

Control of Wing Size and Proportions by *Drosophila* Myc

D. Christine Wu* and Laura A. Johnston^{†,1}

*Department of Biological Sciences and [†]Department of Genetics and Development, Columbia University,
New York, New York 10032

Manuscript received September 29, 2009
Accepted for publication October 28, 2009

ABSTRACT

Generation of an organ of appropriate size and shape requires mechanisms that coordinate growth and patterning, but how this is achieved is not understood. Here we examine the role of the growth regulator dMyc in this process during *Drosophila* wing imaginal disc development. We find that dMyc is expressed in a dynamic pattern that correlates with fate specification of different regions of the wing disc, leading us to hypothesize that dMyc expression in each region directs its growth. Consistent with this view, clonal analysis of growth in each region demonstrated distinct temporal requirements for dMyc that match its expression. Surprisingly, however, experiments in which dMyc expression is manipulated reveal that the endogenous pattern has only a minor influence on wing shape. Indeed, when dMyc function is completely lacking in the wing disc over most of its development, the discs grow slowly and are small in size but appear morphologically normal. Our experiments indicate, therefore, that rather than directly influence differential growth in the wing disc, the pattern of dMyc expression augments growth directed by other regulators. Overall, however, an appropriate level of dMyc expression in the wing disc is necessary for each region to achieve a proportionately correct size.

HOW pattern and growth are coordinated during development to produce an organ of correct size and shape is a central question in biology. The *Drosophila* wing is an elegant, self-organizing system that is ideal for the study of this coordination. Wing growth is coupled to the specification of cell fates, and these processes are regulated by a small number of conserved signaling pathways and selector proteins. The wing develops from the wing imaginal disc, a proliferating epithelium housed in the larva that also gives rise to the dorsal thorax of the adult fly. The adult wing includes the blade, made from wing pouch (WP) cells of the wing disc, and hinge structures, which are formed by cells immediately proximal to the WP.

Wing development proceeds through a series of steps in which regions of fates are specified. Discs begin development composed of cells with either anterior (A) or posterior (P) identity and subsequently undergo several subdivisions. Early in the second larval instar (L2), the action of Wingless (Wg) and the EGF receptor divide the wing disc into large domains that define the body wall and wing (WANG *et al.* 2000; ZECCA and STRUHL 2002). A short time later, a second subdivision segregates dorsal (D) and ventral (V) cells. At the D/V boundary, Notch signaling induces expression of Wg and the wing selector gene *vestigial* (*vg*) in the boundary cells, initiat-

ing the expansion of the WP region (COUSO *et al.* 1993; WILLIAMS *et al.* 1993; DIAZ-BENJUMEA and COHEN 1995; KIM *et al.* 1995, 1997; NEUMANN and COHEN 1996, 1997; ZECCA *et al.* 1996). Near the end of L2, the expression of *homothorax* (*hth*), a selector gene required for hinge development, becomes specifically expressed in proximal cells (AZPIAZU and MORATA 2000; CASARES and MANN 2000). This is followed in early L3 by the appearance of a ring of Wg expression that circumscribes the WP (the inner ring, IR), and in mid-L3 a second, concentric ring (the outer ring, OR) (COUSO *et al.* 1993; WILLIAMS *et al.* 1993; NEUMANN and COHEN 1996). *Hth* is a target of Wg in these cells and is upregulated in cells adjacent to the two rings of Wg expression (CASARES and MANN 2000). These latter events mark the hinge specification of proximal wing cells.

Recent work indicates that Wg and Dpp, a BMP/TGF- β family member, regulate wing growth by engaging the Fat/Hippo tumor-suppressor signaling pathway and by controlling a Vg feed-forward loop that expands the WP (ZECCA and STRUHL 2007a,b; ROGULJA *et al.* 2008). Fat/Hippo signaling regulates the transcription of several genes required for cell survival, cell division, and growth (HARVEY *et al.* 2003; PANTALACCI *et al.* 2003; UDAN *et al.* 2003; WU *et al.* 2003; HUANG *et al.* 2005; CHO *et al.* 2006). The dMyc transcription factor, encoded by the *diminutive* (*dm*) gene, also provides an essential role in controlling growth of the fly and is regulated by Wg and Dpp (JOHNSTON *et al.* 1999; PROBER and EDGAR 2002). Myc is a conserved protein that is essential for growth in both vertebrates and invertebrates. In both mice and Dro-

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.109.110379/DC1>.

¹Corresponding author: Columbia University, 701 W. 168th St., HHSC 704, New York, NY 10032. E-mail: lj180@columbia.edu

sophila, hypomorphic alleles of *myc* result in animals with a smaller body size (JOHNSTON *et al.* 1999; TRUMPP *et al.* 2001). Despite being smaller, *dm* mutant flies appear morphologically normal with no obvious patterning defects (JOHNSTON *et al.* 1999), suggesting tight linkage between the patterning machinery and dMyc. In the wing, Wg and Notch activity repress dMyc expression in the zone of nonproliferating cells that surrounds the D/V boundary to enforce a cell cycle arrest of these cells (JOHNSTON *et al.* 1999; DUMAN-SCHEEL *et al.* 2004; HERRANZ *et al.* 2008). However, how dMyc contributes to wing development and the nature of the relationship between pattern formation and dMyc expression and activity in the growing wing disc is not understood.

In this study, we examine the role of dMyc in the generation of size and shape of the *Drosophila* wing. We find that dMyc expression is regionally patterned and dynamic throughout wing development and provide evidence that its spatial and temporal expression pattern corresponds to a functional requirement in the growth of cells in different regions of the disc. Despite this, our data indicate that the spatial pattern of dMyc expression is not necessary for sculpting the shape of the wing. Furthermore, we find that rudimentary wing growth can occur in the complete absence of dMyc, although its absence prevents the wing from reaching the correct size or proportion. Together, our experiments argue that while not essential to produce a wing, dMyc expression and function permits the wing to grow at a rate that is compatible with the rate of larval development and allows each region to reach its correct size and proportion at the end of development.

MATERIALS AND METHODS

Fly strains and husbandry: The following strains were used: *dm^{po}* (JOHNSTON *et al.* 1999), *yw;Tub>dmyc, y⁺>Gal4/CyO; +*, and *yw;+;Tub>dmyc, y⁺>Gal4* (hereafter called *Tub-dmyc*) (DE LA COVA *et al.* 2004). Sevelin, *yw;+;+, FRT19A;ry⁵⁰⁶*, and *yw; FRT82B N-myc* were obtained from the Bloomington Stock Center. *w dm^{po} FRT19A/FM7c, w dm⁴ FRT19A/FM7*, and *FRT82B Tub-dmyc^{WT}* were gifts of P. Gallant. *yw Ubi-GFP FRT19A;hsflp1* was a gift of G. Struhl. *P{neoFRT}^{82B} M(3)96C, arm-lacZ* was a gift of E. Bach. *yw;VgGal 4, UAS-flp, Tub>CD2>Gal 4 UAS-GFP/CyO* was a gift of M. Crickmore (CRICKMORE and MANN 2006).

