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Drosophila Myc Regulates Organ Size by Inducing Cell Competition

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Summary

Experiments in both vertebrates and invertebrates have illustrated the competitive nature of growth and led to the idea that competition is a mechanism of regulating organ and tissue size. We have assessed competitive interactions between cells in a developing organ and examined their effect on its final size. We show that local expression of the *Drosophila* growth regulator dMyc, a homolog of the c-*myc* protooncogene, induces cell competition and leads to the death of nearby wild-type cells in developing wings. We demonstrate that cell competition is executed via induction of the proapoptotic gene *hid* and that both competition and *hid* function are required for the wing to reach an appropriate size when dMyc is expressed. Moreover, we provide evidence that reproducible wing size during normal development requires apoptosis. Modulating *dmyc* levels to create cell competition and *hid*-dependent cell death may be a mechanism used during normal development to control organ size.

Introduction

Developing metazoan organs possess intrinsic information, determined by their identity, which allows them to reach an appropriate and reproducible shape and size (Bryant and Simpson, 1984; Johnston and Gallant, 2002). However, the mechanism of intrinsic size control remains a mystery despite abundant genetic, transplantation, and regeneration experiments that supply evidence for its existence (Bryant and Simpson, 1984; Stern and Emlen, 1999). An intrinsic program that regulates size presumably employs signals that monitor organ growth during development or regeneration. Genetic studies in vertebrates and invertebrates suggest that signaling from the conserved pattern organizing morphogens BMP/Dpp/TGF-β, Wnt/Wingless, and Hedgehog contributes to this program, but it is unclear how they monitor and regulate growth (Johnston and Gallant, 2002).

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In *Drosophila*, organ size control has been studied primarily in imaginal discs, the larval organs that give rise to adult appendages. Signaling from Decapentaplegic (Dpp) and Wingless conveys global information about cellular identity and each cell's position within the disc and also influences cell survival and disc growth (Burke and Basler, 1996; Johnston and Sanders, 2003; Moreno et al., 2002a; Zecca et al., 1995, 1996). Discs are divided into developmental compartments of cells related by common ancestry, which have been proposed to function as independent units of growth (Lawrence and Struhl, 1996). However, despite constraints imposed by compartments, considerable flexibility is built into discs, since cell lineages exhibit a plasticity illustrated by the random, irregular shapes of clones generated during development (Bryant, 1970). This flexibility is important for the elimination of misspecified or growth-impaired cells and their replacement by more robust neighboring cells.

Several experiments have demonstrated the influence of local interactions in cell survival and growth decisions made during wing disc development. Wing disc cells apparently sense each other's growth rates, and under certain conditions, competitive interactions occur that lead to elimination of the slowest-growing cells. This phenomenon, termed "cell competition," was identified in experiments using *Minutes*, dominant mutations in genes encoding ribosomal proteins that result in defective protein synthesis, slow growth, and developmental delay (Morata and Ripoll, 1975). In *Minute* mosaic wings, clones of wild-type cells overtake much of the mutant wing, but slower-growing cells are eliminated, and the wing's final size is not affected (Morata and Ripoll, 1975; Simpson, 1979). Cell competition is not restricted to mosaic wings harboring *Minute* mutations, as it also occurs when cells are mutant for genes that regulate growth (Burke and Basler, 1996; Johnston et al., 1999; Prober and Edgar, 2000). Thus, competition is a frequent outcome when cell populations differ in growth rate. However, although these experiments and those with *Minutes* suggest that cell competition is an important component of growth control, whether it is required for size control is still unknown. Recently, cell competition induced in mosaic wing discs mutant for one *Minute*, *M(2)60E*, was reported to result from a reduction in Dpp signaling, leading to activation of the stress MAP Kinase Jun N-terminal Kinase (JNK) and ultimately to death of the cells under competitive stress (Moreno et al., 2002a). Whether this cascade of events is a general mechanism operating under all conditions of cell competition in wing discs remains to be determined.

How is organ size intrinsically controlled? In principle, the mechanism imposes homeostatic controls on the three fundamental components of organ growth: cell division, cellular growth (cellular biosynthesis), and cell survival. Experiments altering cell division rates during wing development indicate that absolute cell number may not be as critical as overall mass in determining organ size (Neufeld et al., 1998; Weigmann et al., 1997). Thus, regulators of cellular growth rates may be targets

of a mechanism that controls organ size. Interestingly, localized expression of some growth regulators in imaginal discs, such as the Phosphoinositide 3-Kinase (PI3K) Dp110, override size control and cause wing overgrowth, whereas localized expression of dMyc, another potent growth promoter, allows appropriate wing size (Johnston et al., 1999; Weinkove et al., 1999).

myc, a gene whose deregulation is prominent in cancer, is a critical regulator of growth in flies and in mammals (Iritani and Eisenman, 1999; Johnston et al., 1999; Trumpp et al., 2001). In previous work, we demonstrated that *Drosophila myc* (*dmyc*) mutant cells grow poorly and are outcompeted by their more robust wild-type neighbors in mosaic wing discs (Johnston et al., 1999). These studies and our observation that regional overexpression of dMyc does not alter wing size prompted us to explore cell competition and its requirements and to determine its relationship to control of organ size. For example, is a difference in growth rates between populations of cells sufficient to induce competition? Normally, wing disc cells proliferate with balanced rates of cell division and cellular growth. Expression of the "unbalanced" growth regulators in *Drosophila*, such as dMyc and Dp110, accelerate cellular growth but not cell division, resulting in larger cell size but not cell number (Johnston and Gallant, 2002). Expression of other "balanced" growth regulators—e.g., cyclin D + Cdk4—increases cell number by accelerating both rates of cellular biosynthesis and cell division equally (Datar et al., 2000). This distinction is of interest, since previous work on cell competition has used *Minutes*, in which "balanced" growth occurs (Neufeld et al., 1998).

To carry out a detailed study of cell competition and its involvement in control of organ size, we have utilized assays in the *Drosophila* wing that allow direct measurement of cell competition induced by different growth inputs. We demonstrate that cell competition is not an invariable consequence of differing growth rates between cell populations, that local dMyc expression induces cell competition similar to that in *Minute* mosaics, that dMyc-induced cell competition leads to death of nearby cells via the proapoptotic gene *hid*, and that cell competition, *hid* function, and apoptosis are required for proper control of wing size. In addition, we provide evidence that cell competition and apoptosis are part of the intrinsic genetic program that controls organ size during development.

