Elimination of Oncogenic Neighbors by JNK-Mediated Engulfment in *Drosophila*

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SUMMARY

A newly emerged oncogenic cell in the epithelial population has to confront antitumor selective pressures in the host tissue. However, the mechanisms by which surrounding normal tissue exerts antitumor effects against oncogenically transformed cells are poorly understood. In *Drosophila* imaginal epithelia, clones of cells mutant for evolutionarily conserved tumor suppressor genes such as *scrib* or *dlg* lose their epithelial integrity and are eliminated from epithelia when surrounded by wild-type tissue. Here, we show that surrounding normal cells activate nonapoptotic JNK signaling in response to the emergence of oncogenic mutant cells. This JNK activation leads to upregulation of PVR, the *Drosophila* PDGF/VEGF receptor. Genetic and time-lapse imaging analyses reveal that PVR expression in surrounding cells activates the ELMO/Mbc-mediated phagocytic pathway, thereby eliminating oncogenic neighbors by engulfment. Our data indicate that JNK-mediated cell engulfment could be an evolutionarily conserved intrinsic tumor-suppression mechanism that eliminates premalignant cells from epithelia.

INTRODUCTION

The development and homeostasis of multicellular organisms largely depend on cell-cell communication that coordinates cell proliferation, differentiation, and cell death. An imbalance of this coordination can trigger cancer development. Most cancers arise from a single cell that acquired multiple oncogenic alterations (Fialkow, 1976; Hanahan and Weinberg, 2000; Kinzler and Vogelstein, 1996; Nowell, 1976). Therefore, in the early stages of neoplastic development, premalignant oncogenic cells emerge as clones that are surrounded by normal cells. Although cell-cell communication between oncogenic cells and surrounding normal cells can create a context that promotes tumor growth and progression (Bissell and Radisky, 2001; Hanahan and Weinberg, 2000), surrounding cells often exert antitumor effects. For instance, immune cells, including lymphocytes and natural killer cells, suppress development of epithelial tumors (Bissell and Radisky, 2001; Dunn et al., 2002; Shankaran et al., 2001; Smyth et al., 2000). In addition, embryonic fibroblasts transformed with the Ras oncogene survive poorly when surrounded by wild-type cells (Land et al., 1983). Furthermore, MDCK cells expressing oncogenic Ras (RasV12) are eliminated from a cultured epithelial monolayer when surrounded by normal MDCK cells (Hogan et al., 2009). These observations suggest that normal tissues possess intrinsic tumor-suppression mechanisms that eliminate oncogenic cells via cell-cell communication (Lowe et al., 2004). However, the molecular events at the interface between oncogenic cells and surrounding normal cells are largely unknown.

Loss of apicobasal polarity is frequently associated with epithelial cancer development (Bissell and Radisky, 2001; Fish and Molitoris, 1994). Indeed, evolutionarily conserved apicobasal polarity genes such as *scribble* (*scrib*) and *discs large* (*dlg*), two junction proteins that function together to establish cell polarity (Bilder, 2004; Tepass et al., 2001), have been shown to function as tumor suppressors. For instance the human homolog of *scrib* has been implicated in cancer development; *Scrib* protein is downregulated by proteasome-mediated degradation in tumors associated with human papillomavirus E6 infection (Massimi et al., 2004; Nakagawa et al., 2004), and loss of *Scrib* is correlated with the aggressiveness of late-stage breast and colon cancers (Gardiol et al., 2006; Navarro et al., 2005). Furthermore, depletion of *scrib* gene in mouse mammary epithelia promotes tumorigenesis (Zhan et al., 2008). Similarly, in *Drosophila* epithelia, loss-of-function mutations in *scrib* or *dlg* result in tumorous overgrowths (Bilder, 2004; Hariharan and Bilder, 2006). These *Drosophila* genes are called “neoplastic” tumor suppressors because mutant flies develop multilayered and invasive tumors in their imaginal discs. Intriguingly, in *Drosophila* imaginal epithelia, clones of neoplastic tumor-suppressor mutant cells induced within wild-type tissue do not overproliferate but, instead, are eliminated from the tissue (Agrawal et al., 1995; Brumby and Richardson, 2003; Igaki et al., 2006, 2009; Pagliarini and Xu, 2003; Woods and Bryant, 1991). This elimination of mutant cells occurs only when mutant cells are surrounded by wild-type cells because removal of surrounding wild-type tissue by inducing cell death allows mutant tissue to overgrow (Brumby and Richardson, 2003). This suggests that normal imaginal tissue exerts an antitumor effect that eliminates oncogenic polarity-deficient cells. Previous studies have shown that these neoplastic tumor-suppressor mutant clones undergo JNK-dependent cell death (Brumby and Richardson, 2003; Igaki et al., 2006, 2009; Uhlirova
et al., 2005). However, the mechanism underlying elimination of oncogenic cells by surrounding normal cells, possibly through cell-cell communication, has remained unknown. Here, we show that normal imaginal cells activate nonapoptotic JNK signaling in response to the emergence of neoplastic tumor-suppressor mutant clones. Furthermore, we show that this JNK activation in surrounding cells promotes elimination of oncogenic neighbors by activating the PVR-ELMO/Mbc-mediated engulfment pathway.

