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Tumor suppression by cell competition through regulation of the Hippo pathway

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Homeostatic mechanisms can eliminate abnormal cells to prevent diseases such as cancer. However, the underlying mechanisms of this surveillance are poorly understood. Here we investigated how clones of cells mutant for the neoplastic tumor suppressor gene *scribble* (*scrib*) are eliminated from *Drosophila* imaginal discs. When all cells in imaginal discs are mutant for *scrib,* they hyperactivate the Hippo pathway effector Yorkie (Yki), which drives growth of the discs into large neoplastic masses. Strikingly, when discs also contain normal cells, the *scrib*$^-$ cells do not overproliferate and eventually undergo apoptosis through JNK-dependent mechanisms. However, induction of apoptosis does not explain how *scrib*$^-$ cells are prevented from overproliferating. We report that cell competition between *scrib*$^-$ and wild-type cells prevents hyperproliferation by suppressing Yki activity in *scrib*$^-$ cells. Suppressing Yki activation is critical for *scrib*$^-$ clone elimination by cell competition, and experimental elevation of Yki activity in *scrib*$^-$ cells is sufficient to fuel their neoplastic growth. Thus, cell competition acts as a tumor-suppressing mechanism by regulating the Hippo pathway in *scrib*$^-$ cells.

Animals have evolved homeostatic mechanisms to eliminate abnormal and cancerous cells, protecting the animal from harm (1). A prominent example of an organism removing abnormal cells that have the potential to form tumors is the elimination of *scribble* mutant (*scrib*$^-$) cells from *Drosophila* imaginal discs (2–8). *scrib* is a conserved tumor-suppressor gene that is essential for the establishment of apical–basal cell polarity (8–10). Scrib is a scaffold protein that localizes to basolateral cell junctions and functions together with the Discs large (Dlg) and Lethal giant larvae (Lgl) adaptor proteins to govern apical–basal cell polarity in epithelial cells (8, 10). Imaginal discs from *Drosophila* larvae that are homozygous mutant for *scrib*, *dlg*, or *lgl* grow into large tumorous masses of neoplastic cells that display several hallmarks of carcinomas: They lose apical–basal cell polarity, hyperproliferate, and have defects in differentiation (10). Interestingly, the neoplastic phenotype of *scrib*$^-$ cells depends on their cellular environment. When *scrib*$^-$ cells are produced in patches (clones) of mutant cells that are surrounded by normal cells, they do not hyperproliferate, remain small, and eventually are eliminated (2–7, 11–13). Similar effects are observed for *lgl*$^-$ and *dlg*$^-$ clones, although they may not be eliminated very efficiently (11, 14, 15). Thus, the presence of wild-type cells prevents *scrib*$^-$, *lgl*$^-$, and *dlg*$^-$ cells from manifesting their tumorigenic potential (2–7, 11–15). Several groups have shown that the JNK stress–response pathway is activated in *scrib*$^-$ clones, leading to engulfment and death or extrusion of mutant cells from the epithelium (2–4, 6, 11, 16). Activation of JNK is required for the elimination of *scrib*$^-$ cells because blocking JNK activity in *scrib*$^-$ cells results in massive overgrowth of clones that is reminiscent of the tumorous overgrowth of entirely mutant discs (2–4, 6, 12, 13). However, blocking apoptosis does not cause overproliferation of *scrib*$^-$ clones (2, 3). Therefore, in addition to inducing apoptosis, JNK suppresses the potential of *scrib*$^-$ cells to hyperproliferate (2, 3). However, how *scrib*$^-$ cells are prevented from hyperproliferating is not known.

The presence of normal cells is required for the elimination of tumorigenic *scrib*$^-$ clones because genetically ablating the normal tissue surrounding *scrib*$^-$ cells results in hyperproliferation of the *scrib*$^-$ cells (2, 3). It has been suggested that cell competition, a process by which viable cells of lower fitness are removed from a tissue and replaced through extra proliferation of fitter neighbors (17), is responsible for the elimination of *scrib*$^-$ and *lgl*$^-$ cell clones (2, 14). However, the hypothesis that *scrib*$^-$ and *lgl*$^-$ clones are eliminated by cell competition is in conflict with other reports and thus is controversial.

It has been reported that cells with compromised Scrib or Lgl function exhibit elevated activity of Yorkie (Yki), a transcriptional coactivator and downstream effector of the Hippo growth-control pathway (13, 14, 18–20). The Hippo pathway is a conserved tumor-suppressor pathway that suppresses growth by antagonizing the activity of Yki (21). Thus, loss of Hippo pathway activity or elevated levels of Yki activity result in hyperproliferation of imaginal disc cells and resistance to apoptosis that normally would eliminate extra cells (21). Notably, an increase in Yki activity can rescue weak cells, such as cells heterozygous for *Minute* (*M*) mutations, from being eliminated by cell competition (22). *M* mutations occur in ribosomal protein-encoding genes and were the first class of genes identified as having cell-competition phenotypes (23). Homozygous *M* mutations are lethal, but heterozygous *M* animals are viable, although their cells have reduced growth rates (23). In genetic mosaics, however, interaction between wild-type and $M^{+/-}$ cells leads to the elimination of the $M^{+/-}$ cells and expansion of the wild-type population, a phenomenon termed "cell competition" (17). Thus, $M^{+/-}$ cells are less competitive than wild-type cells. Importantly, elevated levels of Yki can rescue $M^{+/-}$ cells from being eliminated by cell competition and also can transform normal cells into supercompetitors that induce apoptosis in their neighbors and proliferate at their neighbors' expense (22, 24, 25). Yki may increase the competitiveness of cells by inducing the expression of Myc, a known regulator of cell competition (24–27). However, the reports that *scrib*$^-$ cells have high levels of Yki activity and the hypothesis that *scrib*$^-$ cells are eliminated by cell competition present a paradox. If *scrib*$^-$ cells indeed have elevated levels of Yki activity, why does that elevated Yki activity not protect *scrib*$^-$ cells from cell competition?