Results

Previous experiments using *Minute* (*M*) cells to study cell competition used mitotic recombination to induce clones of wild-type cells in a background of heterozygous *M* cells. Under these conditions, the wild-type cells compete with *M* cells and fill large areas of the wing (Morata and Ripoll, 1975; Simpson, 1979; Simpson and Morata, 1981). We employed a clonal assay, which we call the "three-clone assay," to combine regulated Gal4-driven expression with mitotic recombination and "flp-out" recombination technology. This combination allowed us to generate three types of marked clones within a wing disc: a "Gal4 clone" expressing a UAS-linked growth regulator and UAS-GFP, a "sibling clone" generated in the same recombination event and marked with a cell surface marker, and a "neutral" clone expressing β-galactosidase, generated at the same time as the other two clones but in an independent, flp-out recombination (Figure 1A, see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/117/1/107/DC1). The Gal4 clones were generated by mitotic recombination, which removed the Gal4 inhibitor Gal80 (Supplemental Figure S1A) (Lee and Luo, 1999). Cells in the monolayered wing disc remain associated with their siblings after cell division and move little during the disc growth period, permitting clone area measurements to be accurate assessments of growth (Neufeld et al., 1998). We thus measured clonal growth by analyzing the clone area for each of the three clone types (Figure 1A). Our assay worked as follows: if cell competition is induced by Gal4/growth regulator-expressing clones, the size of sibling clones will be smaller than sibling clones in control discs not expressing the growth regulator. Neutral clones are distributed randomly throughout the disc and serve as an internal control, providing information about how proximity to cells expressing a growth regulator affects competition. In control experiments, the Gal4, sibling, and neutral clones were similar in size, indicating that they grew at similar rates and that the presence of Gal80, Gal4/GFP, or β-gal per se did not affect the growth of wing disc cells (Figure 1B, Control).

Cell Competition Is Induced by dMyc but Not Dp110 or Cyclin D + Cdk4

To ask whether cell competition is always induced when populations of cells with different growth rates are generated in a wing disc, we used the three-clone assay to generate Gal4 clones expressing the growth regulators dMyc, Dp110, or cyclin D in combination with its obligate kinase partner, Cdk4, in the developing wing disc. We then compared the size of the Gal4, sibling, and neutral clones to comparable clone types in control discs. As expected, Gal4 clones expressing dMyc, Dp110, or CycD + Cdk4 were significantly larger than control Gal4 clones, due to promotion of cellular growth rates by each regulator (Figure 1B). However, only sibling clones generated in recombination events with Gal4/dMyc clones were smaller than control sibling clones. Sibling clones next to Gal4/Dp110 or Gal4/CycD + Cdk4 clones were similar in size to each other and to control sibling clones (Figure 1B). These results indicate that cells adjacent to Gal4/dMyc clones are at a growth disadvantage, but cells next to Gal4/Dp110 or Gal4/CycD + Cdk4 clones are not.

We wished to compare competition induced by dMyc with that induced in *Minute* mosaics. The *Minute M(3)66D* carries a mutation in the gene encoding the ribosomal protein RpL14 (Saeboe-Larssen et al., 1997). We therefore overexpressed a rescuing UAS-RpL14 transgene in the *M(3)66D* discs using the three-clone assay. The UAS-RpL14 transgene rescues certain *M(3)66D* phenotypes such as bristle size when expressed with a bristle-specific Gal4 driver (J. Merriam, personal communication). In our assay, Gal4/RpL14 clones outgrew control clones, indicating that the transgene rescued the growth of the mutant *M(3)66D* cells (Figure 1D). In addition, Gal4/RpL14 clones competed

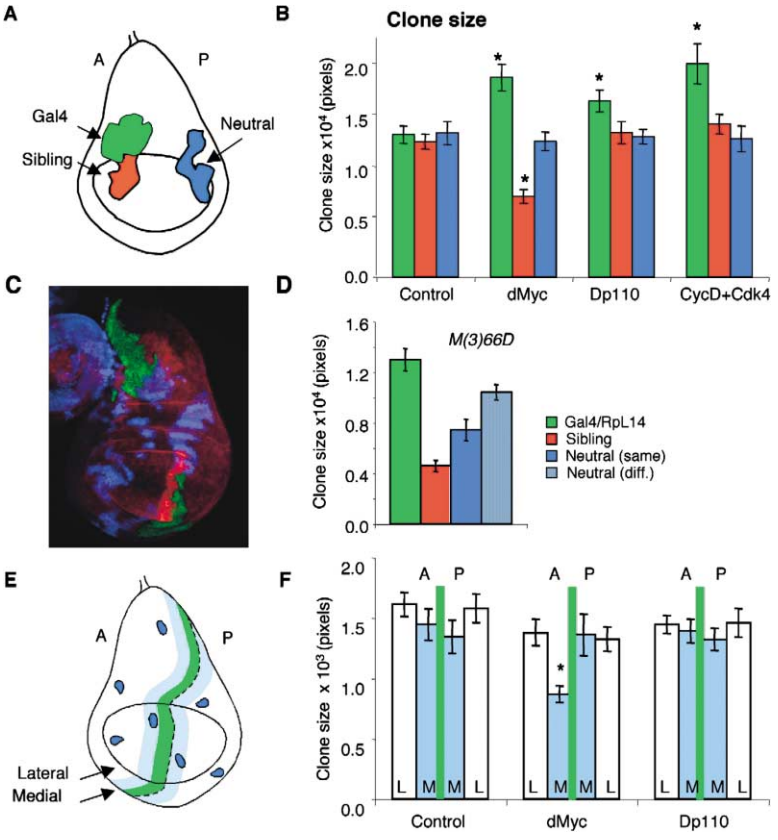

Figure 1. The Growth Regulator dMyc but Not the PI3K-Dp110 or Cyclin D/Cdk4 Induces Cell Competition

(A and C) Clonal assay of cell competition that utilizes three clone types (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/117/1/107/DC1 for details). Mitotic recombination produces a GFP-expressing "Gal4" clone (green) and its "Sibling," marked by a cell surface marker (red). An independent recombination event produces a β-galactosidase-marked clone ("Neutral," blue). A (anterior) and P (posterior) show wing disc orientation in this as well as all subsequent disc images. In control assays, all three clone types were similar in size (B). (B) Clone sizes in wild-type wing discs. Clones expressing dMyc, Dp110, or CycD + Cdk4 were larger than GFP controls ($p = 1.0 \times 10^{-5}$). Siblings of dMyc clones were smaller ($p = 1.29 \times 10^{-9}$), indicating they were at a growth disadvantage. (D) RpL14 expression rescues growth of *M(3)66D* cells and induces cell competition. RpL14-expressing clones are significantly larger than controls in *M(3)66D* discs not expressing RpL14 (Neutral diff., $p = 0.02$). Sibling and neutral clones in the same *M(3)66D* wing discs (Neutral same) were smaller than controls ($p = 4.9 \times 10^{-10}$ and $p = 0.0085$, respectively). (E) A second assay of cell competition uses flp-outs events to produce neutral β-galactosidase-marked clones (blue) in wing discs in which the regional driver DppGa14 is used to overexpress growth regulators (green). DppGal4 is only expressed in imaginal discs and in salivary glands (unpublished data). Clones were categorized as medial (M, light blue) if within 50 pixels (corresponding to approximately eight cell diameters) of the Dpp expression domain. All remaining clones were considered lateral (L, white). (F) Neutral clone sizes in lateral and medial regions of wild-type discs. Clones in the anterior, medial region of dMyc-expressing discs were significantly smaller than those in the same region of GFP controls ($p = 0.0003$). Sizes shown represent the mean of several clone measurements. Error bars, SEM.

