Non-cell-autonomous induction of tissue overgrowth by JNK/Ras cooperation in a Drosophila tumor model

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The role of c-Jun N-terminal kinase (JNK) signaling in cancer is enigmatic, and both tumor-promoting and tumor-suppressing functions have been ascribed to JNK pathway components. We have used the Drosophila eye to investigate the function of the JNK pathway in three different tumor models of increasing malignancy. Benign lesions caused by loss of the neoplastic tumor suppressor gene scribble can efficiently be eliminated by JNK-induced apoptosis. In such a scenario, the eye reverts to a wild-type phenotype, indicating that the JNK pathway prevents tumor formation. The situation changes in the case of aggressive tissue overgrowth, which can be induced by oncogenic activation of the Ras/Raf pathway in the eye, or in malignant invasive tumors resulting when Raf activation is combined with loss of scribble. The growth of these more aggressive tumor types is significantly, yet incompletely, suppressed by JNK-mediated apoptosis. Remarkably, oncogenic Raf and JNK cooperate in these tumors, to induce massive hyperplasia in adjacent wild-type tissue. Thus, depending on the genetic context, JNK signaling can eradicate tumors by removing premalignant cells, or promote aberrant overgrowth in tissues surrounding primary lesions.

Tumor cells have commonly developed mechanisms to evade apoptosis, and loss of proapoptotic JNK signaling function has been connected with the transformed phenotype.

Tumorigenesis is a complex process that is brought about by the combined effect of multiple oncogenic lesions. Several genetic changes, typically gain-of-function mutations in proto-oncogenes and loss-of-function mutations in tumor suppressors loci have to cooperate in order for tumors to form (23). This complexity has hampered a genetic analysis of tumor development in vivo, and the study of oncogene cooperation has mostly been restricted to cell culture models. Recently however, Drosophila systems have been established to recreate and study tumor progression and oncogene cooperation. By using targeted somatic gain- and loss-of-function mutations, tumors of varying malignancy can reproducibly be induced in developing eye tissue. The loss of epithelial polarity genes such as scribble (scrib), lethal giant larvae (lgl), or discs large (dlg) in groups of cells of the eye imaginal disc epithelium results in the formation of nondifferentiating, disorganized, and mildly overproliferating tissue, leading to the designation of these genes as neoplastic tumor suppressors (24).

Homologues of the Drosophila dlg, lgl and scrib genes have been identified in mammals, and several lines of indirect evidence implicate these proteins in tumor suppression as well. For example, dlg expression is consistently lost in gastric cancers, an event that is associated with increased tumor invasiveness. Furthermore, dlg and scrib are targeted for degradation by viral oncoproteins upon infection with high-risk human papillomaviruses (HPV) and retroviruses such as human T-lymphotropic virus 1 (HTLV-1), suggesting a role of these genes in suppressing virally induced transformation (24, 25). The absence of scribble function from clones of developing Drosophila eye imaginal disc epithelium gives rise to localized benign, premalignant lesions in the emerging adult eye (26, 27) (The terms “benign” and “malignant” are used in a broad sense as introduced in refs. 24 and 25 to reflect the phenomenological similarities between clinical pathologies and Drosophila phenotypes.) More aggressive overgrowth can be observed upon expression of activated alleles of Raf or Ras, in the eye imaginal disk. The malformations resulting from such oncogenic activation of the Ras pathway remain, however, restricted to the adult eye (28). Yet more malignant tumors arise when Ras activation and mutations causing loss of tissue integrity are combined in the same cells. These genetic lesions cooperate to induce invasive, malignant, and, ultimately, lethal tumors (26, 27).

To study the complex functions of JNK in the establishment of cancer in an intact organism and to investigate context-specific contributions of JNK to tumor formation, we examined the
effects of manipulating JNK activity in Drosophila tumor models of increasing malignancy.

Materials and Methods

Fly Stocks. All fly culture and crosses were carried out at 25°C. For generation of eye mosaicos, the following mutant and transgenic fly strains were used: (i) eyFLP1; Act>γ+>Gal4, UAS-GFP; FRT82B Tub-Gal80 (a gift of T. Xu and T. Igaki; ref. 27); (ii) w; FRT82B UAS-Rafact (Rafact); (iii) w; FRT82B scrib1/TM6B (or scrib1); (iv) w; FRT82B scrib1 UAS-Rafact/TM6B (a gift from A. M. Brumby; ref. 26), (v) w; FRT82B; (vi) w; UAS-Hepact; FRT82B; (vii) w; UAS-Hepact; FRT82B scrib2/TM6B; (viii) w; UAS-Hepact; FRT82B scrib1 UAS-Rafact/TM6B; (ix) w; FRT82B scrib1 UAS-bskDN/TM6. scrib1 and scrib2 alleles displayed similar phenotypes as reported (26, 29).