Here we investigated this paradox further. We show that *scrib*$^-$ cells are indeed eliminated by cell competition. We found that for this elimination to occur, *scrib*$^-$ cells undergo a JNK-dependent suppression of Yki activity; this suppression of Yki activity

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prevents *scrib*$^{-}$ cells from hyperproliferating and enables their removal. The modulation of Yki activity in *scrib*$^{-}$ cells thus is a critical effect of the JNK-dependent cell-competition process that removes such tumorigenic cells from imaginal discs. Finally we show that the Myc and Ras oncogenes, which can rescue *scrib*$^{-}$ clones from elimination (2, 4, 15), do so by conferring competitive fitness to *scrib*$^{-}$ cells and thereby prevent the down-regulation of Yki activity in *scrib*$^{-}$ cells. Our results thus further characterize the effects of cell-competition pathways in removing tumorigenic *scrib*$^{-}$ cells from imaginal discs.

Results

Normal Cells Inhibit the Proliferation of *scrib*$^{-}$ Clones. *scrib*$^{-}$ clones activate JNK signaling and induce JNK-dependent apoptosis (2–4, 6). However, the induction of apoptosis is not sufficient to explain how *scrib*$^{-}$ clones are eliminated, because blocking apoptosis by coexpression of the caspase inhibitor p35 does not rescue the small clone size of *scrib*$^{-}$ clones to the size observed when JNK activity is inhibited (2, 3). To confirm that apoptosis is not sufficient for the removal of *scrib*$^{-}$ clones, we generated large and consistent numbers of GFP-marked *scrib*$^{-}$ cell clones by combining an eye-specific source of Flippase (*ey-Flp*) using the mosaic analysis with a repressible cell marker (MARCM) system (28) and examined the contribution of these mutant cells to third-instar eye discs as a measure of their proliferation and survival. Corroborating previous observations, *scrib*$^{-}$ clones comprised only a small fraction of eye discs compared with wild-type control clones (Fig. 1 *A* and *B*) (2–5, 7), as did *scrib*$^{-}$ clones that coexpressed p35 or the antiapoptotic gene *Drosophila* inhibitor of apoptosis 1 (*Diap1*) (Fig. 1*C* and Fig. S1 *A*–*C*) (3). Blocking apoptosis thus is not sufficient to rescue the growth defects of *scrib*$^{-}$ clones. In contrast, *scrib*$^{-}$ cells in which JNK signaling was blocked by coexpressing a dominant-negative form of the *Drosophila* JNK *Basket* (*Bsk*^{DN}; overexpression is indicated as +*Bsk*^{DN}) or because they were generated in animals that were homozygous mutants for *eiger* (*egr*$^{-/-}$), an extracellular ligand that activates JNK signaling (16), were no longer eliminated and grew into large clones (Fig. 1 *D* and *G* and Fig. S1*D*) (2–4, 12). In addition to surviving, these clones hyperproliferated, as revealed by an excess of BrdU-incorporating cells in mutant clones, in contrast to *scrib*$^{-}$ clones with normal JNK activity, which did not grow and remained small (Fig. 1 *E*–*G* and Fig. S2 *A*–*F*) (12). Therefore, in addition to triggering apoptosis, JNK signaling counteracts the potential of *scrib*$^{-}$ cells to hyperproliferate (2, 3). Notably, *scrib*$^{-}$ cells that cannot activate JNK still showed defects in photoreceptor differentiation, observed through ELAV expression, and in cell polarity, observed through anti-Patj staining, forming multilayered structures of tumorigenic cells (Fig. 1 *H* and *I*) (12). These data show that *scrib*$^{-}$ cells have the potential to hyperproliferate and in genetic mosaics this potential is counteracted by JNK activity (2, 3).

Cell Competition Regulates Hippo Pathway Activity in *scrib*$^{-}$ Cells. The observation that the proliferation of *scrib*$^{-}$ cells is restricted in the presence of wild-type neighbors raised questions about the role of neighboring cells in maintaining homeostasis and eliminating *scrib*$^{-}$ cells. Removal of *scrib*$^{-}$ clones may depend on cell competition (2), on the presence of neighboring cells with normal apical–basal polarity (3), or on circulating hemocytes that attach to *scrib*$^{-}$ cells and secrete Egr (6). To determine the contribution of cell competition to the elimination of *scrib*$^{-}$ cells, we decreased the fitness of the surrounding *scrib*$^{+}$ cells by making them heterozygous for *M*. *scrib*$^{-}$ cells with *M*$^{+/-}$ neighbors formed large clones of proliferating cells, revealed by high levels of BrdU incorporation, that often resulted in deformed and overgrown imaginal discs (Fig. 1*J* and Fig. S2 *B* and *G*). This result demonstrates that the suppression of the tumorigenic potential of *scrib*$^{-}$ cell clones depends on the fitness of their neighboring cells rather than on the mere presence of cells with normal polarity. Thus, cell competition between *scrib*$^{-}$ cells and neighboring wild-type cells is essential for the elimination of *scrib*$^{-}$ cells.