effectively, since sibling clones were much smaller than clones in control discs (Figure 1D). We asked whether cells at a distance were also affected by the RpL14-expressing clones by examining the size of the neutral clones in the same disc and the size of neutral clones in control *M(3)66D* discs. Neutral clones in the same disc as Gal4/RpL14 clones (Figure 1D, Neutral [same]) were smaller than neutral clones in control *M(3)66D* discs (Figure 1D, Neutral [diff.]). This suggests that Gal4/RpL14 clones competed with distant, neutral clones, although less effectively than closely situated sibling clones. Thus, expression of RpL14 in cells mutant for *rpl14* increases their ability to grow and induces cell competition, leading to slower growth in surrounding cells in a proximity-dependent fashion. These results are consistent with previous experiments showing that wild-type cells compete against *Minute* mutant cells (Morata and Ripoll, 1975; Simpson, 1979). In addition, they show that the three-clone assay successfully measures cell competition and demonstrate that cell competition induced by dMyc and in *Minute* mosaics is similar.

In our experiments with Gal4/dMyc clones, we found that the size of neutral clones in contact with and residing in the same compartment as Gal4/dMyc clones was significantly smaller than those residing in the opposite compartment (see Supplemental Figure S1B at http://www.cell.com/cgi/content/full/117/1/107/DC1). The long growth period (72 hr) required in this assay made it difficult to determine how much time cells in neutral clones actually spent near Gal4/dMyc clones. Thus, we turned to a different assay to investigate the impact of proximity to dMyc-expressing clones.

Distance and a Compartment Boundary Protect Cells from Competition by dMyc

To refine our control over the period of growth of neutral clones, we used the DppGal4 driver to express dMyc or Dp110 in a broad stripe of anterior wing disc cells, induced neutral lacZ-expressing clones randomly throughout the disc, and then scored the size of the clones after defined periods of growth (Figure 1E). To determine how the proximity of neutral clones to fast-growing cells affected their growth, clones were scored according to their location: near (medial, within approximately eight cell diameters from the anterior edge of the dMyc expression domain) or far (lateral) from the dMyc-expressing cells (Figures 1E and 1F). In addition, we made note of whether clones resided in the posterior (P) or anterior (A) compartment, two of the earliest and largest developmental compartments formed in the disc.

In control discs expressing only GFP in the Dpp domain, medial and lateral clones in both compartments were not significantly different in size (Figure 1F). Similarly, clones in discs expressing Dp110 were similar in

size to each other. However, neutral A clones in discs expressing dMyc were significantly smaller than controls when located near the Dpp domain (A medial) (Figure 1F; $p = 0.0003$). The size of clones located far from dMyc-expressing cells (A lateral) was slightly but not significantly smaller than controls (median = 1377.2 versus 1609.9 for control; $p = 0.1153$). As DppGal4 is transiently expressed in many anterior cells (Weigmann and Cohen, 1999), we cannot determine whether neutral clones must have physical contact with dMyc-expressing cells to suffer a competitive disadvantage. Our measurements indicate, however, that cells eight cell diameters from the Dpp domain can sense the competitive effects of dMyc-expressing cells.

Remarkably, in contrast to neutral A clones, the size of neutral clones in the P medial or lateral regions was not affected by the dMyc-expressing cells, even though P medial cells physically abut the dMyc-expressing cells just as A medial cells do. This result indicates that the presence of the A-P compartment boundary protects P cells from cell competition induced by dMyc expression in the A compartment as it does in *Minute* mosaic wings (Morata and Ripoll, 1975).

Cells under Competitive Stress Die due to Induction of the Proapoptotic Gene *hid*

Cell competition could lead to small clone size by a variety of mechanisms, including reduction of cellular growth rates, a decrease in cell division rates, or the elimination of cells through cell death (Morata and Ripoll, 1975; Moreno et al., 2002a; Simpson, 1979). In DppGal4/dMyc wing discs, the number of cells per A medial neutral clone was decreased compared to A medial clones in control discs. In contrast, in these discs, cell number in neutral clones anywhere in the P compartment was similar to controls, consistent with the lack of competition in the posterior of DppGal4/dMyc discs. Nucleolar size, assessed by expression of Nop1, a nucleolar antigen whose expression is sensitive to growth conditions (Aris and Blobel, 1988), was unaffected in cells under competitive stress (data not shown), as was cell size in medial or lateral cells of DppGal4/dMyc discs (data not shown). However, cell death was dramatically increased in discs with clones of Gal4/dMyc. Gal4 clones expressing each of the three growth regulators increased the frequency of death, measured by TUNEL assay, within the clones. A significant increase in TUNEL-positive cells was also found outside of Gal4/dMyc clones, indicating that cells under competitive stress die frequently (Figure 2A). Comparable results were found using an antibody to activated Caspase 3 (data not shown) (Srinivasan et al., 1998). To determine the mechanism of their death, we examined the expression of the proapoptotic genes *reaper* (*rpr*) and *head involution defective* (*hid*). Both *hid* and *rpr* expression were increased in cells expressing dMyc or Dp110 (Figure 2C and data not shown), but *hid* mRNA was also upregulated in A medial cells of DppGal4/dMyc discs, suggesting that cells under competitive stress die as a result of *hid* induction (Figures 2B and 2C, red arrow). Consistent with this idea, mutations reducing the dose of *hid* in discs expressing DppGal4/dMyc reduced cell death of nonexpressing cells to less than 15% of controls (Figures 2D–2F). These

A

Tunel+ cells (fold over control)	Control	dMyc	Dp110	CycD/Cdk4	
In clone	1.0	8.3 *	4.2	4.4	
Outside clone	1.0	3.5 *	0.3	0.8	* p<0.005