Embryos from appropriate crosses were collected on grape plates for 2-hr periods and ≤ 50 first instar larvae were transferred to freshly yeasted, molasses food vials and raised at 25°.

Growth measurements: *Clonal analysis:* Mutant or control (zero-copies GFP) and sibling (two-copies GFP) clones were induced by Flp/FRT-mediated mitotic recombination after larval heat shock in a 37° incubator. Heat shocks were carried out for 40 min at 48 hr after egg laying (AEL) or for 30 min at 72 hr AEL. Clones induced at 48 hr AEL were allowed to grow until either 81 hr AEL or 112 hr AEL. Clones induced at 72 hr AEL were allowed to grow until 112 hr AEL. Discs were stained for either Wg or Hth protein to define the hinge, pouch, and notum. The clonal area was measured using Axiovision software (Zeiss) as described (DE LA COVA *et al.* 2004).

Minute experiments: *Tub-dmyc^{WT}* (STEIGER *et al.* 2008) was recombined onto *FRT82B M(3)96C, arm-lacZ*. Experiments were done with *dm⁴* mutant males rescued with *Tub-dmyc^{WT}*. Animals were heat-shocked mid-second instar (72 hr AEL for *dm⁴; FRT82B Tub-dmyc/+* and 76 hr AEL for *dm⁴; FRT82B M Tub-dmyc/+*) and dissected 64 hr later (136 and 140 hr). *dm⁴* mutant clones were marked with two copies of *pi-myc* or lack of *arm-lacZ*. *FRT82B* control clones were induced at 48 hr AEL and dissected at 112 hr AEL.

Wing disc size measurements: The IR and OR of Wg expression were used to demarcate the WP and hinge (Figure 2A). Hinge size was measured at 82, 96, and 110 hr AEL as the area within the IR and OR, and WP size as the area inside the IR. Given that the hinge region of the wing disc becomes increasingly folded between 96 and 110 hr AEL, this method of size measurement underestimates actual hinge size; however, it allows an assessment of relative size trends between genotypes as the discs gain mass.

Adult wing measurements: Hinge measurements in adult wings were made by tracing the proximal to distal costa, continuing to its intersection with the radius, connecting this intersection to the allula, tracing around the allula and axillary cord, and finishing back at the proximal costa, excluding the tegula. Blade measurements were made by following the distal border of the hinge (as defined above) and encircling the rest of the blade.

Statistics used for size measurements: For size measurements, Student's *t*-test was used to determine significance of $P < 0.05$. When measurements were converted to ratios or normalized to control, a nonparametric test, Mann-Whitney, was used to determine significance of $P < 0.05$. *P*-values were calculated using Excel or MiniTab.

Quantitative PCR: The relative level of *dmyc* expression was determined by quantitative (real-time) RT-PCR on RNA isolated from 30 to 40 late L3 wing discs of each genotype (Table 1). RNA was isolated using TRIzol (Invitrogen), and single-stranded cDNA was produced from 1 μ g RNA using a SuperScript First-Strand Synthesis kit (Invitrogen). PCR reactions were performed using LightCycler FastStart DNA Master-Plus SYBR Green I kit (Roche). *dmyc* expression levels in each genotype were normalized to *act5C* or *nup44A* levels (both genes gave equivalent results) and then normalized to *yw* control. RNA was isolated from whole wing discs; thus the level of *dmyc* mRNA expression is the sum of all regions of the disc. Comparisons between discs with patterned *vs.* ubiquitous *dmyc* expression are therefore an averaging of high- and low-expressing cells.

Immunocytochemistry: RNA *in situ* hybridizations were carried out using digoxigenin-labeled RNA probes (JOHNSTON and EDGAR 1998). Fixation and immunocytochemistry of imaginal discs were carried out as described (JOHNSTON and EDGAR 1998). The following antibodies and dilutions were used: mouse anti-digoxigenin-AP, 1:2000 (Roche); rabbit anti-GFP, 1:200 (Invitrogen); mouse anti-Wg 4D4, 1:30 (Developmental Studies Hybridoma Bank); guinea pig anti-Hth, 1:2000 (gift of R. Mann); rabbit anti- β -gal, 1:2000 (MP Biomedicals); and guinea pig anti-dMyc, 1:1000 (gift of G. Morata). Secondary antibodies used were purchased from Jackson ImmunoResearch and Invitrogen Molecular Probes. Images were taken using a Zeiss Axioplan 2 microscope with an Orca-100 CCD camera (Hamamatsu) or AxioCam (Zeiss) and processed with Photoshop (Adobe) software.

Vg memory experiment: To selectively remove dMyc from wings of animals that otherwise express dMyc, we used a "memory" experiment with flies of the following genotype: *dm⁴; VgGal 4, UAS-flp, Tub>CD2>Gal 4/+ , UAS-GFP; Tub>dmyc>Gal4/+*. Cells that express *VgGal4* at any point during development will express UAS-Flp recombinase and excise