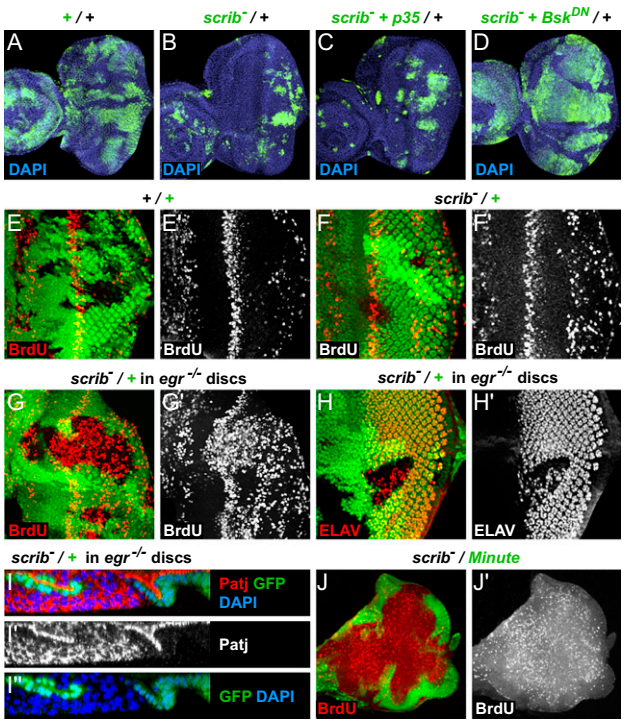

Fig. 1. Activation of JNK restrains the proliferation of *scrib*$^{-}$ cells. Shown are confocal images of mosaic eye imaginal discs. Anterior is to the left in all panels. (*A*–*D*) Clones generated using the MARCM system to label mutant clones by GFP expression (green) and *ey-Flp* to induce recombination in eye discs. Nuclei are labeled with DAPI (blue). (*A*) Wild-type clones. (*B*) *scrib*$^{-}$ clones. (*C*) *scrib*$^{-}$ clones overexpressing p35 (+*p35*) are prevented from undergoing apoptosis. (*D*) *scrib*$^{-}$+*Bsk*^{DN} clones. *scrib*$^{-}$ clones lacking JNK activity overgrow. (*E*–*J*) Mosaic eye imaginal discs containing clones marked by the absence of GFP expression (green in *E*–*J* and *I*″). (*E*) Discs with wild-type clones showing the normal pattern of BrdU incorporation (grey in *E*′). (*F*) *scrib*$^{-}$ clones do not show proliferation defects. (*G*) *scrib*$^{-}$ clones in homozygous *egr*$^{-/-}$ discs have an excess of BrdU-incorporating (grey in *E*′) cells posterior to the second mitotic wave, indicating hyperproliferation. (*H*) *scrib*$^{-}$ clones, marked by lack of GFP, in an *egr*$^{-/-}$ animal stained for ELAV, a marker of differentiated neurons (grey in *H*′). (*I*) Optical cross-section through a wing disc with a *scrib*$^{-}$ clone, marked by absence of GFP, in an *egr*$^{-/-}$ animal stained for Patj (red) and DAPI (blue). Patj (grey in *I*′) is mislocalized, indicating cell polarity defects. (*J*) *scrib*$^{-}$ cells surrounded by *M*$^{+/-}$ cells with BrdU staining (grey in *J*′). Genotypes are listed in *SI Methods*.

To gain insight into the effects of cell competition on *scrib*$^{-}$ cells and to explore how *scrib*$^{-}$ cells are prevented from hyperproliferating, we analyzed the activity of pathways known to regulate imaginal disc growth in *scrib*$^{-}$ cells that were protected from cell competition and then compared that activity with that of *scrib*$^{-}$ cells facing cell competition. Readouts for the Decapentaplegic (Dpp) and Hedgehog (Hh) pathways (29) were not affected significantly in *scrib*$^{-}$ clones in *egr*$^{-/-}$ discs, demonstrating that *scrib*$^{-}$ cells protected from cell competition do not misregulate these signaling pathways (Fig. S3). In contrast, *expanded-lacZ* (*ex-lacZ*), a reporter for the Hippo tumor-suppressor pathway and Yki activity (30), was dramatically upregulated in *scrib*$^{-}$ cells in *egr*$^{-/-}$ discs as well as in *scrib*$^{-}$+*Bsk*^{DN} clones (Fig. 2 *A*–*C* and *E*–*H* and Figs. S2*H* and S4 *A* and *B*) (13). In addition, Yki was more concentrated in the nuclei of *scrib*$^{-}$ cells in *egr*$^{-/-}$ discs than in surrounding *scrib*$^{+}$ cells, a finding that is consistent with elevated Yki activity (Fig. 2*D*). Similarly, *scrib*$^{-}$ clones surrounded by *M*$^{+/-}$ cells also displayed high levels of *ex-lacZ* expression (Fig. 3 *A*–*D*). In addition, *scrib*$^{-}$ homozygous discs, in which all cells are *scrib*$^{-}$ and therefore do not face cell

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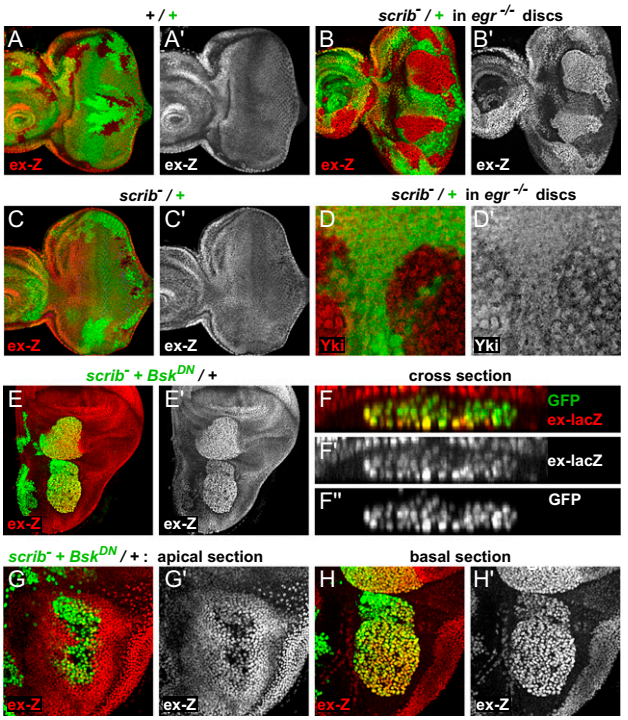