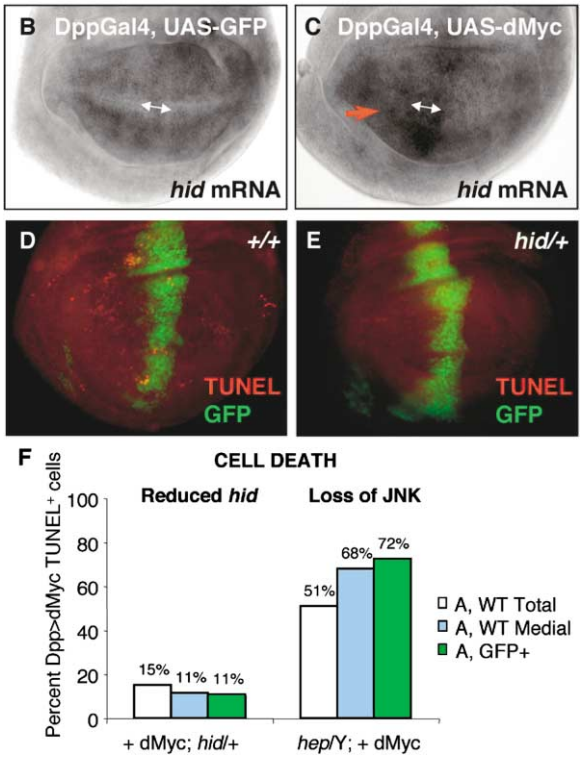

Figure 2. dMyc Expression Induces Autonomous and Non-Cell-Autonomous Cell Death

(A) TUNEL-positive (TUNEL$^+$) cells inside and outside of clones overexpressing dMyc, Dp110, or CycD + Cdk4 or GFP alone as a control. Cells located outside of clones were scored as positive when within 50 pixels (corresponding to about eight cell diameters) of the clone border.

(B and C) *hid* mRNA expression is upregulated in dMyc-expressing cells (white arrowheads) and in nearby A medial cells (red arrow).

(D) Expression of dMyc in the Dpp domain (green) in wild-type discs results in apoptosis, visible by TUNEL (red), primarily within the anterior compartment.

(E) In *hid*/+ heterozygotes expressing dMyc, apoptosis is substantially reduced.

(F) Comparison of TUNEL-positive cells in wild-type (+/+), *hid*/+, and *hep* mutant discs expressing dMyc under DppGal4 control, expressed as a percent of TUNEL$^+$ cells in wild-type discs expressing dMyc. TUNEL$^+$ cells were scored in three regions: all GFP-negative anterior cells (A, wt total), GFP-negative cells next to the dMyc-expressing domain (A, wt medial), and GFP-positive cells expressing dMyc (A, GFP$^+$).

observations indicate that *hid*-induced apoptosis is a frequent outcome of dMyc-induced cell competition and suggest that the small clone size that defines cell competition in our assays is primarily the result of cell death.

Since Dpp is required for wing disc growth and cell survival (Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002; Moreno et al., 2002a), we considered that reduced levels of Dpp signaling in cells under competitive stress might lead to their death. Expression of

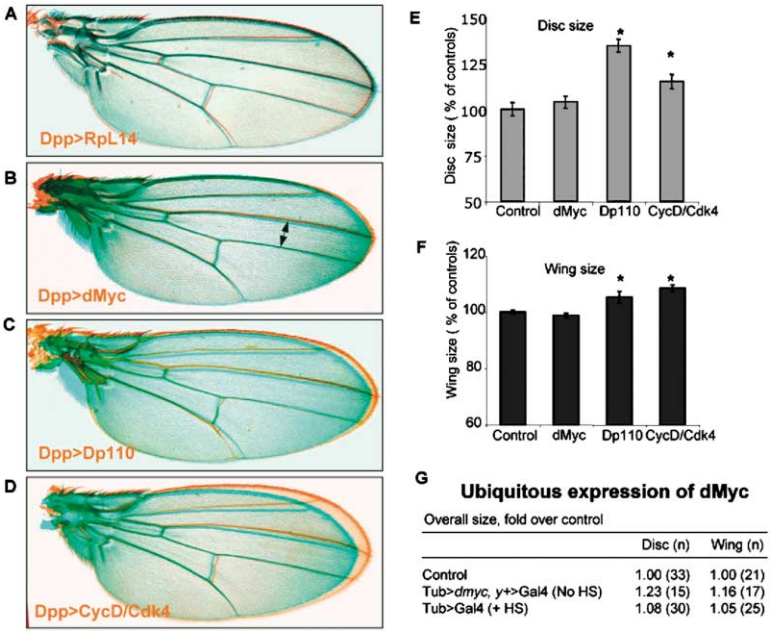

Figure 3. Appropriate Wing Size Requires Cell Competition

(A) Adult wings of *M(3)66D* flies expressing RpL14 in the Dpp domain (orange) were indistinguishable in size from *M(3)66D* controls (blue). (B–D) The size of wild-type wings expressing dMyc, Dp110, or CycD + Cdk4 (orange) compared to controls (blue). (B) dMyc expression resulted in increased cell size (data not shown) and increased the width of the Dpp domain (arrows), but overall wing size was unchanged. In contrast, wings expressing Dp110 ([C], orange) or CycD + Cdk4 ([D], orange) overgrow, particularly within the anterior compartment, where the growth regulator is expressed ($p < 0.001$). (E) Quantification of wing discs sizes. Each is shown as a percent of controls. Discs expressing dMyc are the same size as controls, while those expressing Dp110 or CycD + Cdk4 are larger than controls ($p < 0.004$). (F) Quantification of adult wing sizes. Interestingly, whereas discs expressing Dp110 were larger than those expressing cyclin D + Cdk4, the reverse is true for adult wings. Error bars, SEM. (G) Size regulation requires cell competition. Wing discs and adult wings from animals that ubiquitously overexpress dMyc (Tub > *dmyc, y+*>Gal4 No HS) were approximately 20% larger than controls. Upon clonal removal of the *dmyc, y+* cassette by flp-out recombination (Tub > Gal4 + HS), wing discs and adult wings were similar to the size of controls.

dMyc had no effect on the expression of the Dpp activity marker P-mad, nor did it alter the expression of the positive Dpp target *spalt* or the negative Dpp targets *thickvein* and *brinker*, suggesting that cells suffering the effects of dMyc-induced competition receive sufficient levels of signaling from Dpp (see Supplemental Figures S3A–S3D at http://www.cell.com/cgi/content/full/117/1/107/DC1 and data not shown). We also examined these cells for activation of JNK signaling, since it accompanies reduction of Dpp signaling and cell competition induced in *M(2)60E* mosaic wing discs (Moreno et al., 2002a). However, although cells in both Gal4/dMyc and Gal4/Dp110 clones activate expression of the JNK target *puckered* (*puc*) (Martin-Blanco et al., 1998), *puc* expression was not detected in cells under competitive stress (data not shown). Furthermore, expression of the JNK ligand *eiger*, the single *Drosophila* Tumor Necrosis Factor (TNF) homolog (Igaki et al., 2002; Moreno et al., 2002b), was unaltered in DppGal4/dMyc discs (data not shown). We eliminated JNK signaling using a null mutant of the MKK7 MAP Kinase *hemipterous* (*hep*) (Glise et al., 1995). Under these conditions, death was reduced only about 30% in cells under competitive stress (Figure 2F).