RESULTS

Imaginal Cells Activate Nonapoptotic JNK Signaling in Response to the Emergence of Neoplastic Tumor-Suppressor Mutants

In Drosophila imaginal epithelia, clones of cells mutant for neoplastic tumor-suppressor genes such as scrib or dlg grow poorly and are eliminated through JNK-dependent cell death when surrounded by wild-type tissue (Brumby and Richardson, 2003; Herz et al., 2006; Igaki, 2009; Igaki et al., 2006; Moberg et al., 2005; Pagliarini and Xu, 2003; Thompson et al., 2005; Uhlirova et al., 2005; Vaccari and Bilder, 2005). To study the mechanism by which surrounding normal tissue exerts an antitumor effect against such mutant cells, we analyzed cells juxtaposed to mutant clones. Using anti-phosphorylated JNK (p-JNK) antibodies and the puc-LacZ reporter (Martin-Blanco et al., 1998), an enhancer-trap allele that monitors JNK activation (Adachi-Yamada et al., 1999a; Agnes et al., 1999; Martin-Blanco et al., 1998), we found that JNK signaling was activated not only in mutant clones (scrib or dlg clones) but also in their surrounding wild-type cells, which was occasionally seen at locations away from mutant clones. Scale bar, 10 μm. Genotypes are as follows: yw, eyFLP1/+ or Y; Act>y+>Gal4, UAS-GFP/+; FRT82B, Tub-Gal80/FRT82B, scrib1 (A–B’); and dlgm52, FRT19A/Tub-Gal80, FRT19A; eyFLP5, Act>y+>Gal4, UAS-GFP/+; pucE69, UAS-BskDN/+ (C–D’). See also Figure S1.

Figure 1. Imaginal Cells Activate Nonapoptotic JNK Signaling in Response to the Emergence of Neoplastic Tumor-Suppressor Mutants

(A–B’) GFP-labeled scrib clones were induced in eye-antennal discs and stained with anti-phospho-JNK antibodies. (B)–(B’) are high-magnification images of the boxed area in (A)–(A’). Arrows show JNK activation in surrounding wild-type cells. Clone is outlined with a dashed line (B’). (C–D’’) GFP-labeled dlg clones expressing BskDN were induced in puc-LacZ/+ eye-antennal discs, and caspase-activated dying cells were visualized by anti-active-caspase-3 antibodies (D–D’’). Clones are outlined with dashed lines (D’’). Asterisk indicates a cell doubly positive for activated JNK and activated caspase-3 antibodies, which was occasionally seen at locations away from mutant clones. Scale bar, 10 μm. Genotypes are as follows: yw, eyFLP1/+ or Y; Act>y+>Gal4, UAS-GFP/+; FRT82B, Tub-Gal80/FRT82B, scrib1 (A–B’); and dlgm52, FRT19A/Tub-Gal80, FRT19A; eyFLP5, Act>y+>Gal4, UAS-GFP/+; pucE69, UAS-BskDN/+ (C–D’’).
of Drosophila JNK Basket (BskDN), which resulted in a line of JNK-activated wild-type cells surrounding mutant clones (Figures 1C–1C). This also indicates that neighboring wild-type cells activate JNK signaling independently of JNK activation in mutant clones. Similar pattern of JNK activation was also observed when clones of cells mutant for another neoplastic tumor-suppressor gene vps25, a component of the endosomal sorting complex required for transport–II (ESCRT-II) complex that regulates protein sorting within the endosomal pathway, were induced in the imaginal discs (Figures S1D–S1E). Activated-caspase and TUNEL staining revealed that JNK activation surrounding neoplastic tumor-suppressor mutants did not cause cell death (Figures 1D–1D). Interestingly, JNK activation in normal cells was observed specifically for cells surrounding neoplastic tumor-suppressor mutants because clones of cells mutant for other apicobasal polarity genes such as bazooka (baz) (a Par-3 homolog), stardust (sdt) (a PALS-1 homolog), or cdc42, as well as of cells mutant for cell adhesion molecules such as shotgun (shg) (an E-cadherin homolog), neither of which shows oncogenic potential (Genova et al., 2000; Müller and Wieschaus, 1996; Tepass et al., 1996; Uemura et al., 1996), did not induce JNK activation in their neighboring wild-type cells (Figures S1F–S1I).

It has recently been shown that clones of the neoplastic tumor-suppressor mutants activate JNK signaling by endocytic activation of Eiger, the Drosophila member of tumor necrosis factor (TNF) (Igaki et al., 2009). We found that nonapoptotic JNK activation in surrounding wild-type cells also depends on Eiger because removal of eiger from the entire imaginal tissue abolished JNK activation in both mutant clones and surrounding cells (Figures S2A–S2B). Because Eiger is a cell-surface TNF ligand (Igaki et al., 2002), which could potentially act in both cell-autonomous and noncell-autonomous manners (Aggarwal et al., 2006; Szlosarek et al., 2006; Wu et al., 1993), we examined the cell autonomy of Eiger function in this phenomenon. Intriguingly, clones of cells overexpressing Eiger (by a weak UAS-Eiger transgene UAS-EigerWG, which causes moderate JNK activation without affecting cell viability; Igaki et al. [2006], [2009]) (Figure S2H, quantified in Figure S2J) activated JNK signaling strictly in a cell-autonomous manner (Figures S2A–S2B). Furthermore, blocking JNK signaling within Eiger-expressing clones completely abolished JNK activation (Figures S2C–S2C′), confirming the cell autonomy of Eiger function. Together, these results indicate that normal imaginal cells activate nonapoptotic JNK signaling in response to the emergence of neoplastic tumor-suppressor mutants by cell-autonomous activation of Eiger-JNK signaling.