Fig. 2. JNK suppresses elevation of Yki activity in *scrib*⁻ cells. Shown are confocal images of mosaic eye (*A*–*C*) and wing (*D*–*H*) imaginal discs. Discs are stained for β-galactosidase (β-Gal) to show *ex-lacZ* (ex-Z) expression (grey in *A*′–*H*′) in all panels except *D*, where anti-Yki staining is shown in red. (*A*–*D*) Clones are marked by the absence of GFP expression (green). (*A*) Wild-type clones. (*B*) *scrib*⁻ clones in an *egr*$^{-/-}$ disc have high levels of *ex-lacZ*. (*C*) *scrib*⁻ clones in a wild-type eye disc show no changes in *ex-lacZ* levels. (*D*) Yki is more concentrated in the nuclei of *scrib*⁻ cells. (*E*–*H*) *scrib*⁻+*Bsk*^{DN} clones marked by the presence of GFP. (*E*) *scrib*⁻+*Bsk*^{DN} clones show induction of *ex-lacZ* (grey in *E*′). (*F*) Optical cross-section through a *scrib*⁻+*Bsk*^{DN} clone shows multilayering. (*G* and *H*) Apical and basal sections of the disc in *E* at higher magnification. Genotypes are listed in *SI Methods*.

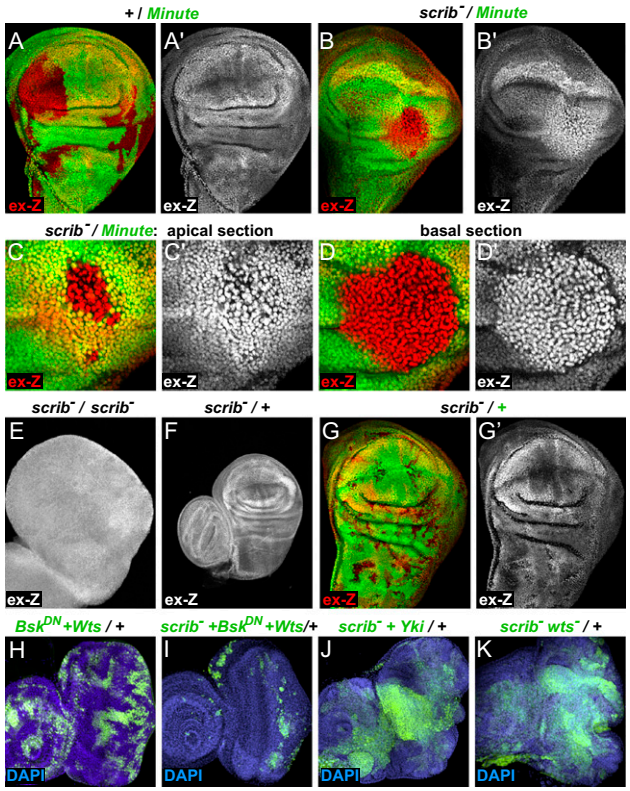

Fig. 3. Cell competition regulates Hippo signaling in *scrib*⁻ cells. Shown are confocal images of mosaic wing (*A*–*G*) and eye (*H*–*K*) imaginal discs. (*A*–*D*) Discs are stained for β-Gal to show *ex-lacZ* expression (grey in *A*′–*G*′), and clones are marked by the absence of GFP expression. (*A*) Wild-type clones in an *M*$^{+/-}$ disc have no changes in *ex-lacZ* expression. (*B*) *scrib*⁻ clone in a *M*$^{+/-}$ disc is large and has high levels of *ex-lacZ* expression. (*C* and *D*) Apical and basal optical sections of the *scrib*⁻ clone in *B*. (*E* and *F*) Confocal images of wing imaginal discs showing *ex-lacZ* expression (grey). The discs were scanned at the same magnification and setting, and single images, rather than maximal projections, are shown. (*E*) Disc homozygous mutant for *scrib* shows uniform induction of *ex-lacZ*. (*F*) Wing disc heterozygous for *scrib* maintains normal *ex-lacZ* expression. (*G*) *scrib*⁻ clones in a wild-type wing disc marked by absence of GFP expression, showing ex-Z expression (grey in *G*′). (*H*–*K*) Eye imaginal discs with clones marked by expression of GFP. DAPI is shown in blue. (*H*) Clones overexpressing *Bsk*^{DN} and *Wts*. (*I*) *scrib*⁻+*Bsk*^{DN}+*Wts* clones are small. (*J*) *scrib*⁻ clones overexpressing Yki (+Yki) and (*K*) *scrib*⁻*wts*⁻ clones evade elimination by cell competition and hyperproliferate. Genotypes are listed in *SI Methods*.

competition, displayed high levels of the Yki activity reporters *ex-lacZ* and *Diap1-GFP* (Fig. 3 *E* and *F* and Fig. S4 *C* and *D*) (31). Thus, *scrib*⁻ cells not facing cell competition have abnormally high levels of Yki activity.

To test whether these elevated levels of Yki activity are required for the hyperproliferation phenotype of "noncompeted" *scrib*⁻ cells, we decreased Yki activity in *scrib*⁻+*Bsk*^{DN} cells by coexpressing Warts (*Wts*), a Hippo pathway serine threonine kinase that phosphorylates Yki and inactivates it (Fig. 3 *H* and *I* and Fig. S5*A*) (21). We found that such cells made only small contributions to third-instar eye discs, indicating that Yki is important for the proliferation of noncompeted *scrib*⁻ clones. Thus, *scrib*⁻ cells not facing cell competition have high levels of Yki activity, which is required for their hyperproliferation.