Mosaic Analysis. Positively marked GFP clones of the desired genotype were generated in eye discs by using the MARCM system (mosaic analysis with a repressible cell marker) (30) by crossing eyFLP1; Act>γ+>Gal4, UAS-GFP; FRT82B Tub-Gal80 (27) females with males of the genotypes listed above. eyeless promoter-driven FLP recombinase (eyFLP) catalyzes mitotic recombination between FRT sites, resulting in loss of Gal80 expression in clones and subsequent derepression of Gal4-dependent transcription. In this manner, only clones in which both recombination events occur become positively marked with GFP (to distinguish them from surrounding tissue) and express other UAS transgenes.

Immunohistochemistry. Eye imaginal discs were dissected from third instar larvae in PBS and fixed with 4% paraformaldehyde in PBST (0.1% Triton X-100) for 30 min. The following antibodies were used: rabbit anti-active caspase 3 (1:100) (Cell Signaling Technology, Beverly, MA); rat anti-Elav (1:200) (Developmental Studies Hybridoma Bank at the University of Iowa). Rhodamine (TRITC)-conjugated or CY5-conjugated secondary antibodies were used: rabbit anti-active caspase 3, is prevalent (Fig. 3).

Results and Discussion

To investigate the possible contribution of JNK signaling to cell transformation in an intact tissue we used the Drosophila eye as a model. First, we induced clones of GFP-labeled scrib-deficient cells in larval eye imaginal discs by using the MARCM (mosaic analysis with a repressible cell marker) system (30) in combination with eye-specific expression of FLP recombinase (32). Consistent with previous observations (26), such scrib−/− clones form benign lesions in the adult eye. The GFP-labeled clonal areas in such eyes seem undifferentiated and disorganized (Figs. L4 and 2C). To examine the influence of JNK at such a premalignant stage of tumorigenesis, we generated scrib mutant clones in which JNK signaling was increased by the expression of an activated version of the JNK kinase, Hemipterous [Hepact, (33)]. Adult eyes of this genotype reverted to an essentially wild-type appearance and never showed the marked lesions resulting from scrib-deficient tissue (Figs. 1A and 2D). Significantly, these phenotypically rescued eyes did not contain any GFP-positive cells (Fig. 2D), suggesting that JNK activity counteracts the outgrowth of scrib−/− tissue or causes the removal of the mutant cells.

To elucidate the cellular basis for the observed phenotypes, we examined the behavior of scrib−/− as well as scrib−/−, hepadax cells in larval eye imaginal discs. Consistent with previous reports (26, 34), the scrib−/− cells in the eye imaginal disc grow into sizable clones of disorganized appearance and do not express neuronal markers, indicating their failure to differentiate (Fig. 3A). In contrast, scrib−/− clones that also express Hepact do not grow appreciably. In the small clones that can occasionally be detected, expression of apoptotic markers, such as activated caspase 3, is prevalent (Fig. 3B; for higher magnification images, see Fig. 5, which is published as supporting information on the PNAS web site). This finding indicates that JNK signaling efficiently eliminates scrib-deficient tissue by an apoptotic mechanism. Consistent with this interpretation, we find that downregulation of JNK signaling in clones of scrib mutant tissue by overexpression of the dominant negative form of Drosophila JNK, Basket (BskDNS) suppresses apoptosis and, presumably as a
consequence of that, increases the overgrowth of this tissue (Fig. 3G and ref. 26).

To assess the effect of JNK signaling on apoptosis in scrib mutant tissue quantitatively, we dissected third instar eye-antennal imaginal discs bearing clones of the relevant genotypes, separated them into cell suspensions, and analyzed them by flow cytometry. Control discs in which GFP-expressing (but otherwise wild-type) clones were induced contained ~41.2% of clonal GFP-positive cells. Very few (2%) of these normal eye disc cells underwent apoptosis as measured by the fraction of cells that display a DNA content lower than 2n, registering in the subG1 region of the FACS profile. The analysis of cells from scrib mutant clones revealed that their contribution to the eye-antennal disc was reduced to 12.4%. Consistently, the number of apoptotic cells in the clonal fraction was significantly increased (16.6%, Fig. 3H). Apoptosis of scrib mutant cells is mediated by JNK, because coexpression of BskDN in scrib mutant clones reduced the proportion of apoptotic cells (2%) and resulted in a significant growth advantage of the mutant tissue, even compared with wild-type cells. Strikingly, when JNK activity is thus suppressed, scrib mutant clones can contribute >70% of the tissue in third instar eye imaginal discs. Conversely, expression of Hepact in scrib mutant tissue reduces the fraction of GFP-labeled mutant cells to undetectable levels (Fig. 3B and H).