Cell Competition Is Required to Control Appropriate Wing Size

Cell competition leading to the death and elimination of some cells may be necessary to prevent overgrowth of the wing when a growth promoter such as dMyc is expressed. To examine this possibility, we looked at the effect of large, fast-growing populations of cells on the regulation of wing disc and adult wing size. By both the three-clone assay and the DppGal4 assay, wing discs and adult wings expressing dMyc were the same size as controls. Similarly, adult wings from *M(3)66D* flies expressing DppGal4/RpL14 were the same size as control *M(3)66D* wings (Figures 3A–3F and data not shown). In striking contrast, both wing discs and adult wings expressing Dp110 or CycD + Cdk4 by either assay were significantly larger than controls (Figures 3C–3F and data not shown). Thus, large populations of cells overexpressing dMyc do not cause the wing to overgrow. Rather, both the developing wing disc and the mature adult wing achieve normal size. Since cell competition is induced by localized expression of dMyc or RpL14 but not by Dp110 or CycD + Cdk4, these results suggest that the process of cell competition is important for the wing disc to regulate its size properly.

Based on these results, we hypothesized that if competition between wild-type cells and those expressing dMyc was necessary for the wing disc to reach the right size, then expression of dMyc in every cell—creating a disc with no overt competition—should allow the disc to overgrow. To test this, we expressed dMyc ubiquitously under control of the Tubulin α-1 promoter in wild-type flies (hereafter called Tub > dMyc flies). Consistent with our hypothesis, Tub > dMyc adult flies were significantly larger than controls and weighed about 15% more than control flies (see Supplemental Figure S2 at http://www.cell.com/cgi/content/full/117/1/107/DC1). The size of wing discs and of adult wings was approximately 20% larger than controls (Figure 3G). Thus, when all cells express dMyc, the wing overgrows, suggesting that in the absence of overt cell competition, the normal control imposed on wing size was overridden.

We further postulated that since wings locally expressing dMyc grow to normal size (Figure 3), then Tub > dMyc wings should reduce their size upon reintroduction of wild-type cells in mosaics. The Tub > dMyc construct contained the *dmyc* cDNA and an adult marker, *yellow* (*y*), flanked by FRT sites and followed

by the Gal 4 gene. This allowed us to remove the *dmyc, y* cassette by inducing Flp recombinase and heritably express UAS-transgenes, including UAS-GFP to mark the cells (see Experimental Procedures). Since the Tub > dMyc transgene was expressed in wild-type animals, excision of the *dmyc, y* cassette would yield cells that lacked the added expression of dMyc but still expressed *dmyc* from its endogenous locus. This situation should create competition between the different cell populations, lead to the elimination of cells lacking Tub > dMyc, and reduce the overall mass of the disc.

We generated cells lacking the *dmyc, y* cassette by heat shock induction of flp recombinase. Clones in adult wings were marked by the absence of *y*. When recombination was induced during the first half of disc development (e.g., 48 hr after egg laying), Tub > Gal4 clones of GFP-marked disc cells were not recovered, nor were y^- patches in adult wings. Marked tissue in discs and in adult wings could be observed, although infrequently, when clones were induced later (72–96 hr after egg laying) or when a stronger heat shock was used (data not shown and Figure 3G). Thus, normally viable wild-type cells lacking the *dmyc, y* cassette were subject to intense cell competition from surrounding Tub > dMyc cells and were rapidly eliminated. Remarkably, despite the scarce recovery of clones, the wing discs and adult wings from these animals were reduced in size compared to Tub > dMyc controls in which no heat shocks were given or Tub > dMyc controls lacking flp-recombinase but subjected to heat shocks (Figure 3G). Taken together, our data suggest that when all cells express dMyc, the wing cannot control its size properly and overgrows. However, the introduction of even small clones of wild-type cells in the background of Tub > dMyc-expressing cells produces intense cell competition, resulting in the elimination of wild-type cells and in smaller wing discs and smaller adult wings. Together, these experiments indicate that when growth rates are increased in developing wings, competitive interactions between cells allows appropriate size control.

Wing Size Control Requires *hid*-Induced Apoptosis

Since dMyc induces both growth and apoptosis in cells in which it is expressed (Figures 2A and 2F) (Johnston et al., 1999), we wished to rule out the trivial possibility that the increased death of dMyc-expressing cells themselves leads to the death of neighboring cells. We therefore blocked cell death specifically in the cells that expressed dMyc by coexpressing dMyc and the caspase inhibitor P35 (Hay et al., 1994; Johnston et al., 1999). When measured by both our three-clone and DppGal4 competition assays, cell competition still occurred when P35 was coexpressed with dMyc (data not shown). Consistent with the link between competition and wing size control, the overall disc size was not increased, despite the presence of additional dMyc-expressing cells (data not shown).

Our experiments thus suggested that competitive interactions leading to *hid*-induced apoptosis are necessary for the wing to maintain an appropriate size when dMyc is expressed. If so, preventing apoptosis of cells suffering from competition should cause discs to overgrow. To test this prediction, we introduced one copy of either a *hid* null allele or the *H99* deletion, which removes *hid* and two other proapoptotic genes, *rpr* and *grim*, into animals to reduce the dose of *hid*. Under both of these conditions, cell death is substantially suppressed throughout the wing disc (Figure 2F and data not shown). In the *hid*/+ background, DppGal4/dMyc expression did not cause a growth disadvantage in neighboring cells. Neutral clones in *hid*/+, DppGal4/dMyc discs were similar in size whether near or far from dMyc-expressing cells (Figure 4A). These clones were also similar to the size of neutral clones in control *hid*/+, DppGal4 discs (Figure 4A). Moreover, DppGal4/dMyc expression in either the *hid*/+ or *H99*/+ backgrounds resulted in a significantly larger than normal anterior compartment (Figures 4B–4D). Thus, *hid* is required for cell death resulting from dMyc-induced competition, and competition and cell death are required for control of disc size. Furthermore, the full complement of *hid* gene dose is necessary to allow apoptosis due to competitive effects of dMyc, indicating that during this type of stress, disc cells are extremely sensitive to levels of *hid* expression.