The finding that Yki activity is elevated in noncompeted *scrib*⁻ cells raised the question of what happens to Yki in *scrib*⁻ cells that do face cell competition. Elevation of Yki levels is sufficient to protect *M*$^{+/-}$ cells from cell competition and can even transform normal cells into supercompetitors (22, 24, 25). Remarkably, *ex-lacZ*, which was up-regulated in noncompeted *scrib*⁻ clones, was not induced in *scrib*⁻ clones surrounded by wild-type cells in most regions of eye and wing discs (Figs. 2*C* and 3*G*). Thus, *ex-lacZ* generally was not elevated in *scrib*⁻ cells that faced cell competition, whereas *scrib*⁻ clones rescued from cell competition (*scrib*⁻+*Bsk*^{DN} clones) had elevated *ex-lacZ* levels in all regions of eye and wing discs (Fig. 2, quantified in Fig. S6). The failure of competed *scrib*⁻ clones to up-regulate Yki activity may be caused by the perdurance of Scrib, because competed *scrib*⁻ clones generally were much smaller than rescued clones. However, *ex-lacZ* also was up-regulated in noncompeted *scrib*⁻ clones that were small (Figs. S7 and S8*B*). Such small clones also had the polarity and differentiation defects seen in big clones, indicating that it is not Scrib perdurance that prevents the up-regulation of Yki activity in *scrib*⁻ clones subject to cell competition. Thus, these data show that cell competition prevents the up-regulation of Yki activity in *scrib*⁻ cells.

We noted that a minority of *scrib*⁻ clones in the hinge region of wing discs and in the posterior region of eye discs displayed some increase in *ex-lacZ* expression, which has been observed by other groups (13, 20). Thirty-one percent of clones in the hinge and 16% of clones in the posterior eye had at least one cell in which *ex-lacZ* was up-regulated (Fig. S6). Notably, the hinge region has been proposed to be a less competitive environment than the wing pouch, and the posterior region of eye discs may similarly be a less competitive environment, since cells in that region start to differentiate earlier than those located more anteriorly (15, 26, 27). Therefore, some *scrib*⁻ clones may face less cell competition in these regions, allowing them to elevate *ex-*

lacZ levels. However, even in the wing hinge region and posterior eye disc region there was a significant difference in *ex-lacZ* expression profiles between *scrib*$^{-}$ clones that were subjected to or protected from cell competition (Fig. S6).

To test whether the suppression of Yki activity by cell competition is required for the elimination of *scrib*$^{-}$ clones, we experimentally increased Yki activity in *scrib*$^{-}$ cells by overexpression of Yki (+*Yki*) or loss of *wts*. Both these manipulations were sufficient to rescue *scrib*$^{-}$ clones from being outcompeted (Fig. 3 *J* and *K* and Fig. S5 *B* and *C*). Therefore, the prevention of Yki up-regulation is key to the elimination of *scrib*$^{-}$ clones. We conclude that cell competition acts as a tumor-suppression mechanism by preventing Yki activation in *scrib*$^{-}$ cells.

scrib^{-} Cells Not Subjected to Cell Competition Have Enhanced Non–Cell-Autonomous Effects on the Hippo Pathway. *scrib*$^{-}$ clones can cause non–cell-autonomous up-regulation of *ex-lacZ* in neighboring wild-type cells (Fig. 3*G*) (20). This non–cell-autonomous effect on Hippo signaling also was observed around *scrib*$^{-}$ clones rescued from elimination: *scrib*$^{-}$ clones in $M^{+/-}$ tissues showed non–cell-autonomous effects on *ex-lacZ* (Fig. 3 *B–D*). Such non–cell-autonomous induction of *ex-lacZ* was observed most dramatically around *scrib*$^{-}$ clones that coexpressed oncogenic Ras^{V12}, which also can rescue *scrib*$^{-}$ cells from being outcompeted and acts synergistically with loss of *scrib* to form tumors (Figs. S7 *A–D* and S8*A*) (2, 4, 7). Clones of *scrib*$^{-}$ cells overexpressing Ras^{V12} (*scrib*$^{-}$+Ras^{V12}) expressed high levels of *ex-lacZ* and also showed strong non–cell-autonomous up-regulation of *ex-lacZ* expression (Figs. S7 *A–D* and S8*A*) (13, 14). Such rescued *scrib*$^{-}$ clones grew into multilayered masses that expanded beyond the epithelial monolayer. This effect, combined with extra growth caused by non–cell-autonomous Hippo pathway regulation, caused noncompeted *scrib*$^{-}$ clones to distort the morphology of the discs (Fig. S7 *A–D*). Non–cell-autonomous regulation of Hippo signaling by abnormal or damaged cells has been observed previously and has been suggested as a mechanism for ensuring that compensatory growth restores the tissue (19, 20).

This regenerative signal has been proposed to depend upon JNK signaling (19, 20). In contrast to these reports, however, we observe non–cell-autonomous effects on *ex-lacZ* in *scrib*$^{-}$+Bsk^{DN} clones and in *scrib*$^{-}$ clones in $egr^{-/-}$ animals (Fig. 2 *E–H* and Fig. S4*B*). Therefore, *scrib*$^{-}$ cells that are not cleared efficiently from imaginal discs are competent to elevate Yki activity in their normal neighbors via a JNK-independent signal.