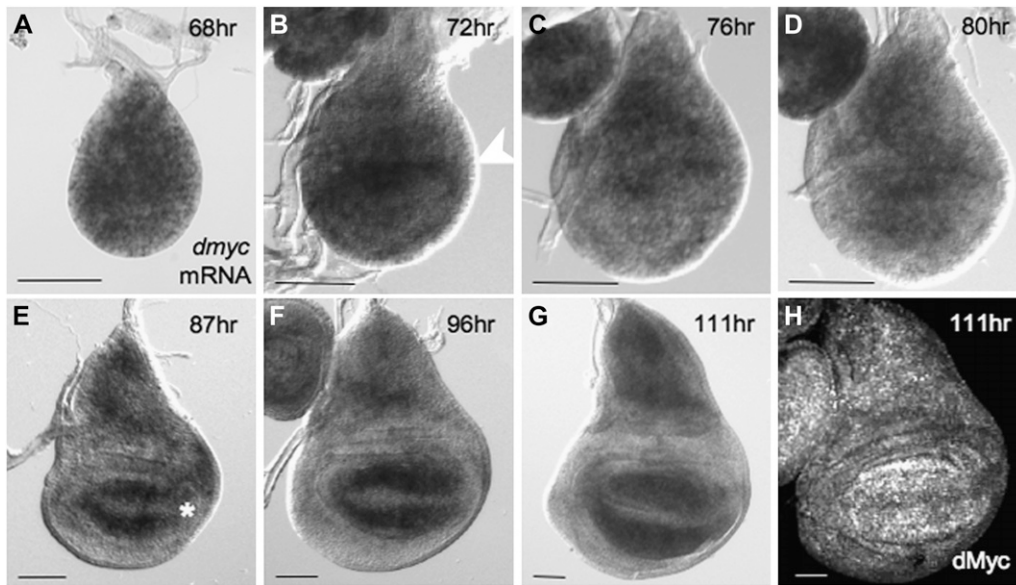


FIGURE 1.—dMyc is expressed in a dynamic pattern during wing disc development. (A–G) *dmyc* mRNA by RNA *in situ* hybridization. (A) *dmyc* is fairly uniformly expressed at early stages of wing disc development. (B and C) Early in the third instar, *dmyc* expression increases in hinge cells (arrowhead in B; also see Figure S1). (D–G) Later, *dmyc* expression gradually decreases in hinge cells. At the same time, *dmyc* expression in WP cells intensifies. Still later, *dmyc* expression is repressed in cells at the D/V boundary as they undergo cell cycle and growth arrest (asterisk in E). (H) dMyc protein expression is similar to that of *dmyc* mRNA. Bars, 50 μ m.

the $>CD2>$ cassette and the $>dmyc>$ cassette. Once the $>CD2>$ cassette is excised, the cells will heritably express GFP from the tubulin promoter. Loss of *dmyc* expression occurs only from cells that have expressed *VgGal4* in their lifetime, while the rest of the animal retains the *Tub>dmyc>Gal4* cassette. The *VgM* driver is activated prior to when dMyc expression becomes patterned in the wing (supporting information, Figure S5, A and B). In addition to the wing disc, GFP-positive cells can be found in the haltere, salivary glands, and a few cells of the leg and brain in the *VgM* experiment (data not shown).

RESULTS

dMyc is expressed in a dynamic pattern during wing disc development: To determine how dMyc contributes to the growth of proximal and distal regions of the wing, we first examined its mRNA and protein expression during the developmental transitions that specify the fates of each region. *dmyc* mRNA is expressed in all cells early in L2 (Figure 1A). As hinge development begins at the transition to L3, *dmyc* expression transiently increases in proximal cells that also initiate expression of the IR of Wg (Figure 1B, Figure S1). As L3 proceeds, *dmyc* expression gradually decreases throughout the dorsal and ventral hinge region, while remaining at high levels in the dorsal body-wall primordium, the notum. At the same time, *dmyc* expression increases in distal cells (Figure 1, C–F). By the end of larval development *dmyc* expression is very low in hinge cells but high in cells of the notum and WP. With the onset of proneural specification at the wing margin, *dmyc* expression is repressed by the activities of Wg and Notch in cells flanking the D/V boundary as these cells arrest growth and division (Figure 1G) (JOHNSTON *et al.* 1999; JOHNSTON and SANDERS 2003; DUMAN-SCHEEL *et al.* 2004; HERRANZ *et al.* 2008). dMyc protein expression is similar to *dmyc*

mRNA at all stages examined (Figure 1H, Figure S1, and data not shown). These observations indicate that the expression of *dmyc* is closely allied with the subdivision of the disc into regions of proximal and distal cell fates.

The dynamic expression of dMyc correlates with changes in disc growth: The dynamic temporal and regional expression pattern of dMyc led us to hypothesize that the functional requirement for dMyc changes during growth of the wing disc. To test this idea, we used Flp/FRT-mediated mitotic recombination to remove *dmyc* function in cell clones at specific times during disc growth (XU and RUBIN 1993). Clones of wing disc cells mutant for either the hypomorphic allele *dm^{po}* or the null *dm⁴* allele were marked by the absence of GFP, as were control clones induced in parallel experiments. The area of each mutant clone was measured after defined periods of growth and compared to controls. As cells remain in close proximity after division, this provides a reasonable measure of growth (NEUFELD *et al.* 1998; JOHNSTON *et al.* 1999; DE LA COVA *et al.* 2004).

We used the transitions in *dmyc* expression as a guide for the initiation and duration of clone growth. To examine the dMyc requirement from the early period of uniform *dmyc* expression to its upregulation in hinge cells, clones were induced at the onset of L2, 48 hr AEL, and allowed to grow until 81 hr AEL, early in L3. The requirement for dMyc during later transitions in *dmyc* expression was examined with mutant and control clones grown from the onset of L3 at 72 or 81 hr AEL until 112 hr AEL, late in L3. Comparison of mutant and control clone size revealed a clear requirement for *dmyc* function at each time point (Figure 2D, Figure S2). Throughout L2 and L3, *dm^{po}* and *dm⁴* mutant clones grew at substantially reduced rates compared to control clones and were significantly smaller than controls at