To assess the contribution of JNK signaling in cell competition and control of wing disc size, we eliminated JNK signaling, again using the *hep* null mutant in discs with DppGal4/dMyc, and found that wing discs were normal in size (see Supplemental Figures S3E and S3F at http://www.cell.com/cgi/content/full/117/1/107/DC1). Only 30% of the death induced by cell competition was eliminated in the *hep* mutant (Figure 2F), indicating that these discs were still able to regulate their size by eliminating cells. The ability to regulate size and lack of disc overgrowth when JNK signaling is blocked is in striking contrast to the effect of reducing *hid* dose by half in DppGal4/dMyc discs. Together, these results demonstrate a critical role for *hid* in competition-induced death and suggest that the role of JNK signaling in cell competition and size control is comparatively minor.

Apoptosis Is Required to Limit Wing Disc Size Variability

Our results implicate *hid*-induced cell death as an important dose-dependent regulator of wing disc size during dMyc-induced cell competition. To determine whether regulated cell death is a general sizing mechanism used in wing development, we blocked apoptosis completely by expressing P35 throughout the wing disc. To our surprise, rather than causing disc overgrowth, the size of discs expressing P35 was on average similar to controls (Figure 4F). However, this genetic background revealed a role for apoptosis in limiting disc size variability (Figure 4H). Normally, wing disc size control is manifested by a distribution of sizes with a distinct peak representing the majority of the population. This pattern of size distribution is unchanged when DppGal4/dMyc is expressed in wild-type discs (Figures 4E and 4G), and a similar pattern is observed during overgrowth, in *hid*/+ or *H99*/+ discs expressing DppGal4/dMyc, or wild-type discs expressing Dp110 or CycD + Cdk4 (data not shown). In striking contrast, discs in which cell death was prevented were widely variable in size and exhibited a relatively flat size distribution (Figure 4H). Thus, in the absence of apoptosis, the mechanism ensuring uniformity of wing size is severely compromised.

Together, our data suggest that apoptosis is required

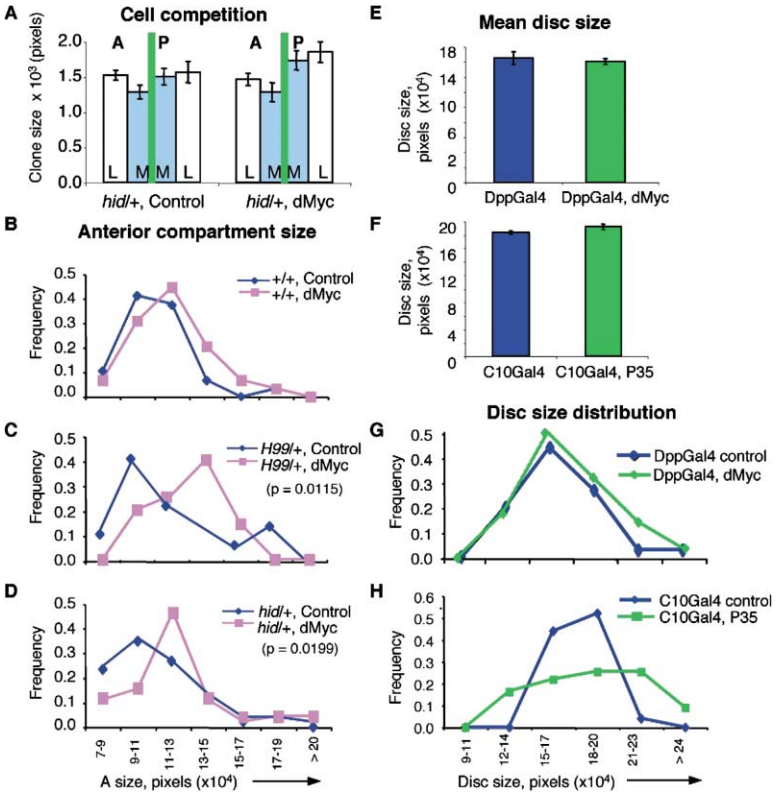

Figure 4. *hid*-Induced Apoptosis Is Required for Cell Competition to Prevent Overgrowth

(A) In a *hid*/+ background, cell competition induced by dMyc is no longer evident. Cell competition assay as shown in Figures 1E and 1F. Neutral clones were scored for size as in Figure 1F in control *hid*/+ discs expressing GFP alone or coexpressing GFP and dMyc. Median values are shown. Error bars, SEM. There is no significant difference between the size of clones in any region, either close to or far from dMyc-expressing cells. (B–D) Frequency distributions of anterior compartment size in control and dMyc-expressing discs in wild-type, *hid*/+, or *H99*/+ backgrounds. DppGal4 is expressed in a subset of anterior cells (see Figure 2D). In wild-type wing discs, the distribution of anterior compartment sizes in discs expressing dMyc was similar to controls. The mean anterior size of DppGal4/dMyc discs was significantly increased in *hid*/+ and *H99*/+ backgrounds (p values in [C] and [D]). (C and D) Reduction of *hid* dose leads to compartment overgrowth when dMyc is expressed under DppGal4 control. In both *H99*/+ and *hid*/+ animals, the distribution of dMyc-expressing anterior sizes was shifted toward larger sizes. Numbers on x axes in (B)–(D) equal pixels × 10^4. (E–H) Blocking apoptosis in the wing disc limits variability of disc size. (E) Wing discs expressing dMyc under DppGal4 control are similar in size to controls. (F) Similarly, the average size of wing discs is unchanged relative to controls when apoptosis is blocked by expression of P35 under C10Gal4 control. Error bars, SEM. (G) Frequency distributions of total wing disc sizes show that most discs fall into a narrow size range, even when dMyc is expressed. (H) In contrast, blocking apoptosis with P35 expression in the wing disc results in a broad distribution of disc sizes compared to control discs, indicating loss of uniform size. Numbers on the x axis equal pixels × 10^4. C10Gal4 is expressed throughout the wing disc.

for limiting variation and promoting reproducibility of wing size. Since *hid* is required for the death of cells under competitive stress, our results raise the intriguing possibility that modulation of *hid* expression by signals that sense growth rate differences between cells may be a mechanism used during normal development to regulate wing size.

Discussion

We have employed assays designed to directly measure cell competition in the developing wing and then assessed its effect on organ size. Our work leads to three major conclusions. First, expression of the c-*myc* protooncogene homolog dMyc in small populations of wing disc cells induces cell competition, leading to the elimination of nearby cells via induction of the proapoptotic gene *hid*. Second, the competition induced by dMyc and the elimination of cells that results is required for control of proper wing size. Finally, our studies reveal that apoptosis is required for the fidelity of size during normal wing development, suggesting that the modulation of *hid* expression by competitive interactions between cells may be used as an endogenous mechanism of size control.