**JNK Activation in Surrounding Cells Promotes Elimination of Neoplastic Tumor-Suppressor Mutant Clones**

The JNK pathway is a pleiotropic-signaling cascade that regulates a variety of biological processes, including proliferation, differentiation, morphogenesis, and cell death (Chang and Karin, 2001; Davis, 2000; Igaki, 2009; Kanda and Miura, 2004; Stronach, 2005). Thus, we asked whether the nonapoptotic JNK activation in surrounding normal cells had any kind of effect on the development of tumors. Blocking JNK signaling only in surrounding normal cells by bsk-RNAi or BskDN significantly suppressed elimination of scrib mutant clones (compare Figures 2E and 2F, quantified in Figure 2H; Figure S2C, quantified in Figure S2I), whereas bsk-RNAi or BskDN expression alone did not affect tissue growth (Figures S2E and S2F, quantified in Figure S2J). Similarly, knocking down of DTRAF2 (Drosophila TNF receptor-associated factor 2), the adaptor protein that mediates Eiger-JNK signaling (Xue et al., 2007), in surrounding cells suppressed the elimination of scrib clones (Figure S2D, quantified in Figure S2I), whereas dtraf-RNAi expression alone did not affect tissue growth (Figure S2G, quantified in Figure S2J). Conversely, forced activation of JNK signaling only in surrounding cells by overexpression of Eiger (EigerWG) enhanced elimination of scrib clones (compare Figures 2E and 2G, quantified in Figure 2H). These results indicate that Eiger-JNK signaling in surrounding cells positively regulates elimination of scrib clones. Because Eiger-JNK signaling within scrib clones promotes their elimination by inducing cell death (Igaki et al., 2009), we examined the relative contribution of Eiger-JNK activations in scrib mutant cells and their surrounding wild-type cells. Analysis of phenotypic severity revealed that knock down of eiger only in scrib mutant clones resulted in 30.7% pupal lethality, whereas knock down of eiger in both mutant and surrounding wild-type clones resulted in 51.3% pupal lethality (Figure 2I). These results suggest that cell-autonomous Eiger-JNK activations in both mutant and surrounding wild-type cells cooperate to give rise to the full activation of the cell-elimination machinery.

The enhancement of cell elimination by neighboring JNK activation was strikingly evident when endogenous eiger was removed from the entire imaginal tissue. In eiger mutant background, scrib clones are no longer eliminated but grow aggressively and develop into tumors due to loss of JNK activation (Igaki et al., 2009) (Figure 3C, quantified in Figure 3F). In this background, overexpression of Eiger-WG only in surrounding cells reversed the phenotype; scrib clones did not overgrow but were outcompeted by surrounding Eiger-expressing cells (compare Figures 3C and 3D, quantified in Figure 3F). Similar to the results obtained in scrib mosaic tissue, clones of vps25 neoplastic tumor-suppressor mutants were also eliminated from the tissue in a manner dependent on Eiger expression in surrounding cells. vps25 clones generated in eye-antennal discs grew poorly, as reported previously (Herz et al., 2006; Thompson et al., 2005; Vaccari and Bilder, 2005) (compare Figures S2K and S2M). We found that removal of eiger gene only in surrounding tissue allowed vps25 clones to overgrow, whereas loss of eiger alone did not confer any disadvantage in tissue growth (Figures S2K–S2N). Together, these results reveal that Eiger-JNK signaling in surrounding normal cells promotes elimination of neoplastic tumor-suppressor mutants. It is important to note that this activity is not directly related to classical cell competition because the elimination of Minute/+ clones is not affected by loss of eiger (Figures S2O–S2Q).

**PVR Acts Downstream of JNK Signaling in Eliminating Premalignant Neighbors**

We next sought to identify downstream components of JNK signaling that mediate the antitumor effect against neoplastic tumor-suppressor mutants. JNK signaling has been implicated in epithelial morphogenetic processes that involve reorganization of the actin cytoskeleton, including cell migration, invasion,
Figure 2. Cell Autonomous Activation of Eiger-JNK Signaling in Surrounding Cells Promotes Elimination of Premalignant Mutant Cells