We conclude that loss of scribble from developing epithelia has two opposing effects: it promotes cell proliferation, but at the same time induces JNK-dependent apoptosis. Our results suggest that the balance between growth and death can be shifted by increasing or suppressing JNK signaling. When the JNK pathway is suppressed, overgrowth prevails so that scribble mutant clones persist and overgrow, ultimately causing pupal death (data not shown and ref. 26). Conversely, strong activation of JNK as delivered by Hepact expression shifts the balance toward apoptosis, and the mutant cells are eliminated.

Interestingly, eyes in which the growth of scrib mutant tissue is thus impeded by JNK activation display the same size as those of wild-type flies (Figs. 1B and 2A and D). This result suggests the existence of a corrective mechanism to maintain normal eye size even when significant fractions of the eye anlage are removed by JNK-dependent apoptosis. Similar processes have been proposed to readjust organ size after cellular injury caused by ionizing irradiation (35–37) or when cells whose growth is compromised, for example, by minute mutations are replaced by their wild-type neighbors (38). Recent studies by Ryoo et al. (39) have suggested that, in imaginal disc cells that are committed to

Fig. 2. Activation of the JNK-signaling pathway by expression of hepact in scrib mutant tissue causes elimination of mutant tissue from the adult eyes. Eyes carrying mutant clones of the indicated genotypes are shown in normal illumination (Left image of each panel) or by using epifluorescence to visualize GFP marked clonal areas (Center image). The image on the Right of each panel illustrates the growth of GFP-labeled clonal tissue of the respective genotypes during larval development. Eye imaginal discs of the scrib-/-, rafact larvae fuse with the brain and cannot be distinguished from other organs. Eighty percent of the scrib-/-, rafact animals cannot pupate and die as giant larvae. Note that the tissue of scrib-/- tumors (C) and of the Hepact-expressing tumors (E) are all labeled by GFP, whereas the overgrowth in animals in which rafact, hepact (F) or rafact, scrib-/-, hepact (H) clones had been induced is GFP-negative and thus is not clonal in origin.
apoptosis, JNK signals the release of a transient mitogenic signal to their neighbors. The ensuing growth would serve to replace the dying cells and restore the affected tissue to its original size and form.

The data described above show that JNK signaling can suppress a benign, premalignant tumor state by apoptotically removing the affected cells. Next, we asked how JNK activation might influence the behavior of more aggressive tumors. Clones of eye imaginal disc cells that express activated Ras or Raf autonomously develop into vigorously proliferating tissue during larval stages and ultimately overgrow much of the adult eye (Fig. 2E). Such Raf-induced tissue overgrowth remains restricted to the tissues derived from the eye-antennal disc and, consistent with previous findings (Pagliarini and Xu, ref. 27), is apparently not invasive. The animals survive until pharate adult stages, with rare adult escapers (Fig. 2E). If, however, the activated Raf allele is combined with a loss-of-function condition for scribble, tumors become invasive and lethal to the affected animals. When GFP-labeled Rafact, scribb−/− clones are generated in the eye disc, massive and invasive overgrowth occurs in larval stages, which kills the animal before pupation, presumably because the transformed cells functionally impair tissues other than the eye (Fig. 2G). These results confirm similar observations by Brumby and Richardson (26) and by Pagliarini and Xu (27).

We investigated whether aggressive Rafact or Rafact, scribb−/−-induced tumors might be cured by JNK signaling, as seen in the case of the benign scribb−/− lesions. In Rafact, hepx2, as well as Rafact, scribb−/−, hepx2 clones, aggressive expansion is suppressed, and clones of this genotype, although easily detectable (as opposed to scribb−/−, hepx2 clones, see Fig. 3), remain much smaller than clones in which Hepact is not coexpressed. This size reduction is likely a consequence of apoptosis induced by Hepact. Although the overgrowing clones of the Rafact, scribb−/− and Rafact clones show little or no signs of apoptosis, JNK activation in such genetic backgrounds elicits prominent caspase 3 activation (Fig. 3D and F). This conclusion is further supported by FACS analyses of the respective eye-antennal imaginal discs. These data indicate that Hepact expression can elevate the occurrence of apoptosis among Rafact (activated version of the Raf oncoprotein)-expressing cells (from 6.0% to 17.3%). However, unlike scribb mutant clones, which are virtually eliminated by Hepact expression, a significant fraction of the Rafact, hepx2 cells (6.4%) survive (Fig. 3H).