Cell Competition Induced by dMyc

Our experiments demonstrate that expression of dMyc in some cells of a developing organ leads to elimination of nonexpressing cells through apoptosis. We find that the growth disadvantage induced by dMyc-expressing cells fulfills the classic definition of cell competition (Simpson and Morata, 1980): viable but slower-growing cells in an organ are eliminated by an encroaching faster-growing cell population, proximity to the fast-growing cell population dictates the severity of the disadvantage in the slow-growing cells, cells are protected from cell competition by developmental compartment boundaries, and appropriate organ size is reached at the end of development. We find that relative differences in dMyc levels lead to competitive situations between cells: *dmyc* mutant cells are outcompeted by neighboring nonmutant cells (Johnston et al., 1999), and wild-type cells, with a normal complement of endogenous *dmyc*, are also subject to competition if surrounded by cells expressing a dMyc transgene. However, wild-type cells appear to be subject to competition only if they lie within about eight cell diameters of dMyc-expressing cells, and they must reside in the same developmental compartment. Thus, proximity, compartmental provenance (Lawrence and Struhl, 1996), and the relative levels of *dmyc* are particularly important aspects of the competitive effects of dMyc.

We have demonstrated that during the process of cell competition induced by dMyc, the proapoptotic gene *hid* is induced in the growth-disadvantaged cells. Since a reduction of *hid* function protects cells from competition-induced death, we believe that *hid* upregulation is

a consequence of the sensing of competitive stress. An intriguing question that remains is how cells are able to sense competition. One possibility is that cells compete for sufficient levels of a survival factor that normally blocks *hid* expression. Dpp signaling promotes cell survival in the wing disc (Moreno et al., 2002a) but appears to be unaffected in discs expressing dMyc. Alternatively, some cells in competition may be deprived of adequate nutrients, although in our experiments, cells at a growth disadvantage retain a normal nucleolar size, arguing that their biosynthetic rates are not abnormally low. However, our results suggest that dMyc provokes competition and *hid* expression via a short-range signal, since close proximity is required for the perception of competitive effects. Perhaps the most intriguing feature of this signal is that it is not perceived by nearby cells across a compartment boundary, although dMyc induces competition between cells within the posterior compartment as well as within the anterior (data not shown). One possibility is that cells expressing dMyc acquire adhesive properties that transmit a competitive signal to neighboring cells, which is not compatible with the adhesive barrier that maintains the compartment boundary.

Growth Requirements for Cell Competition

Our studies reveal that cell competition is not invariably induced whenever rapidly growing cells populate regions of a developing organ. Both the PI3K Dp110 and cyclin D/Cdk4 potently promote growth when overexpressed, yet they do not induce competition in any of our assays. These observations also demonstrate that balanced growth—growth that simultaneously drives cell division and cellular growth—is not required to induce cell competition. dMyc expression increases clonal mass solely by increasing cell size (Johnston et al., 1999). Thus, this trait of cell competition may be related to a size-measuring mechanism that recognizes total mass rather than cell number (Neufeld et al., 1998). However, Dp110 also promotes growth primarily by increasing cell size, indicating that qualitative differences exist in the cellular response to expression of dMyc and Dp110. Although both growth regulators increase protein synthesis, dMyc probably does so by increasing components of the protein synthetic machinery (initiation factors and ribosomal proteins, etc.) (Orian et al., 2003), whereas PI3K signaling is thought to function by increasing the utilization of existing machinery (Stocker and Hafen, 2000; Thomas, 2000). Regardless of the mechanism, our experiments argue against the notion that apposed populations of fast- and slow-growing cells always result in cell competition.

Appropriate Size Control Requires Apoptosis

We have provided three lines of evidence that indicate that cell competition leading to cell death is required for control of wing size. First, growth induced by local expression of either Dp110 or cyclin D + Cdk4 does not induce competition and causes wing overgrowth. Second, when dMyc is expressed in all cells of the wing disc, the wing overgrows, whereas the introduction of clones lacking dMyc leads to cell competition and to wings approaching normal size. Finally, genetic reduction of *hid* prevents the cell death associated with competition and leads to overgrowth of the compartment in which the dMyc-expressing cells reside.

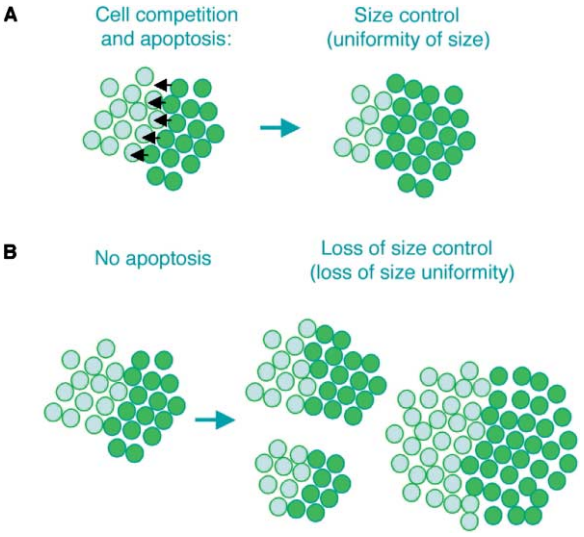

Figure 5. Cell Competition and Apoptosis as Components of Organ Size Control

(A) During cell competition, cells with a growth advantage (green) signal to wild-type cells (gray), inducing the expression of the pro-apoptotic gene *hid* and eventually their death. During subsequent growth, cell competition continues until the fast-growing cells have populated most of the organ while eliminating most of the wild-type cells. Due to elimination of cells through apoptosis, normal and uniform organ size is reached at the end of the development.

(B) When apoptosis is blocked by genetic elimination of *hid* function or ubiquitous expression of P35, wild-type (gray) cells populate portions of the organ, but a wide variation of final organ sizes is observed. Thus, elimination of apoptosis prevents uniformity of organ size.

An important conclusion of this work is that apoptosis is critical for appropriate wing development. Our experiments demonstrate that apoptosis has two roles in regulating wing size. One role is uncovered when the disc is challenged by local changes in dMyc levels, conditions in which cells are exceptionally sensitive to *hid* gene dosage: the full *hid* complement is necessary for the disc to respond to competition properly and eliminate cells. However, a second role of apoptosis is revealed when it is abolished: this role regulates uniformity of disc size, and its loss is manifested as a widening of the range of disc sizes within a given population. This second role of apoptosis indicates that organ overgrowth is distinct from loss of organ size control. Wing overgrowth—observed when cell competition is not executed during local growth perturbations—occurs such that, although larger than normal, wing size still falls within a uniform range. In contrast, loss of size control is the absence of a discrete and reproducible size population and results from a failure to induce apoptosis during the process of growth (Figure 5). Based on our observations, we propose that *hid*-regulated apoptosis contributes to a disc-intrinsic mechanism that limits variation in size by allowing elimination of cells. This mechanism may serve as negative feedback to the positive aspects of growth during development. Loss of feedback control could allow stochastic variation in size,

as we have observed. Although it has been proposed that overall organ mass rather than cell number is sensed by the intrinsic size mechanism, our experiments imply that size control is implemented at least in part by reduction of cell number via apoptosis.