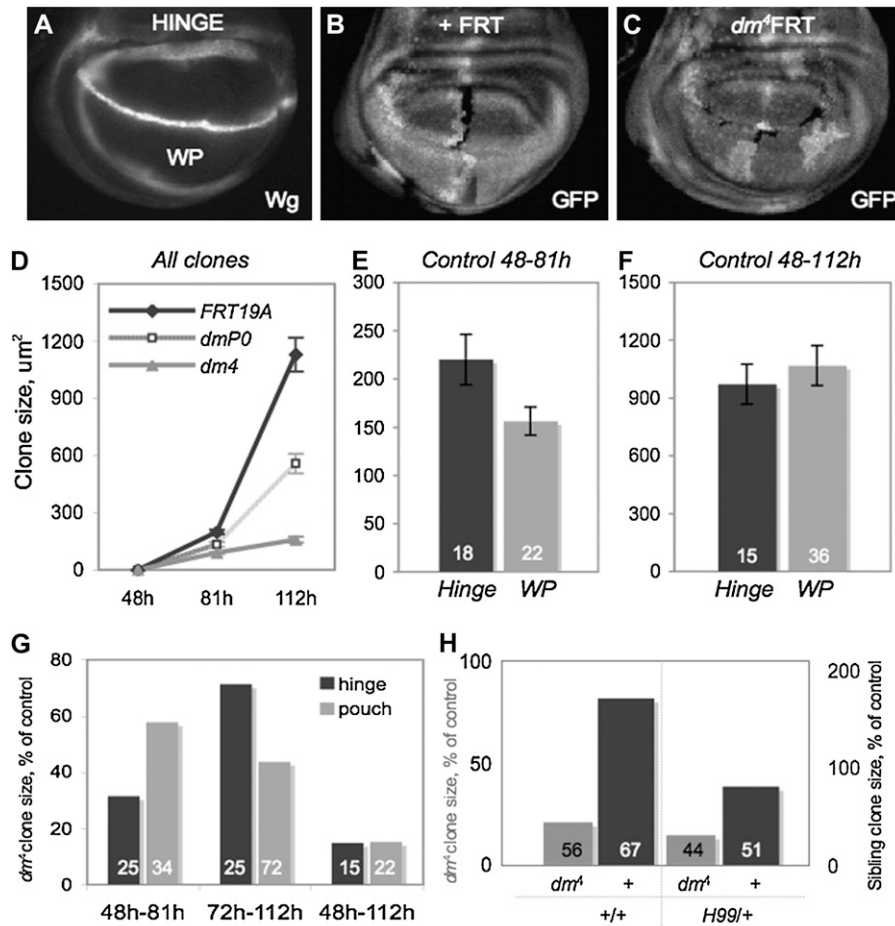


FIGURE 2.—Regional and temporal growth requirements for dMyc reflect its expression pattern during wing disc growth. (A) Analysis of control and *dm⁴* mutant clones in female larvae to determine growth requirements for dMyc. Clones were induced at specific times and scored on the basis of location: hinge (region within Wg rings) and WP (region within inner ring of Wg). Different regions of the disc were scored using either Wg or Hth staining. (B and C). Mitotic recombination produces a GFP-negative clone and a sister clone with two copies of GFP. The GFP-negative *dm⁴* mutant clones (*dm^{P0}*, hypomorphic allele, or *dm⁴*, null allele) (C) were compared to GFP-negative control clones (B). (D) Clonal growth of *dm^{P0}* mutant cells and *dm⁴* mutant cells show a dose-dependent requirement for *dm⁴* in cell proliferation and growth. Clones were induced at 48 hr AEL and dissected at either 81 hr AEL or 112 hr AEL to assess growth over time (where clone growth is the product of cellular growth, cell division, and cell survival). (E) Hinge cell clones grow significantly larger than WP clones early ($P = 0.04$). (F) WP clones later grow more to reach the same size as hinge clones by 112 hr AEL. (G) *dm⁴* mutant clones are significantly smaller than control clones in all regions both early and late in development ($P < 10^{-3}$ for all *dm⁴* mutant clones compared to

corresponding control). Hinge cells are more sensitive to loss of *dm⁴* early in development, whereas WP cells are more sensitive to loss of *dm⁴* late in development. Early, *dm⁴* hinge clones grow to 32% of control hinge clone size while *dm⁴* WP clones reach 58% of control WP clone size (Mann–Whitney test, $P < 10^{-4}$). Later, *dm⁴* WP clones grow to only 44% of control WP clone size while *dm⁴* hinge clones reach 71% of control hinge clone size (Mann–Whitney test, $P < 10^{-4}$). By the end of development, *dm⁴* clones in either region are $< 20\%$ of corresponding control clone size. (H) Wild-type sibling clones (GFP++) of *dm⁴* mutant clones that have grown from 48 hr AEL to 112 hr AEL are significantly bigger than corresponding control GFP++ clones ($P < 10^{-9}$). In a *H99/+* background, wild-type sibling clones of *dm⁴* mutant clones are no longer significantly bigger than corresponding control GFP++ clones ($P = 0.17$). The *H99/+* background does not alter the size of the *dm⁴* mutant clone (combined from all regions: $P = 0.83$). Bar graphs are labeled with number of clones measured.

the end of the growth phase (Figure 2D). The amount of growth that occurred was dMyc dose dependent, such that *dm^{P0}* clones grew more at each time point than *dm⁴* clones. Despite their significantly reduced size, *dm⁴* null mutant clones proliferate to some extent (median number of cells = 17, 48–112 hr AEL clones), indicating that cells lacking *dm⁴* grow at a rate set by other growth regulatory mechanisms.

We then examined the functional requirement for dMyc in distal and proximal regions of the disc at specific times. Control and *dm⁴* mutant clones were generated as above and scored on the basis of their location in either hinge or WP (Figure 2A). We focused on these regions in particular because their counterparts are easily measured in adults. The early L3 increase in *dm⁴* expression in cells fated to be hinge (Figure 1, B–D; Figure S1) predicted that its loss at that time would compromise the growth of these cells. We

therefore allowed *dm⁴* and control clones to grow from 48 to 81 hr AEL and compared the extent of their growth (Figure 2D). During this period, control clones in the hinge grew significantly larger than those in the WP (Figure 2E). *dm⁴* hinge clones grew slowly and reached only 32% of control clone size at the end of the growth period (Figure 2G). *dm⁴* clones located in the WP also grew slowly, but were not as compromised as hinge clones and reached 58% of control WP clone size (Figure 2G). Thus, hinge cells are particularly sensitive to loss of *dm⁴* during L2–early L3. This suggests that the increase in *dm⁴* expression in hinge cells during this time contributes to their relatively faster rate of growth (Figure 2G).

Midway through L3, *dm⁴* expression again changes: it increases in WP cells and is reduced in hinge cells (Figure 1, E–G). These changes suggest that the requirement for dMyc in hinge cells is reduced as de-