(A–C) GFP-labeled mitotic clones expressing Eiger<sup>+</sup> (A–B<sup>0</sup>) or Bsk<sup>DN</sup>+Eiger<sup>+</sup> (C–C<sup>0</sup>) were induced in wild-type (A–A<sup>0</sup>) or puc-LacZ/+ (B–C<sup>0</sup>) eye-antennal discs. JNK activation was visualized by anti-phospho-JNK (A–A<sup>0</sup>) or anti-b-galactosidase (B–C<sup>0</sup>) antibodies. Scale bars, 10 μm. (D–G) Wild-type or scrib clones marked by the absence of GFP were induced in wild-type background eye-antennal discs, and bsk-inverted repeat (RNAi) (F) or Eiger<sup>+</sup> (G) was expressed in GFP-labeled surrounding tissue. Manipulation of gene expression only in surrounding tissue was achieved by the Gal4/UAS expression system with the Gal4...
and cell shape changes (Davis, 2000; Xia and Karin, 2004). Therefore, we performed a candidate screen for downstream targets of JNK signaling by searching molecules regulating the cell cycle, the actin cytoskeleton and found that PVR, the Drosophila ortholog of PDGF/VEGF receptor (Cho et al., 2002; Duchek et al., 2001), was upregulated. PVR expression was upregulated in both mutant clones and surrounding wild-type cells (Figures 3G–3H, arrows and arrowheads, respectively), which correlated with the pattern of JNK activation (Figures S3A–S3A”). In addition, PVR was upregulated in clones of cells overexpressing Eiger”W in a cell-autonomous manner (Figures 3I–3I”). This upregulation of PVR was completely canceled by blocking JNK signaling within the Eiger-expressing clones (Figures 3J–3J”).

We found three AP1 consensus sequences (TGAG/CTCA) at the upstream region of the pvr gene within 10 kb from the coding region (data not shown). Consistent with these results, we found that pvr expression was upregulated by Eiger signaling at the transcriptional level in a JNK-dependent manner (Figure S3B). All of these data indicate that PVR is a downstream target of the JNK pathway.

To examine the contribution of surrounding PVR expression to elimination of mutant clones, we blocked PVR expression only in surrounding normal cells. Downregulation of PVR expression only in surrounding cells significantly suppressed elimination of scrib clones to an extent similar to blocking JNK signaling (Figure 3A, quantified in Figure 3F), whereas pvr-RNAi expression alone did not affect tissue growth (Figure S3E, quantified in Figure S3I). In addition, knocking down of PVR in Eiger”W-expressing clones generated in eiger mutant background cancelled their ability to outcompete scrib clones (compare Figures 3D and 3E, quantified in Figure 3F), placing PVR downstream of JNK signaling in this phenomenon. Furthermore, ectopic expression of PVR in surrounding cells markedly promoted elimination of scrib clones (Figure 3B, quantified in Figure 3F), which also reversed the elimination-defective phenotype caused by loss of eiger (Figure S3G, quantified in Figure S3J), whereas overexpression of PVR alone in a wild-type tissue context conferred no obvious growth advantage (Figure S3F, quantified in Figure S3I). On the other hand, knocking down of PVR within scrib mutant clones had no effect on their elimination (compare Figures S3C and S3D, quantified in Figure S3H). Together, these results indicate that JNK activation in surrounding cells promotes elimination of neoplastic tumor-suppressor mutants through PVR.

**Premalignant Mutant Cells Are Killed by Neighboring Normal Cells by Engulfment**

To understand the mechanism by which JNK-PVR signaling in premalignant mutant cells, we analyzed the morphology and the spatial pattern of cell elimination in scrib mosaic tissue. Cell death occurring within scrib clones was visualized by a genetically encoded activated-caspase reporter DB:::PARP::Venus (Williams et al., 2006) in combination with antibodies against cleaved PARP (poly-ADP-ribose polymerase-1), a peptide that serves as a substrate for Drosophila effector caspsases. Using this system, we found that dying cells in scrib clones were largely restricted to boundaries between scrib and wild-type populations (87.2% of dying cells, n = 675) (Figures 4A–4C”). We also noticed that most of these dying cells were being detached from their clones and incorporated into neighboring wild-type population (Figures 4C–4C”, arrows and arrowheads). To directly analyze the dynamics of cell elimination, we established a live-imaging system of organ-cultured imaginal discs. Using this system, we found that a significant number of scrib cells were fragmented after they were incorporated into neighboring wild-type population (Figure 4D; Figure S4 and Movies S1 and S2), suggesting that scrib cells are killed through engulfment by surrounding cells. Intriguingly, an analogous pattern of cell death in imaginal epithelia has been reported during Minute/+ cell competition, the phenomenon whereby faster-dividing wild-type cells kill slower-dividing Minute/+ neighbors (Li and Baker, 2007). Indeed, we found that scrib cells exhibited a morphology similar to Minute/+ cells that are surrounded by wild-type cells because double labeling of scrib and wild-type clones showed that scrib cells were being completely internalized into neighboring wild-type population (Figures 4E–4E”, enlarged in Figures 4F–4G”). Furthermore, these internalized scrib cells were stained with LysoTracker, a phagosome maturation marker that labels the completion of the phagocytosis process (Awasaki and Ito, 2004; Kurant et al., 2008). These observations suggest that scrib cells are killed by surrounding wild-type cells by engulfment.