Consistent with the reduction in clonal growth, flies in which Hepact has been introduced into the tumorigenic genotypes overcome lethality and in most cases yield viable adults. Strikingly, however, the eyes of such flies display massive overgrowth. The heads are significantly bigger (Fig. 1C) and the retina of these mutants is dramatically larger than that of a wild-type eye.
In many cases, the retina is folded and bunched to accommodate the surfeit of tissue (Fig. 2 F and H). In contrast to scrib−/− lesions or tumors induced by rafact alone, these severely hyperplastic eye structures are well patterned and show a distinctive ommatidial organization (Fig. 2 F and H). Remarkably, inspection of GFP fluorescence shows that the overgrown tissue was not derived from clonal cells (compare Fig. 2 E, F, and H). Evidently, the tumorous overgrowth was induced cell non-autonomously in the phenotypically wild-type cells surrounding the clones of Rafact- and Hepact-expressing cells.

How can the extraordinary overgrowth of wild-type tissue in flies carrying clones of scrib mutant and Rafact/Hepact-expressing cells be explained? Pérez-Garijo et al. (40) have recently demonstrated a proapoptotic function of the JNK pathway in wing imaginal disc cells exposed to cytotoxic stress. Interestingly, JNK signaling not only triggers apoptosis in the stressed cells, it also causes adjacent cells to undergo compensatory proliferation. By this mechanism, neighboring cells re-store tissue size after the apoptotic removal of damaged cells. The signal for compensatory growth (in the wing disc delivered by Wingless and Dpp) is normally transient in nature, because the signal-producing cells are slated to die shortly after JNK signaling engages. If, however, apoptosis is artificially abrogated, for example by the expression of p35 or by inhibition of hid/rpr/grim expression, cells that are prevented from dying (“undead” cells) continue to induce cell proliferation in their surroundings. Such a situation can induce significant non-autonomous tissue overgrowth. Based on these reports and our findings, we propose the following mechanism to explain the observed effects of JNK activation in the Drosophila tumor progression model (Fig. 4). In wild-type or scribble mutant cells, a strong JNK signal, as it is delivered by Hepact, will initiate apoptosis. Consistently, clones in which Hepact is expressed in a wild-type or scribble−/− background do not survive larval development and do not contribute to the adult eye. In dying, the Hepact−/− expressing cells prompt adjacent wild-type cells to undergo compensatory growth, which restores the adult eye to a wild-type appearance. Thus, JNK signaling acts as a suppressor of a premalignant state. In more aggressive and invasive tumors of the rafact or rafact, scribble−/− genotypes, however, JNK signaling changes its role from an antagonist of cell transformation to a promoter of tumor growth. Hepact promotes apoptosis in the clones, which significantly reduces the expansion of primary tumors. At the same time, however, the apoptotic effect of JNK is attenuated by activated Raf, and cell death is delayed. It has been reported that Ras/Raf signaling can act anti-apoptotically in imaginal disc cells. This effect may occur by the inhibition of the death protein Hid through direct ERK-mediated phosphorylation (41). This effect of Rafact gives surviving cells more time to produce mitogenic factors. So, although Raf and Hepact antagonize each other with regard to the growth of primary tumors, they cooperate in stimulating overgrowth in a cell non-autonomous manner.

A combination of hyperactive JNK and Raf as we created it in the clones of Drosophila eye imaginal disc might conceivably occur frequently in clinically relevant settings of Ras-driven tumorigenesis. Many circumstances may activate the JNK pathway in the course of tumor progression. In addition to biological signals resulting from the neoplasia, such as cytokine secretion or hypoxia, external stimuli in the form of chemotherapeutic drugs can potentially activate the JNK pathway. It is thus important to explore potential beneficial or adverse effects of JNK in cancer. The results described here indicate that JNK can act both as an antagonist of cell transformation, or as an oncogenic factor that can cooperate with components of the Ras pathway in tumor formation. The specific role of JNK signaling with regard to tumor formation thus seems to be highly dependent on the cellular and genetic context. This multifaceted function of the JNK pathway in cell transformation has to be considered in evaluating the system as a target for therapeutic intervention.

The surprising finding that cooperation between JNK and Ras signaling causes non-autonomous tumor growth emphasizes the relevance of tissue interactions in tumorigenesis. Tumor–stroma interactions are known to make relevant contributions to the pathology of cancer, for example by inducing vascularization or inflammatory responses (23). The potentially very important role of stromal tissue in cancer pathology is obviously difficult to study in cell culture, and Drosophila seems to offer a facile and genetically versatile model system in which relevant studies can be performed.

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