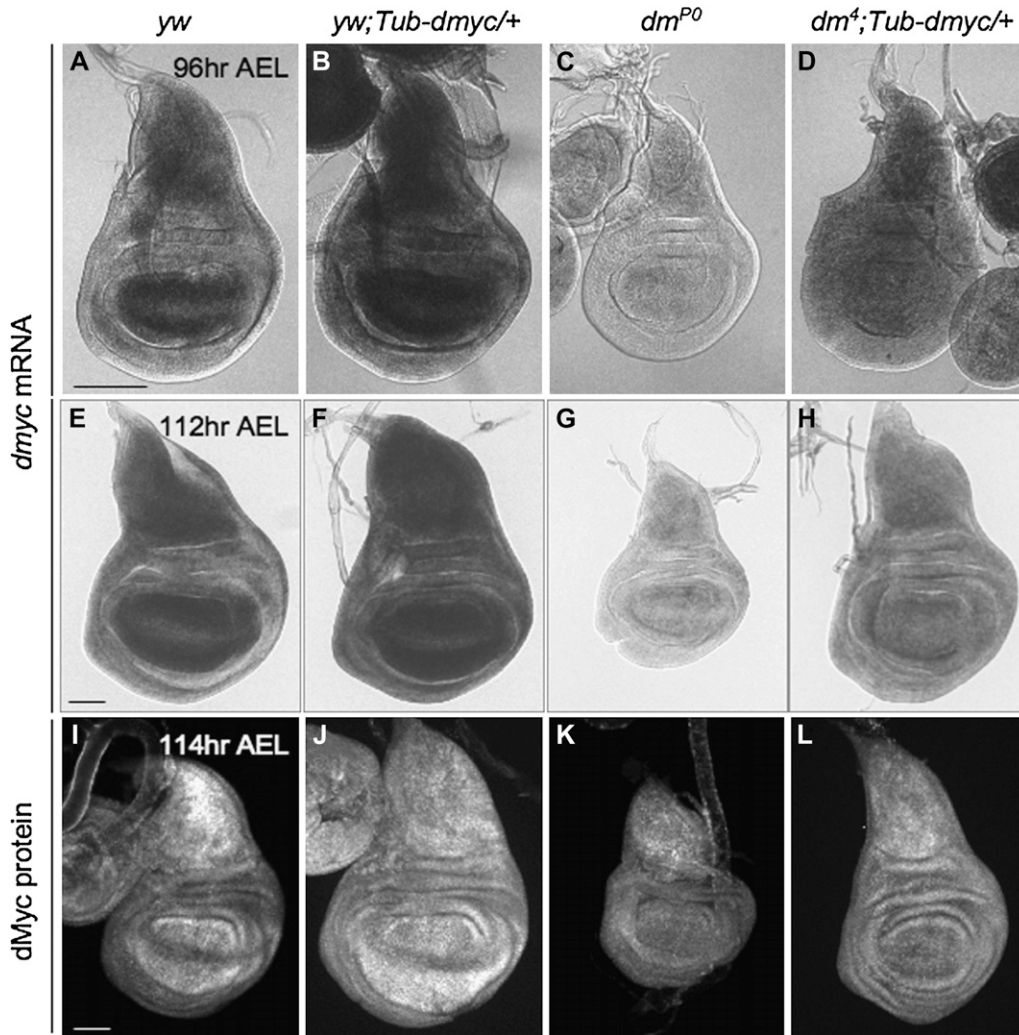


FIGURE 3.—dMyc expression after experimental manipulation. *dmyc* mRNA in wing discs at 96 hr AEL (A–D) and 112 h AEL (E–H) of male larvae. (A and E) *yw*: wild-type level and patterned *dmyc* expression. (B and F) *yw; Tub-dmyc/+; +; +*: wild type pattern plus additional ubiquitous expression driven by *Tub-dmyc* (condition 1). (C and G) *dm^{P0}*: low-level, but wild-type pattern, dMyc expression (condition 2). (D and H) *dm⁴; Tub-dmyc/+; +; +*: low-level, ubiquitous, dMyc expression (condition 3). The additional staining in the WP appears to coincide with the folds in this region and is less evident at 96 hr when the disc is less folded. (I–L) dMyc protein in wing discs at 114 hr AEL. (I) *yw*. (J) *yw; Tub-dmyc/+; +; +*. (K) *dm^{P0}*. (L) *dm⁴;Tub-dmyc/+; +; +*.

velopment proceeds, whereas in WP cells it increases. Whereas significantly smaller at 81 hr AEL ($P = 0.04$), control WP cell clones reached the same size as hinge clones at the growth period's end, suggesting that they grew more during 81–112 hr AEL (Figure 2, E and F). Growth of *dm⁴* mutant WP clones was significantly impaired and reached only 44% of control clone size by the end of the growth period (Figure 2G). In contrast, *dm⁴* clones in the hinge were less compromised and reached 71% of control hinge clone size (Figure 2G). These data indicate that the temporal changes in the pattern of *dmyc* mRNA and protein expression are accompanied by dynamic regional requirements for dMyc function during wing development.

Even though hinge and WP cells appear to have temporally different requirements for dMyc, proliferation of cells in both regions was significantly impaired by its loss. The average size of a *dm⁴* mutant clone that grew from 48 to 112 hr AEL was <20% of the size of a corresponding control clone (Figure 2G). The reduced growth could be due to cell-autonomous loss of *dmyc* function or to nonautonomous cell death induced by cell competition (JOHNSTON *et al.* 1999; DE LA COVA *et al.*

2004). We assessed the contribution of cell competition in these experiments by examining the size of wild-type sibling clones, marked by two copies of GFP (GFP++), that were generated along with the mutant clones by recombination. Sibling GFP++ clones of *dm⁴* mutant clones (GFP–) were considerably larger than control GFP++ clones induced in parallel (Figure 2H). Stimulation of faster growth of “winner” cells in response to slow growth of “loser” cells is a hallmark of cell competition (JOHNSTON 2009); thus these results indicate that competition occurs between wild-type sibling cells and *dm⁴* mutant cells (and also the nonclonal *dm⁴/+* cells). This was confirmed by removing one copy of the proapoptotic genes *hid*, *grim*, and *rpr* with the *H99* deficiency (*H99/+*), which prevents 90% of competition-induced cell death due to dMyc overexpression (DE LA COVA *et al.* 2004). *dm⁴* clones generated in a *H99/+* background prevented the extra sibling clone growth. However, it did not appreciably alter the ability of *dm* null mutant cells to proliferate ($P = 0.83$) or to increase the frequency of their recovery (16% of GFP++ sibling clones were not accompanied by *dm⁴* clones; this frequency was 14% in the *H99/+* background) (Figure

TABLE 1
Modulation of the *dmyc* expression pattern alters wing disc size in late-L3 wing discs

Condition	Genotype	% <i>yw</i> WP	% <i>yw</i> H	WP:H ratio	<i>dmyc</i> mRNA	<i>n</i>
Wild type	<i>yw</i>	100	100	1.4	1.0	19
1	<i>yw, Tub-dmyc/+</i>	105	109	1.1	1.7	31
2	<i>dm^{po}</i>	46	56	1.2	0.2	24
3	<i>dm^t; Tub-dmyc/+</i>	63	74	1.2	0.8	30
	<i>dm^t;+;Tub-dmyc/+</i>	62	77	1.2	ND	19

Wing disc size from male larvae at late L3 (110-hr time point in Figure 4). Conditions 1–3 are as described in the text. *yw* is used as a wild type (WP) and hinge (H). *dmyc* mRNA level per wing disc cell is relative to *yw*. *dm^t;+;Tub-dmyc/+* measurements were measured in a separate experiment; this genotype is delayed by 23 hr compared to *yw* and *dm^t; Tub-dmyc/+*.