Therefore, we asked whether JNK-PVR-mediated elimination of premalignant neighboring clones was indeed due to increased engulfment of these clones. scrib cells at the boundaries between scrib and wild-type populations were frequently engulfed by surrounding cells, as visualized by LysoTracker staining (8.0% LysoTracker-positive cells/boundary) (compare Figures 5A–5A” and 5B–5B”, quantified in Figure 5G). The number of LysoTracker-positive scrib cells was markedly increased when Eiger”W was expressed in surrounding cells (15.0% cells/boundary) (Figures 5C–5C”, quantified in Figure 5G). Similarly, overexpression of PVR in cells surrounding scrib clones increased the number of LysoTracker-positive scrib cells (13.4% cells/boundary) (Figures 5D–5D”, quantified in Figure 5G). In contrast the number of LysoTracker-positive scrib cells was significantly reduced when scrib clones were

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Additional experiments and data are available in Supplemental Data. See also Figure S2.
Figure 3. JNK-PVR Signaling in Surrounding Cells Promotes Elimination of Premalignant Mutant Clones

(A–E) scrib clones marked by the absence of GFP were induced in wild-type (A and B) or eiger mutant (C–E) background eye-antennal disc, and pvr-RNAi (A), PVR (B), Eiger+W (D), or Eiger+W+ pvr-RNAi (E) was expressed in GFP-labeled surrounding tissue as described in Figure 2. (F) Quantification of relative size of clones in surrounding tissue.
generated in eiger mutant discs (0.3% cells/boundary) (Figures 5E–5E′, quantified in Figure 5G). In this background, reintroduction of EigerW only in surrounding cells significantly increased LysoTracker-positive scrib cells (22.6% cells/boundary) (Figures 5F–5F′, quantified in Figure 5G). On the other hand, overexpression of neither EigerW alone nor PVR alone increased LysoTracker-positive cells at the interface between wild-type clones (Figure S5), indicating that Eiger-JNK-PVR signaling is not sufficient for exhibiting the antitumor response and might require additional signals from the neoplastic clones. Together, these results indicate that activation of JNK-PVR signaling in surrounding cells leads to enhanced phagocytic activity, which promotes elimination of neighboring premalignant mutant clones by actively killing these cells through engulfment. Consistent with this conclusion, we found that surrounding normal cells that were activating JNK signaling frequently locate adjacent to LysoTracker-positive mutant cells (71.8% of total LysoTracker-positive cells, n = 103) (Figure 6; Figures S6A–S6C). Given that most of LysoTracker-positive scrib cells were within wild-type cells (Figures 4E–4G), these results indicate that the LysoTracker-positive mutant bodies lie within JNK-activating wild-type cells. Indeed, our analysis revealed that 46.8% of JNK-activating wild-type cells were engulfing neighboring mutant cells (Figure S6D).

**JNK-PVR Signaling in Surrounding Cells Promotes Elimination of Premalignant Neighbors by ELMO/Mbc-Mediated Engulfment**

One of the essential cellular events that drives cell engulfment during phagocytosis is cytoskeletal rearrangement mediated by the signaling pathway involving ELMO (engulfment and cell motility, a Ced-12 homolog) and Mbc (myoblast city, a Ced-5/DOCK180 homolog) (Brugnera et al., 2002; Nolan et al., 1998), both of which act together to form a guanine-nucleotide exchange factor (GEF) complex for Rac GTPase. Therefore, we examined whether these molecules are involved in elimination of neoplastic tumor-suppressor mutants from imaginal epithelia. Knocking down of ELMO only in surrounding wild-type cells significantly suppressed elimination of scrib clones (Figure 7A, quantified in Figure 7D), whereas elmoRNAi expression alone did not affect tissue growth (Figure S7D, quantified in Figure S7H). In addition, loss of mbc function only in surrounding cells suppressed the elimination (Figure S7A, quantified in Figure S7F), whereas loss of mbc alone did not affect growth (Figure S7E, quantified in Figure S7H). Furthermore, loss of mbc or knock down of elmo in surrounding cells that were expressing EigerW strongly reduced the ability of these cells to engulf scrib cells (from 15.0% to 1.4% or 2.3% LysoTracker-positive cells/boundary, respectively; Figures S7I–S7J′, quantified in Figure S7K). These results indicate that ELMO/Mbc-mediated cell engulfment is required for the elimination of scrib clones.

From the results presented so far, we postulated that downstream events of JNK-PVR signaling in surrounding wild-type cells could be activation of the ELMO/Mbc-mediated engulfment pathway. Interestingly, it has been reported that ELMO and Mbc act downstream of PVR in Dro sophila egg chambers during both F-actin accumulation in follicle cells and the early phase of border-cell migration (Bianco et al., 2007). Furthermore, genetic and biochemical analyses have shown that ELMO and Mbc function downstream of PVR signaling during thorax closure (Ishimaru et al., 2004), a morphogenetic process that involves JNK-mediated control of cytoskeleton dynamics (Martin-Banco et al., 2000). We found that reduction in elmo function in EigerW- or PVR-expressing clones cancelled their ability to outcompete scrib clones (Figures 7B and 7C, quantified in Figure 7D), suggesting that ELMO also functions downstream of PVR in eliminating neoplastic tumor-suppressor mutants. Similarly, loss of mbc function in EigerW- or PVR-expressing clones cancelled their ability to outcompete scrib clones (Figures 7B and S7C, quantified in Figure S7G). Together, these results indicate that activation of JNK-PVR signaling in surrounding cells promotes elimination of premalignant neighbors through ELMO/Mbc-mediated engulfment (Figure 7E).