Increased Relative Myc Levels Protect _scrib_$^{-}$ Cells from Cell Competition. To test further the importance of cell competition in the elimination of *scrib*$^{-}$ cells, we increased their fitness by overexpressing Myc (+*Myc*), which turns cells into supercompetitors (26, 27). We found that overexpression of Myc in *scrib*$^{-}$ cells rescued their poor growth and resulted in strong up-regulation of *ex-lacZ* expression (Fig. 4 *A* and *C* and Fig. S8*B*). This result is striking because overexpression of Myc in wild-type cells did not cause up-regulation of *ex-lacZ* expression; rather, it slightly suppressed *ex-lacZ* expression levels (Fig. 4 *B* and *D*) (25). This indicates that the increase of Yki activity in *scrib*$^{-}$+*Myc* clones is an indirect consequence of these cells being able to evade cell competition due to the increased fitness conferred by Myc overexpression, rather than Myc directly inducing Yki activity. Thus, Myc has different effects on Hippo signaling in *scrib*$^{-}$ and wild-type cells and the oncogenic potential of Myc is more dramatically realized in *scrib*$^{-}$ cells than in wild-type cells. This suggests that elevated Myc may most potently increase the proliferation of tumorigenic cells by counteracting the growth suppressing effects of cell competition that they may face.

This result could be explained by two different kinds of effects. One possibility is that the absolute level of Myc in *scrib*$^{-}$ cells determines whether *scrib*$^{-}$ cells can survive in the presence of normal neighbors. Alternatively, it could be that high levels of Myc in *scrib*$^{-}$ cells transform them into supercompetitors. In the latter case, the relative levels of Myc between *scrib*$^{-}$ cells and their neighbors would determine whether the *scrib*$^{-}$ cells will be eliminated. To distinguish between these two possibilities, we overexpressed Myc throughout the posterior wing compartment and produced *scrib*$^{-}$ clones in this uniformly high-Myc environment. If Myc contributes to the absolute growth ability of *scrib*$^{-}$ clones rather than to relative growth ability, we would expect that *scrib*$^{-}$ clones would not be eliminated and would be able to grow when Myc is overexpressed in the entire tissue. We observed that *scrib*$^{-}$ clones generated in compartments in which Myc is overexpressed are not rescued from elimination (Fig. 4*E*). This result indicates that high levels of Myc are insufficient to rescue *scrib*$^{-}$ clones from being eliminated by cell competition if surrounding normal cells also have high levels of Myc. Thus, the effects of Myc on the survival of *scrib*$^{-}$ clones are not a simple result of a cell-autonomous increase in proliferation rate. Rather, the relative level of Myc in *scrib*$^{-}$ cells compared with their normal neighbors is important. When *scrib*$^{-}$ cells have more Myc

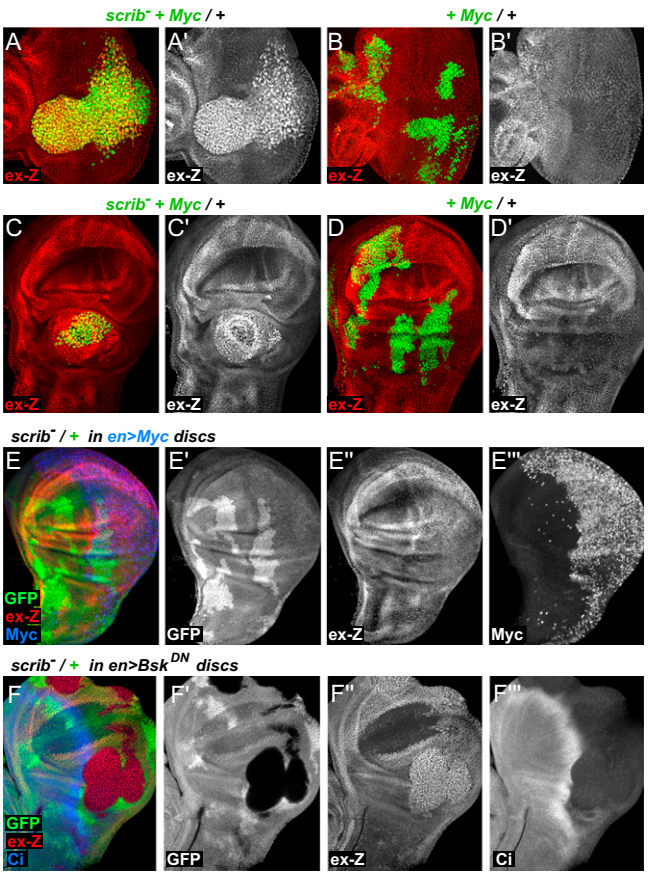

Fig. 4. Myc overexpression promotes tumorigenesis of *scrib*$^{-}$ clones. Shown are confocal images of mosaic eye and wing imaginal discs. (*A–D*) Clones of cells are positively marked by GFP expression (green), and discs are stained for β-Gal to reveal *ex-lacZ* expression (red in *A–D* and grey in *A′–D′*). (*A*) *scrib*$^{-}$ clones overexpressing Myc (+*Myc*) in an eye disc are not eliminated by cell competition and induce *ex-lacZ*. (*B*) *Myc* clones in an eye disc show no notable defects. (*C*) *scrib*$^{-}$+*Myc* clones in a wing disc elevate *ex-lacZ* expression. (*D*) +*Myc* clones in a wing disc suppress *ex-lacZ*. (*E* and *F*) *scrib*$^{-}$ clones marked by the absence of GFP expression were generated in discs in which *engrailed-Gal4* drove overexpression of transgenes in the posterior compartment. (*E*) *scrib*$^{-}$ clones were generated in a wing disc in which *Myc*, tagged with *c-Myc* (grey in *E′′′*), was overexpressed in the posterior compartment. No *scrib*$^{-}$ clones are observed, but a large GFP^{+} twinspot in the posterior compartment shows that *scrib*$^{-}$ tissue was eliminated. (*F*) *scrib*$^{-}$ clones overproliferate and induce ex-Z (grey in *F′*) in a wing disc in which Bsk^{DN} was expressed. The posterior compartment is marked by the absence of Cubitus interruptus (Ci) (grey in *F′′′*). Genotypes are listed in *SI Methods*.

than their neighbors, they are protected from elimination; when both populations have high Myc levels, the *scrib*$^-$ cells are eliminated. This result confirms that *scrib*$^-$ cells are eliminated by cell competition. In contrast to these results with Myc overexpression, *scrib*$^-$ clones were rescued from elimination when Bsk^{DN} was overexpressed in entire posterior compartments, showing that the overexpression in this system is early enough to rescue *scrib*$^-$ clones (Fig. 4*F*). Altogether, we conclude that Myc acts as an oncogene in *scrib*$^-$ cells by increasing their relative fitness.