2H). We also assessed the growth potential of *dm^t* mutant clones by inducing them in a *Minute* heterozygous background (*M/+*). We found that, although the additional growth advantage in a *M/+* background significantly increased *dm^t* mutant clone size ($P = 1.0 \times 10^{-4}$), these clones were still only an eighth of the size of a control clone grown for the same period of time (Figure S3). We interpret these results collectively to mean that cells carrying the null *dm^t* allele have a very limited growth potential. We therefore conclude that the slow growth of *dm^t* clones is primarily due to a cell-intrinsic requirement for dMyc activity.

The sum of these experiments suggest that during L2 and L3 the distal and proximal regions of the wing disc grow with distinct characteristics that correlate with changes in dMyc expression. After an initial period in which dMyc is expressed uniformly in the disc, its expression transiently increases in cells fated to become hinge. This increase correlates with an increase in the proliferation rate of hinge cells compared to WP cells. A short time later, dMyc expression changes again, now decreasing in hinge cells but increasing in cells of the WP. This change, which occurs mid-L3, is correlated with a relative increase in WP cell proliferation. Also during this period, WP cells straddling the D/V boundary lose dMyc expression and exit the cell cycle (JOHNSTON and EDGAR 1998; JOHNSTON *et al.* 1999). We conclude that the level of dMyc expression is correlated with the differential growth rates of cells in the wing disc.

The endogenous pattern of dMyc expression is dispensable for wing growth: Thus far our results indicate that the patterned expression of dMyc is correlated with the regional growth rate differences observed during wing disc development and suggest that dMyc expression might contribute growth instructions for wing shape. We tested this hypothesis by experimentally manipulating the expression pattern and the level of dMyc expression during wing development. We created three genetic conditions in which the pattern and/or intensity of *dmyc* expression differed from our wild type control (*yw*, Figure 3, A, E, and I), using *dm* mutants and

strains containing a transgene that ubiquitously expresses dMyc at low levels under the *Tubulin α -1* promoter (*Tub-dmyc*) (DE LA COVA *et al.* 2004) as follows:

Condition 1 (*yw;Tub-dmyc/+*): These flies express the wild-type *dmyc* expression pattern plus additional ubiquitous expression driven by *Tub-dmyc* (Figure 3, B, F, and J). The transgene increased *dmyc* expression ~65% over control wing discs (percentages of each condition were measured by quantitative RT-PCR and normalized to control *yw* wing discs; Table 1).

Condition 2 (*dm^{po}*): *dmyc* is expressed in the wild-type pattern but at 15% of control wing disc levels (Figure 3, C, G, K; Table 1).

Condition 3 (*dm^t;Tub-dmyc/+;+*, or *dm^t;+;Tub-dmyc/+*): The endogenous pattern of expression is abolished due to the *dm^t* null mutation. This is replaced by ubiquitous expression of *dmyc* at ~77% of control wing disc levels (Figure 3, D, H, and L; Table 1). The results of each of these experiments are summarized in Table 1 and described below.

The *Tub-dmyc* transgene rescues the L2 lethality of hemizygous *dm^t* male larvae to pharate adulthood, but only 2% of these animals eclosed (Table S1). Expression of mRNA and protein from the *Tub-dmyc* transgene was verified by RNA *in situ* hybridization and by immunofluorescence, respectively (Figure 3), and the relative level of *dmyc* mRNA was quantified by RT-PCR for each genetic condition (Table 1). We measured hinge and WP size in wing discs from each condition at three time points during L3 and in adult wings (see MATERIALS AND METHODS for details). Although absolute scale differs between the genetic conditions, during their growth the discs of each condition take on appropriate and characteristic folds in the hinge and pleural regions, allowing size comparisons between them (Figure 3, Figure S4).

In control wing discs, the hinge and WP are similar in size at 82 hr AEL (Figure 4, A and C). During the subsequent 28 hr, isometry is lost and WP size increases faster than hinge size (Figure 4A), resulting in a significant rise in the ratio of WP to hinge (WP:H) size over time from 1.1 to 1.4 at the latest time point (Figure 4C). The