**DISCUSSION**

Loss of epithelial integrity, particularly apicobasal polarity, is often associated with tumor development and malignancy (Bissell and Radisky, 2001; Fish and Moi toris, 1994). To counteract this, normal epithelial tissue seems to exert antitumor effects against such oncogenic cells. Here, we report a series of observations indicating that normal imaginal epithelial cells exert an antitumor effect against premalignant mutant cells through activation of the JNK-mediated engulfment pathway.

**An Intrinsic Tumor Suppression that Eliminates Highly Malignant Cells**

Our data show that normal imaginal cells exert a JNK-mediated antitumor effect against “neoplastic” tumor-suppressor mutants such as scrib, dig, and vps25 cells. Intriguingly, this intrinsic tumor suppression seems to be specifically effective against neoplastic tumor-suppressor mutants because clones of cells mutant for “hyperplastic” tumor suppressors, such as the Hippo pathway (hippo, salvador, mats, and warts/lats), PTEN, and Tsc1/Tsc2 genes, grow over and develop into tumors even in the presence of surrounding wild-type tissue (Hariharan and each genotype shown in (A)–(E)). Relative size of scrib–/– clones was determined as described in Figure 2, scrib clones induced in wild-type background (Figure 2E) is shown as a control. Six to eight imaginal discs were examined using ImageJ (Student’s t test, *p < 0.01), (D–H) GFP-labeled scrib clones were induced in eye-antennal disc and stained with anti-PVR antibodies. (H–I′) are high-magnification images of the boxed area in (G)–(G′). Arrowheads indicate PVR expression in surrounding wild-type cells. Arrows indicate PVR expression within a scrib clone. (I–J) GFP-labeled clones expressing EigerW (I–I′) or BskON+ElmoW (J–J′) were induced in eye-antennal discs and were stained with anti-PVR antibodies. A weak signal of endogenous PVR expression was observed in the region posterior to the morphogenetic furrow. Genotypes are as follows: UAS-pvrRNAi+/+ or Y; eyFLP5, Act>y-Gal4, UAS-GFP++; FRT82B/FRT82B, Tub-Gal80, scrib1 (A); eyFLP5, Act>y-Gal4, UAS-GFP/UAS-pvr; FRT82B/FRT82B, Tub-Gal80, scrib1 (B); eyFLP5, Act>y-Gal4, UAS-GFP, egr1, UAS-EigerW, egr1; FRT82B/FRT82B, Tub-Gal80, scrib1 (D); eyFLP5, Act>y-Gal4, UAS-GFP, egr1, UAS-EigerW; FRT82B/FRT82B, Tub-Gal80, scrib1 (E); eyFLP5, Act>y-Gal4, UAS-GFP++; FRT82B/FRT82B, Tub-Gal80, scrib1 (F); eyFLP5, Act>y-Gal4, UAS-GFP/UAS-pvr; FRT82B/FRT82B, Tub-Gal80, scrib1 (G–H′); eyFLP5, Act>y-Gal4, UAS-GFP/UAS-EigerW; FRT82B/FRT82B, Tub-Gal80 (I–I′); and UAS-bskD; eyFLP5, Act>y-Gal4, UAS-GFP/UAS-EigerW; FRT82B/FRT82B, Tub-Gal80 (J–J′). See also Figure S3.
Figure 4. Imaginal Cells Engulf Premalignant Neighbors

(A–C”) The activated-caspase-3 indicator CD8-PARP-Venus was expressed within scrib clones, and dying cells were visualized by anti-cleaved PARP antibodies. The nuclei were stained with DAPI. Arrows in (C)–(C”) indicate dying scrib cells at the boundaries between scrib and wild-type populations.
Bilder, 2006). These hyperplastic tumors consist of overproliferating imaginal cells that are normally shaped and maintain the characteristics of an epithelial monolayer, ultimately differentiating into adult tissues. This contrasts with the characteristics of “neoplastic” tumors composed entirely of neoplastic tumor-suppressor mutant cells; neoplastic mutant tissues become rounded and multilayered, lose the ability to differentiate, and exhibit signs of invasive and metastatic behaviors, resulting in host lethality (Hanharan and Bilder, 2006). Thus, fly “neoplastic” tumors show a striking overlap with the physiological changes seen in human malignant epithelial tumors (Hanharan and Weinberg, 2000). Thus, our findings suggest that JNK-mediated epithelial tumor suppression may have evolved to specifically eliminate highly malignant neoplastic cells from the tissue. Consistent with this notion, we found that JNK signaling was activated in neither the Hippo pathway mutant clones nor their surrounding wild-type cells (data not shown). Furthermore, forced activation of JNK signaling in surrounding normal cells did not eliminate Hippppo pathway mutant clones (data not shown).

Given that scrib mutant cells have been shown to be eliminated through cell competition (Brumby and Richardson, 2003; Rhiner et al., 2010) and that Minute/+ cells have been shown to be killed by neighboring wild-type cells through mbc-mediated engulfment during cell competition (Li and Baker, 2007), an important question is whether or not Eiger-JNK signaling also drives cell competition between wild-type and Minute/+ cells. Intriguingly, we found that Minute/+ cells are still eliminated by cell competition in eiger-000 background (Figures S2O–S2Q), indicating that the signaling pathway in surrounding cells activated against oncogenic neighbors is different from the one observed in classical cell competition. Future studies on the mechanism by which surrounding wild-type cells “sense” oncogenic neighbors would address the molecular basis of this difference. Interestingly, we found that wild-type cells neighboring neoplastic mutant cells elevate endocytosis (data not shown), which could trigger the endocytic activation of Eiger-JNK signaling in imaginal cells (Igaki et al., 2009). Thus, the early events that occur in imaginal cells in response to the emergence of oncogenic neighbors include the elevation of endocytosis that triggers autonomous Eiger-JNK signal activation.

