU (Becton-Dickinson, 1:50) and rabbit anti-β-galactosidase (Rockland, 1:400). F-actin was detected with phalloidin–tetramethylrhodamine isothiocyanate (TRITC; Sigma, 0.3 μM). For detection of apoptotic cells, discs were dissected in phosphate-buffered saline (PBS) and mounted in 10 μg/ml acridine orange (Sigma). For the detection of S phase cells, a 1 h BrdU pulse was followed by fixation, immunodetection of LacZ, further fixation, acid treatment and detection of the BrdU epitope. All fluorescent labelled samples were analyzed by confocal microscopy (Bio-Rad MRC1000).

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