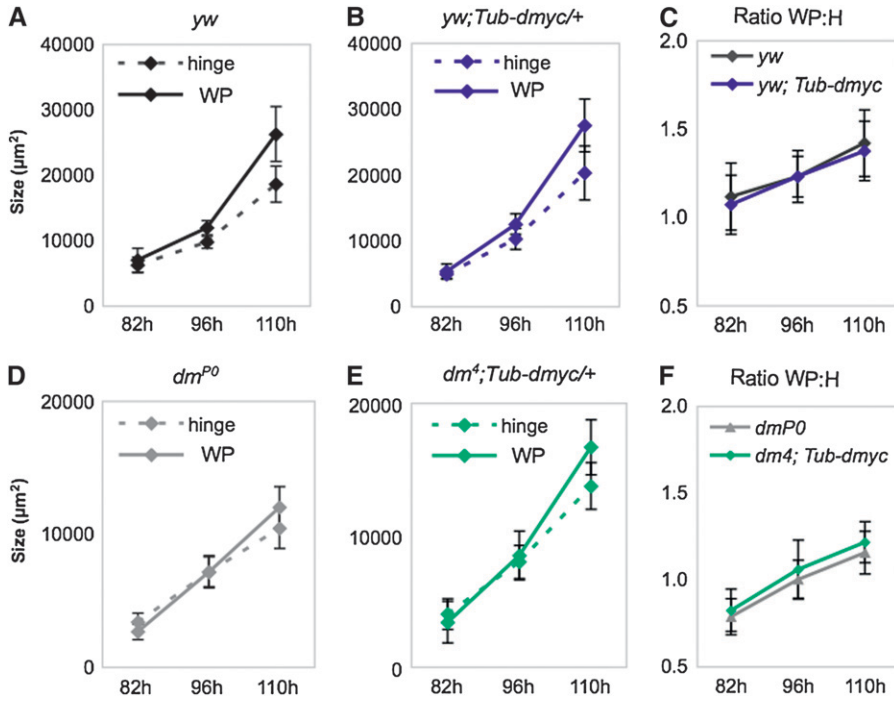


FIGURE 4.—Proportional growth in the wing disc does not require patterned expression of dMyc, but growth rate is reduced in its absence. Hinge and WP sizes throughout development in male larvae. (A) Our wild type strain, *yw*. (B) *yw* plus *Tub-dmyc/+* wing discs (condition 1). (A and B) At 82 hr AEL, hinge and WP sizes do not differ in either *yw* or *yw; Tub-dmyc/+* wing discs (hinge vs. WP 82 hr: *yw*, $P = 0.18$; *yw; Tub-dmyc/+*, $P = 0.23$), but are different at 96 and 110 hr for both genotypes (hinge vs. WP 96 hr: *yw*, $P = 4.0 \times 10^{-8}$; *yw; Tub-dmyc/+*, $P = 2.2 \times 10^{-4}$; hinge vs. WP 110 hr: *yw*, $P = 2.2 \times 10^{-7}$; *yw; Tub-dmyc/+*, $P = 2.2 \times 10^{-9}$). (C) Ratios of wing WP to hinge (WP:H) in control *yw* and *yw; Tub-dmyc/+* wing discs throughout development. WP:H significantly increases over time in both *yw* and *yw; Tub-dmyc/+* wing discs (Mann-Whitney test—*yw*: 82 vs. 96 hr, $P = 0.027$; 96 vs. 110 hr, $P = 5.0 \times 10^{-4}$; *yw; Tub-dmyc/+*: 82 vs. 96 hr, $P = 4.8 \times 10^{-3}$; 96 vs. 110 hr, $P = 8.0 \times 10^{-3}$), but WP:H ratios of *yw*

and *yw; Tub-dmyc/+* wing discs are not different from each other at any time point (Mann-Whitney test—*yw* vs. *yw; Tub-dmyc/+*: 82 hr, $P = 0.47$; 96 hr, $P = 0.91$; 110 hr, $P = 0.41$). (D) *dm^{po}* (condition 2). (E) *dm⁴; Tub-dmyc/+* (condition 3). (D and E) At 82 hr, hinge and WP size are significantly different in *dm^{po}* wing discs ($P < 10^{-3}$) but are not different in *dm⁴; Tub-dmyc/+* wing discs ($P = 0.27$). At 96 hr AEL, hinge and WP size are not significantly different in either *dm^{po}* or *dm⁴; Tub-dmyc/+* wing discs (hinge vs. WP at 96 hr: *dm^{po}*, $P = 0.99$; *dm⁴; Tub-dmyc/+*, $P = 0.26$) but are different at 110 hr in both genotypes (hinge vs. WP at 110 hr: *dm^{po}*, $P = 8.0 \times 10^{-4}$; *dm⁴; Tub-dmyc/+*, $P = 2.2 \times 10^{-7}$). (F) Ratios of WP:H in *dm^{po}* and *dm⁴; Tub-dmyc/+* wing discs significantly increase over time (Mann-Whitney test—*dm^{po}*: 82 vs. 96 hr and 96 vs. 110 hr, $P = 1.0 \times 10^{-4}$; *dm⁴; Tub-dmyc/+*: 82 vs. 96 hr and 96 vs. 110 hr, $P < 10^{-4}$) and were consistently smaller than wild type at every time point (Mann-Whitney test—*yw* vs. *dm^{po}* at 82, 96, 110 hr: $P < 10^{-4}$; *yw* vs. *dm⁴; Tub-dmyc/+* at 82 and 96 hr: $P = 1.0 \times 10^{-4}$ and at 110 hr: $P = 2.0 \times 10^{-4}$). WP:H ratios of *dm^{po}* and *dm⁴; Tub-dmyc/+* wing discs were not significantly different at any time point (Mann-Whitney test—*dm^{po}* vs. *dm⁴; Tub-dmyc/+* at 82 hr: $P = 0.44$; at 96 hr: $P = 0.91$; and at 110 hr: $P = 0.41$). Measurements were done with animals carrying a *Tub-dmyc* cassette on chromosome 2. All P -values are derived from Student's t -tests unless otherwise indicated. Error bars show standard deviation. Three additional experiments showed similar trends.

late increase in WP:H ratio coincides with the upregulation of *dmyc* expression in WP cells and the concomitant expression decrease in hinge cells (Figure 1).

Condition 1: Although the *Tub-dmyc* transgene in *yw* wing discs increases dMyc expression by 65%, the growth of the discs does not deviate from the normal trend. Hinge and WP size are similar at 82 hr AEL but subsequently diverge due to faster growth of the WP (Figure 4B). As in controls, the WP:H size ratio increased from 1.1 to 1.4 over the 28 hr of growth in our experiments (Figure 4C).

Condition 2: Wing discs from the hypomorphic mutant *dm^{po}* express *dmyc* in the endogenous pattern but at substantially reduced levels (Figure 3, C, G, and K; Table 1). The reduced *dmyc* expression significantly impairs disc growth and leads to a smaller overall disc size at each time point (Figure 4D). We noted several differences in the kinetics of growth of these discs. In contrast to controls, hinge size is significantly larger than WP size at 82 hr AEL ($P = 0.007$), and parity of size between the two regions is reached sometime after 82 hr but prior to 96 hr AEL (Figure 4D). Moreover, WP size

does not exceed hinge size until 110 hr AEL, ~12 hr later than controls (Figure 4D). Although the WP:H size ratio of *dm^{po}* discs increases incrementally (0.8 at 81 hr, 1.2 at 110 hr), it is consistently smaller than controls (Figure 4, C and F). In general, the hinge region grows more than the WP at all time points, but both regions are ~50% smaller than *yw* controls at 110 hr. Larvae of this genotype are significantly delayed in their development and grow for an additional 10 hr beyond our last measuring point (data not shown). These observations suggest that the 85% reduction in *dmyc* expression in *dm^{po}* wing discs leads to a growth program whose trend is virtually identical to controls, but which occurs at a significantly slower rate.