Discussion

In this study we show that tumorigenic *scrib*$^-$ cells are removed from *Drosophila* imaginal discs by a cell–cell signaling event that suppresses elevated Yki activity in *scrib*$^-$ cells. Previous reports implicated JNK as a mediator of cell competition of *scrib*$^-$ clones, where it induces apoptosis and suppresses proliferation (2–5, 7). However, it was not known how JNK prevents *scrib*$^-$ clones from hyperproliferating. We now provide evidence that JNK prevents *scrib*$^-$ clones from hyperproliferating by regulating the activity of the Hippo pathway effector Yki. First, *scrib*$^-$ clones that do not face cell competition up-regulate Yki activity, which drives their hyperproliferation. Second, when *scrib*$^-$ clones do face cell competition, then JNK signaling prevents the up-regulation of Yki activity. Third, experimental up-regulation of Yki activity is sufficient to rescue *scrib*$^-$ clones from being eliminated by cell competition. Fourth, experimental suppression of Yki activity in *scrib*$^-$ clones not subjected to cell competition is sufficient to suppress their hyperproliferation. Therefore, cell competition suppresses up-regulation of Yki activity in *scrib*$^-$ cells, and this suppression is important for the elimination of *scrib*$^-$ clones by cell competition. Previous reports showed that Hippo pathway reporters can be up-regulated in *scrib*$^-$ and *lgl*$^-$ mutant discs and clones (13, 14, 18, 20) and that Yki is required for the overgrowth of *scrib*$^-$+*Bsk*^{DN} cells not subjected to cell competition (13). However, these studies did not analyze the effects of cell competition on Yki activity in *scrib*$^-$ cells. Our analysis now shows that *scrib*$^-$ cells facing cell competition do not up-regulate Yki activity and thereby identifies a mechanism that is critical for the elimination of *scrib*$^-$ cells.

Although it was reported that *scrib*$^-$ and *lgl*$^-$ clones can up-regulate *ex-lacZ* expression and Yki activity (13, 14, 18, 20). However, upon quantification we found that the majority of *scrib*$^-$ clones have normal or reduced levels of *ex-lacZ* expression, and only a small percentage of *scrib*$^-$ clones have elevated levels of *ex-lacZ* expression. Clones with elevated *ex-lacZ* expression were observed mainly in the hinge region of wing discs, which may provide an environment of reduced cell competition (15, 26, 27). Thus, outcompeted *scrib*$^-$ clones do not have elevated levels of Yki activity. In contrast, when *scrib*$^-$ clones are rescued from cell competition, they show highly elevated levels of *ex-lacZ* expression (this study and refs. 13 and 14). Similarly, discs that are entirely mutant for *scrib,* thereby creating an environment that does not have competing normal cells, show hyperactivation of Yki (this study and ref. 13). Cell competition thus prevents the hyperactivation of Yki in *scrib*$^-$ clones and turns a potential high-Yki "supercompeting" *scrib*$^-$ cell into a cell of lower fitness and less resistance to apoptosis. Importantly, *scrib*$^-$*wts*$^-$ and *scrib*$^-$+*Yki* clones show greatly increased growth and survival compared with *scrib*$^-$ clones. These results show that elevated levels of Yki are sufficient to protect *scrib*$^-$ cells from being outcompeted. Thus, if Yki activity already was high in *scrib*$^-$ cells facing cell competition, those cells would not be outcompeted, and overexpression of Yki or loss of *wts* would not cause such dramatic effects on the survival and growth of *scrib*$^-$ clones. Apparently, Yki levels in *scrib*$^-$ cells facing cell competition are not high enough for these cells to evade cell competition. Thus, the amount of Yki activity in *scrib*$^-$ cells is a critical determinant of whether *scrib*$^-$ clones are eliminated or form tumorous tissue, and the suppression of Yki activity in *scrib*$^-$ clones is important for the elimination of *scrib*$^-$ clones by cell competition.

Our studies show that JNK activity is required in *scrib*$^-$ cells for the suppression of Yki activity by cell competition. In contrast, JNK signaling can induce Yki activity during regeneration and compensatory proliferation in imaginal discs (19, 20). Therefore, the effects of JNK signaling on Yki activity in *scrib*$^-$ cells are different from those in normal cells: JNK signaling activates Yki in normal cells promoted to regenerate but suppresses Yki in *scrib*$^-$ cells induced to be eliminated. Interestingly, both these effects are observed in discs with *scrib*$^-$ clones. In *scrib*$^-$ cells, JNK activity suppresses the hyperactivation of Yki, but in neighboring cells that are stimulated to proliferate and compensate for the loss of *scrib*$^-$ cells, the activities of both JNK and Yki are elevated (11, 19, 20). However, we still observed non–cell-autonomous effects on Yki reporters in *egr*$^{-/-}$ animals and in discs that ubiquitously inhibited JNK signaling by *Bsk*^{DN}. Therefore, JNK-independent signals contribute to the non–cell-autonomous induction of Yki activity around *scrib*$^-$ clones. The regulation of Yki by JNK signaling thus is complex and context dependent and may involve several mechanisms.