Tumor Suppression by a Context-Dependent Switch of JNK-Signaling Outputs

In this and our previous studies, we have found that JNK signaling facilitates elimination of neoplastic tumor-suppressor mutant clones through its activity in both mutant clones and surrounding wild-type cells (Figure 7E). JNK induces apoptosis in mutant clones, whereas in surrounding wild-type cells JNK mediates the engulfment of neighboring mutant cells. This suggests that a context-dependent “switch” of JNK-signaling outputs drives efficient elimination of dangerous cells from epithelia. A possible mechanism by which different JNK-signaling outcomes are produced is the context-dependent sensitization of cells to JNK-dependent cell death. It has been shown that moderate activation of JNK signaling by EigerW in normal imaginal cells causes no cell death, whereas combination of EigerW and loss of cell polarity synergistically induces cell death (Igaki et al., 2006). Furthermore, downregulation of E-cadherin shg, which could be caused by loss of scrib (Pagliarini and Xu, 2003), also cooperates with EigerW-induced moderate JNK activation to strongly induce cell death (Igaki et al., 2006). Thus, the proper establishment of apicobasal polarity or cell-cell junctions may prevent JNK-mediated epithelial cell death, whereas neoplastic tumor-suppressor mutant cells, which lose normal epithelial organization, may be sensitized to JNK-dependent cell death signaling. It would be interesting to investigate whether this context-dependent switch of JNK-signaling outcomes is also involved in the phenomenon that Ras signaling turns JNK’s apoptotic role into a tumor-promoting program (Corredo et al., 2010; Igaki et al., 2006).

It has recently been shown that scrib clones cause their neighboring Ras-activating clones to exhibit metastatic behavior possibly through a propagation of JNK activation from scrib cells to Ras-activating cells (Wu et al., 2010). Although our data indicate that JNK activity is not propagated from scrib cells when these mutant cells are surrounded by wild-type tissue because surrounding cells still activate JNK signaling when JNK is blocked within scrib clones (Figures 1C–1C), our observations raised an interesting possibility that the PVR-ELMO/Mbc pathway, which is involved in cytoskeletal rearrangement, could contribute to the invasive behavior of Ras-activating cells neighboring scrib clones. Indeed, we found that blocking pvr, elmo, or mbc function in neighboring Ras-activating cells attenuated metastatic behavior of these tumors (data not shown), suggesting the context-dependent role of the PVR-ELMO/Mbc pathway during the process of intercellular oncogenic cooperation between neoplastic and Ras-activating cells.

Tumor Suppression by Cell Engulfment

Several lines of evidence have indicated that engulfment is required not only for clearance of dead cells but also for active cell killing. For instance, in C. elegans mutants homozygous for hypomorphic allele of the caspase gene ced-3, cell death is further reduced by loss of the engulfment genes (Hoeppner et al., 2001; Reddien et al., 2001). In addition, Drosophila imaginal cells have been shown to kill neighboring Minute/+ cells by engulfment (Li and Baker, 2007). Furthermore, it has been shown in mammalian epithelial cell culture systems that
a nonapoptotic cell death process, called entosis, works by cell-in-cell invasion and subsequent lysosomal degradation of live internalized cell (Overholtzer et al., 2007). Interestingly, evidence of entosis can be seen in many types of human tumors (Overholtzer et al., 2007), suggesting that entosis could function as an intrinsic tumor-suppression mechanism. Indeed, the presence of a tumor-suppressive cell engulfment has been previously postulated based on observations of cell-in-cell phagocytic processes called “cell cannibalism” in lung tumor cells (Brouwer et al., 1984). In this study we found that normal epithelial cells actively engulf oncogenic neighbors to eliminate them from imaginal tissue. Given that components of the Drosophila antitumor cell engulfment pathway (such as Eiger, JNK pathway components, PVR, ELMO, and Mbc) are conserved from flies to humans, it is possible that this machinery is an evolutionarily conserved intrinsic tumor-suppression mechanism installed in epithelium.

EXPERIMENTAL PROCEDURES

Fly Strains and Generation of Clones

Fluorescently labeled mitotic clones (Lee and Luo, 1999; Xu and Rubin, 1993) were produced in larval imaginal discs using the following strains: y,w, eyFLP1; Act>y+>Gal4, UAS-GFP; FRT82B, Tub-Gal80 (82B tester-1), eyFLP5, Act>y+>Gal4, UAS-GFP; FRT82B, Tub-Gal80 (82B tester-2), y,w, eyFLP1; G454, Act>y+>Gal4, UAS-mmRFP; FRT82B, Tub-Gal80 (82B RFP-tester), y,w, eyFLP1; Tub-Gal80, FRT43D; Act>y+>Gal4, UAS-BSKDN; Act>y+>Gal4, UAS-mmRFP; Act>y+>Gal4, UAS-PVR; Act>y+>Gal4, UAS-PVRDN; Act>y+>Gal4, UAS-PVR-RNAi (Rosin et al., 2004); UAS-elmo-RNAi (Ishimaru et al., 2004); and UAS-straf2-RNAi (Xue et al., 2007).