Cell Competition: An Endogenous Mechanism of Size Control?

Is cell competition also part of the intrinsic size control program? If cell competition has a role in normal development, growth rate variations should be observed within developing organs. Indeed, both spatial and temporal differences in cell proliferation rates exist in the wing disc (Johnston and Sanders, 2003; Neufeld et al., 1998), and cell size also varies across the disc, suggesting differences in cellular growth rates (L.A.J., unpublished data). *dmyc* is regulated both by Wingless (Johnston et al., 1999) and Dpp (unpublished data; Prober and Edgar, 2002), which direct the majority of disc patterning. Minor alterations in their signaling could plausibly cause subtle competitive effects by influencing levels of *dmyc* expression, which in turn would modulate *hid* expression and allow for the correction of patterning mistakes that occur during development. In this sense, cell competition, on a small scale, might be a surveillance or "quality control" mechanism to guarantee that organs reach a body-proportional, reproducible size with the appropriate complement of cell fates.

Cell competition is likely a common mechanism used in organs under many conditions, including the adverse. Competitive mechanisms are known to be important to reestablish homeostasis in lymphoid tissue after an immune response (Plas et al., 2002). During tumorigenesis, cancer cells may compete with normal tissue and ultimately overtake the organ, leading to overgrowth of the tumor. In addition, cell competition could prove important therapeutically for many diseases. For example, when liver cells are transplanted into a diseased host liver, cell competition would be critical for the replacement of viable but damaged liver cells with the regenerating donor cells. Although of the three growth regulators we tested only dMyc induced cell competition, other growth-promoting genes that induce cell competition probably exist. The identification of these genes holds promise for a further elucidation of the role of cell competition in organ development.

Experimental Procedures

Fly Strains

The following strains were used: *yw* hsflp; UAS-dMyc (Johnston et al., 1999), *yw* hsflp; UAS-Dp110 (Weinkove et al., 1999), *yw* hsflp; UAS-CycD, UAS-Cdk4 (Datar et al., 2000), *yw* hsflp; UAS-P35 (Hay et al., 1994), *w*; Act>CD2>Gal4, UAS-GFP (Neufeld et al., 1998). Act>stop>lacZ, *yw* hsflp, Tub-Gal4, UAS-GFP; hsCD2 Tub-Gal80 FRT2A/TM6B, *yw* hsflp, Tub-Gal4, UAS-GFP; FRT42D hsCD2 Tub-Gal80/CyO, yw; C10Gal4; and *yw* hsflp; FRT2A/TM6B were gifts of G. Struhl. *yw* hsflp; FRT42D/CyO; and *w*; Act>stop>lacZ were obtained from the Bloomington stock center. Dpp^{blk}Gal4, UAS-GFP/SM6-TM6B was a gift of M. Zecca. puc^{e69}(puc-lacZ)/TM6B and hep^{r75}/FM6 were gifts of M. Mlodzik. *yw*; UAS-GFP; *M(3)66D* BII-Gal4; and *yw*; *M(3)66D* UAS-RpL14/TM6B were gifts of J. Merriam. hid^{X14}/TM3,Sb and *Df(3L)H99*/TM6B were gifts of K. White. *yw* hsflp; Tub > *dmyc*, y^+>Gal4/CyO was generated in this work.

Fly Husbandry

Eggs from appropriate crosses were collected on yeasted grape plates for short periods (2–4 hr). After hatching, larvae were transferred to standard molasses food vials (≤50/vial) supplemented with fresh yeast and raised at 25°C for defined periods of time.

Growth Measurements

Three-Clone Assay

The MARCM (Lee and Luo, 1999) system was used to generate random mitotic clones that express UAS-transgenes of interest and UAS-GFP as a lineage marker. Clones were induced by larval heat shocks carried out at 37°C for 20 min at 30 hr after egg laying, and clones were allowed to grow for 72 hr. The flp-out lacZ cassette was used to generate random clones that express β-gal as a marker. After clone induction, full expression of UAS transgenes took up to 40 hr, suggesting that perdurance of Gal80 can last for days (data not shown). However, the increased size of Gal4 clones indicates that each regulator was able to overcome any initial disadvantage due to Gal80 perdurance. Clonal growth was determined by measuring the two-dimensional area of clones (average n = 40 per experimental condition) after fixing wing discs at the end of the growth period. Clonal area and linear distance measurements were made at 200× magnification (at 200× magnification, 1 pixel = 0.53 microns). Measurements of Neutral clone size in Figure 1 included clones located within the same compartment as a Gal4 clone as well as those outside of that compartment. Clones were also analyzed by compartment (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/117/1/107/DC1).

DppGal4 Assay

Flp-out clones in animals carrying the DppGal4 driver were induced by heat shock at 37°C for 20 min at 60 hr after egg laying and were allowed to grow for 40 hr. Clonal growth was determined as above. Whole disc areas were measured at 100× magnification. Student's t tests were used to determine significance. Relative cell size was determined by dividing the mean number of cells/clone by the mean clone area.

Molecular Biology

dMyc was expressed constitutively in all cells from a transgene constructed by fusing the α-Tubulin promoter to FRT sequences, followed by the entire open reading frame of *dmyc*, followed by the *yellow* open reading frame fused to a tubulin trailer, followed by FRT sequences and the Gal4 cDNA in a four -step cloning procedure, to generate Tub > *dmyc*, y^+ > Gal4. Details of the plasmid construction are available upon request. The plasmid was injected into embryos and transformants isolated according to standard procedures.

Histology

Fixation and immunocytochemistry of imaginal discs were carried out as described (Johnston and Edgar, 1998). RNA in situ hybridizations were carried out using digoxigenin-labeled RNA probes (Johnston and Edgar, 1998). TUNEL assays were carried out using Apoptag Red (Intergen). Images were acquired using Openlab software and a Zeiss Axioplan 2 microscope with an Orca-100 CCD camera (Hammatsu) or a BioRad 1240 Confocal microscope and processed with Photoshop (Adobe) software. The following antibodies and dilutions were used: rabbit anti-Spalt, 1:500 (R. Barrios); rabbit anti-PS1, 1:2000 (K.-E. Heldin); mouse anti-Digoxigenin, 1:2000 (Roche); rabbit anti-CM1, 1:10,000 (Becton-Dickenson); mouse anti-rat CD2 (1:400; Serotec), rabbit anti-β-gal (1:2000; Cappel); and mouse anti-Engrailed 4D9 (1:10, Developmental Studies Hybridoma Bank). Secondary antibodies used were purchased from Jackson Immunoresearch and Molecular Probes.

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