The observation that *wts*$^-$ *scrib*$^-$ clones overgrow indicates that JNK and Wts function in parallel to regulate Yki or that JNK regulates the Hippo pathway upstream of Wts. JNK can phosphorylate and activate Yap1 to regulate apoptosis in mammalian cells (32, 33). Notably, the JNK phosphorylation sites of Yap1 are different from the Lats phosphorylation sites (21), supporting

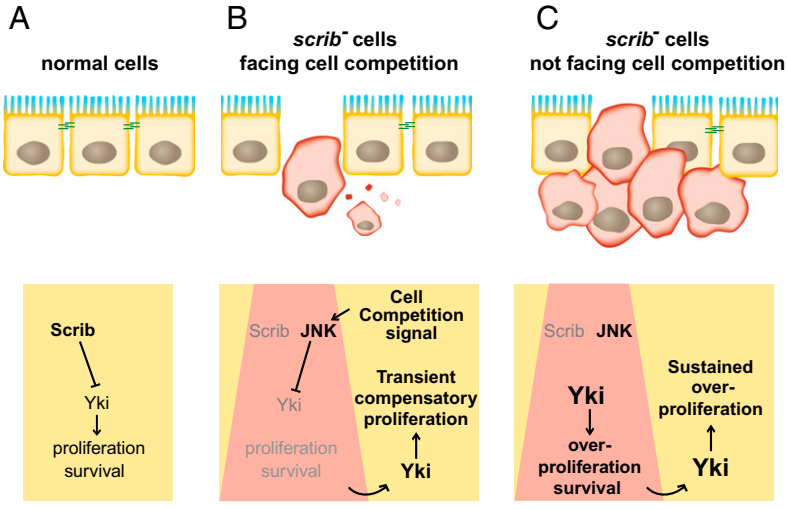

Fig. 5. Model for cell competition acting as a tumor-suppressor mechanism. (*A*) Wild-type cells have normal apical basal polarity (*Upper*). In such cells, Scrib limits the amount of Yki activity (*Lower*). (*B*) When *scrib*$^-$ cells arise in a disc, they face cell competition, which leads to their elimination (*Upper*). In such tissues, cell competition leads to activation of JNK in *scrib*$^-$ cells; JNK activation antagonizes Yki activity, leading to the elimination of the clone. The presence of *scrib*$^-$ cells leads to a non–cell-autonomous activation of Yki activity in neighboring cells, which promotes compensatory proliferation (*Lower*). (*C*) *scrib*$^-$ cells surrounded by poorly competing *M*$^{+/-}$ cells (*Upper*) do not suppress the high levels of active Yki caused by loss of Scrib (*Lower*). These *scrib*$^-$ cells are not eliminated, hyperproliferate, and produce a sustained signal that activates Yki in neighboring cells, stimulating overproliferation.

a model in which JNK functions in parallel with Wts to regulate Yki activity. However, it is not known whether the same sites also act to suppress the activity of Yki in other contexts.

Although several models have been proposed to explain how cell–cell interactions between *scrib*$^-$ and normal cells lead to the elimination of *scrib*$^-$ clones from epithelia, it was not clear what properties normal cells must possess to perform this tumor-suppressive role (16, 17). Our data demonstrate that for *scrib*$^-$ cells to be eliminated they must be juxtaposed with cells that have higher levels of competitive fitness, not just proper cellular architecture. Overexpression of the Myc or Ras^{V12} oncogenes in *scrib*$^-$ clones increases their fitness. As a result, in *scrib*$^-$ clones cell competition does not suppress Yki activity, which protects these clones from being eliminated. Interestingly, Myc expression also synergizes with loss of *scrib* to form tumors in mammals (9), and our data offer a model to explain this phenomenon.

In addition to the cell-autonomous hyperproliferation, *scrib*$^-$ cells that are not removed from imaginal discs have profound non–cell-autonomous effects on the Hippo pathway. This non–cell-autonomous Hippo pathway-regulating signal may serve normally as a regenerative growth signal that facilitates the replacement of eliminated or dying cells, such as outcompeted *scrib*$^-$ cells (19, 20). If *scrib*$^-$ clones are not eliminated efficiently, however, this signal may persist longer than required to restore the tissue, thereby causing overgrowth and deformation of neighboring tissue. Thus, continued residence of tumorigenic cells can stimulate growth beyond that needed for compensation, essentially hijacking the proliferation and regeneration programs of their normal neighbors. Therefore, the non–cell-autonomous activation of Yki by *scrib*$^-$ cells may have important implications for tumor–stromal interactions in human cancers.

In summary, we conclude that cell competition is crucial in suppressing the tumorigenic capacity of *scrib*$^-$ cells and does so by regulating their Yki activity (Fig. 5 *A* and *B*). Loss of this regulation results in overproliferation of both tumorigenic cells and neighboring wild-type cells (Fig. 5*C*). Efficient elimination of *scrib*$^-$ clones by cell competition prevents Yki-fueled overgrowth of mutant cells and prevents them from disrupting proliferation control of their normal neighbors. Thus, we identified a tumor-suppression mechanism that depends on signaling between normal and tumorigenic cells. These data identify evasion of cell competition as a critical step toward malignancy and illustrate a role for wild-type tissue in preventing the formation of cancers.

Methods

***Drosophila* Stocks and Culture.** All crosses were maintained at 25 °C. Mutant clones were induced by mitotic recombination using the Flippase/Flippase recognition target (Flp/FRT) system. Flp recombinase was expressed in a tissue-specific manner using *ey-Flp* and *ubx-Flp* or was induced conditionally using *hs-Flp*. The Upstream Activation System (UAS)-Gal4 system was used to overexpress genes of interest. The *scrib*^{2}-null allele was flipped against corresponding *ubi-GFP*–marked *FRT* chromosomes to generate *scrib*$^-$ clones. To express GFP and other genes of interest in mutant clones, the MARCM system was used (28). Heat shocks were performed at 37 °C for 30 min during the first or second larval stage. Information regarding immunostaining procedures and *Drosophila* strains used is given in *SI Methods*.

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