Quantification of Relative Size of Clones

Size of clones relative to the whole area of wild-type eye-antennal disc was examined using ImageJ. Mitotic clones were generated in eye-antennal discs using the MARCM system (with the Gal4/UAS expression system, its inhibitory protein Gal80, and the UAS-GFP construct; Lee and Luo [1999]), which allows us to visualize GFP-positive homozygous (TG80−/TG80−) clones as well as...
GFP-negative homozygous and heterozygous (TG80+/TG80+ and TG80+/TG80−/C0) mixed population. Therefore, the relative size of GFP-negative homozygous (TG80+/TG80+) clones was calculated by subtracting the size of heterozygous (TG80+/TG80−/C0) clones from the total GFP-negative area. The relative size of heterozygous (TG80+/TG80−/C0) clones (22.4%) was determined by generating wild-type clones using FRT82B and eyFLP5; it produced 38.8% ± 3.5% GFP-positive homozygous (TG80−/C0/TG80−/C0) clones in eye-antennal disc, together with putative 38.8% homozygous (TG80+/TG80+) and 22.4% heterozygous (TG80+/TG80−) clones. The negative number for the relative size of GFP-negative homozygous (TG80+/TG80+) clones indicates that GFP-positive homozygous (TG80−/C0/TG80−/C0) clones have outcompeted both GFP-negative homozygous (TG80+/TG80+) and heterozygous (TG80+/TG80−) clones. Data were collected as mean ± SE (%).

Histology

Larval tissues were stained with standard immunohistochemical procedures using rabbit anti-Eiger polyclonal antibody R1 (1:250–500) (Igaki et al., 2009), mouse anti-phospho-JNK monoclonal antibody G9 (Cell Signaling; 1:100), rat anti-PVR antibody (1:250) (Rosin et al., 2004), mouse anti-β-galactosidase antibody (Sigma; 1:500), rabbit anti-cleaved PARP antibody (Cell Signaling; 1:200), rabbit anti-GFP antibody (Molecular Probes; 1:200), and rat anti-GFP antibody (Nacalai Tesque; 1:1000), and were mounted with DAPI-containing SlowFade Gold Antifade Reagent (Molecular Probes). For LysoTracker staining, larvae were dissected in Schneider’s media and were incubated in the same media with 2.5 μM LysoTracker Red DND-99 (Molecular Probes) for 20 min. Although LysoTracker could label both phagosomes and autophagosomes, these can be distinguished by the size of LysoTracker-positive spots; we focused on analyzing the case that the whole cell is LysoTracker positive, which exhibits the cell as being engulfed by a neighboring cell. Images were taken with a Zeiss LSM510 META confocal microscope. Data were collected as mean ± SE (%).

Time-Lapse Imaging

A dissected eye-antennal disc was put on a plastic dish and cultured in Schneider’s Drosophila medium, which allowed us to analyze the dynamics of cell elimination in proliferating imaginal epithelium for more than 3 hr. Images were acquired at 5-min intervals for up to 5 hr on an upright confocal microscope (FV1000; Olympus) equipped with an Olympus 60×NA1.1 LUMFL water-immersion objective. Image processing was done with ImageJ.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two movies and can be found with this article online at doi:10.1016/j.devcel.2011.02.007.

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Figure 7. JNK-PVR-ELMO/Mbc Signaling in Normal Cells Promotes Elimination of Premalignant Neighbors

(A–C) scrib clones (GFP negative) were induced in eye-antennal discs with surrounding elmo-RNAi (A), Eiger\(^{\text{W}}\)+elmo-RNAi (B), or PVR+elmo-RNAi (C)-expressing tissue (GFP positive). (D) Quantification of relative size of clones in each genotype shown in (A)–(C). Relative size of scrib\(^{-/-}\) clones was determined as described in Figure 2. Six to eight imaginal discs were examined using ImageJ (Student’s t test, \(p < 0.01\)). (E) A model for intrinsic tumor suppression caused by dual functions of JNK signaling in Drosophila imaginal epithelia. JNK promotes cell death in neoplastic tumor-suppressor mutant clones, whereas in surrounding cells JNK promotes elimination of neighboring mutant cells through the PVR-ELMO/Mbc engulfment pathway. Whether these two JNK-activation processes are linked in a single pathway or act in parallel pathways, to our knowledge, is currently unknown. Genotypes are as follows: eyFLP5, Act>y+>Gal4, UAS-GFP/+; FRT82B, Tub-Gal80, scrib\(^{1}\)/FRT82B, UAS-elmo-RNAi (A); eyFLP5, Act>y+>Gal4, UAS-GFP/UAS-Eiger\(^{\text{W}}\); FRT82B, Tub-Gal80, scrib\(^{1}\)/FRT82B, UAS-elmo-RNAi (B); and eyFLP5, Act>y+>Gal4, UAS-GFP/UAS-PVR; FRT82B, Tub-Gal80, scrib\(^{1}\)/FRT82B, UAS-elmo-RNAi (C).
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