Condition 3: Wing discs in which endogenous expression of *dmyc* is completely replaced by a low, ubiquitous level of *dmyc* expression (*dm⁴; Tub-dmyc*; Figure 3, D, H, and L) are severely reduced in size at all stages (Figure 4E). As with discs from condition 2, the relative growth changes between hinge and WP in these discs are delayed. In this case, isometry between hinge and WP size exists at 82 hr and also at 96 hr AEL. Regional

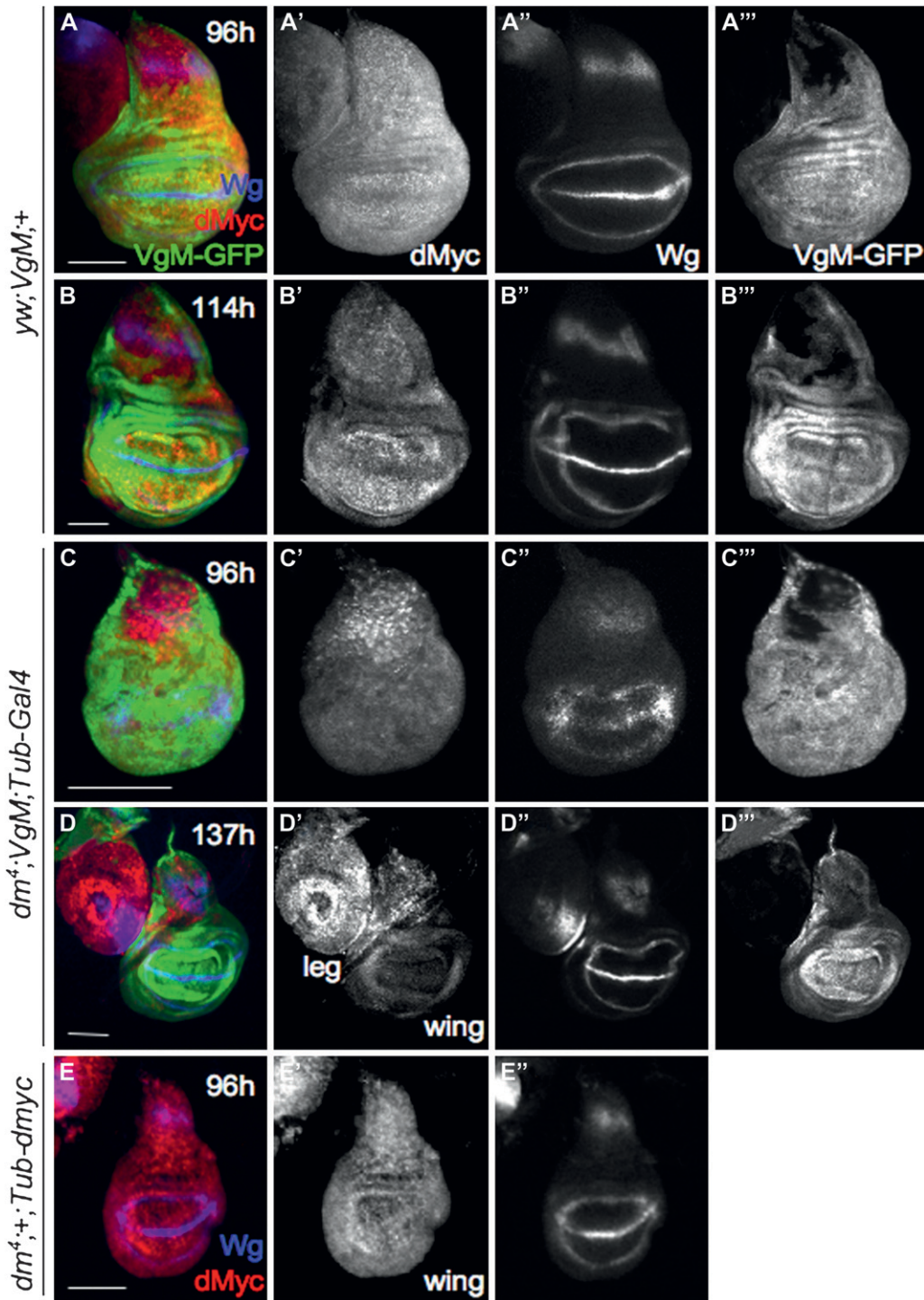


FIGURE 5.—Loss of *dMyc* in wing discs slows their growth and patterning. *dMyc* (red) and *Wg* (blue) antibody staining and *VgM-GFP* expression (green) in 96-hr and late-L3 wing discs. (A) Ninety-six-hour *yw;VgM/+* wing disc: (A') *dMyc*, (A'') *Wg*, and (A''') *GFP*; (B) 114-hr (late-L3) *yw;VgM/+* wing disc: (B') *dMyc*, (B'') *Wg*, and (B''') *GFP*; (C) 96-hr *dm⁴;VgM;Tub-Gal4/+* wing disc: (C') *dMyc* and (C'') *Wg* expression in 96-hr *dm⁴;VgM;Tub-Gal4/+* wing discs (immature relative to *dm⁴;Tub-dmyc/+* wing discs of the same chronological age; compare to E''). The pattern in C'' is instead comparable to control *yw* wing discs at 72 hr AEL (Figure S1, A''–C''); (C''') *GFP*; (D) 137-hr (late-L3) *dm⁴;VgM;Tub-Gal4/+* wing disc: (D') *dMyc* and (D'') *Wg*. By late-L3, *Wg* expression has matured (compare to B''): (D''') *GFP*; (E) 96-hr AEL *dm⁴;Tub-dmyc/+* wing disc: (E') *dMyc* and (E'') *Wg* expression for comparison with discs in C–C''.

growth rates diverge only after 96 hr AEL, and by 110 hr AEL the WP is significantly larger than the hinge ($P = 2 \times 10^{-7}$). The increase in WP:H ratio of these discs is similar to *dm⁴* wing discs (0.8 at 81 hr to 1.2 at 110 hr; Figure 4F) and is significantly smaller than the ratio of *yw* controls at all time points (Figure 4, F and C; Table 1).

Taken together, these experiments demonstrate that wing development can occur in the absence of the endogenous pattern of *dmyc* expression. In wild type, distal and proximal regions of the wing disc switch from

isometric to allometric growth late in L3, and this switch is not prevented when the normal pattern of *dmyc* expression is altered. This switch occurs even when the endogenous pattern of *dmyc* expression is completely replaced by ubiquitous expression, although reduced levels of *dmyc* expression significantly delay its onset. These data suggest that the dynamic pattern of *dmyc* expression does not instruct the regional growth changes in the wing disc. Instead, they imply that the absolute level of *dmyc* expression is critical to set the rate at which

TABLE 2
Rudimentary, disproportionate wings form in the absence of dMyc

Genotype	A. Male wing discs		WP:H ratio	n
	% <i>yw</i> WP	% <i>yw</i> H		
<i>yw</i> ;+;+	100	100	1.5	6
<i>dm⁴</i> ;+; <i>Tub-dmyc</i> /+	62	77	1.2	9
<i>dm⁴</i> ;VgM/+; <i>Tub-Gal4</i> /+	41	56	1.1	24
Genotype	B. Adult male wings		B:H ratio	n
	% <i>yw</i> blade	% <i>yw</i> hinge		
<i>yw</i> ;+;+	100	100	9.6	34
<i>yw</i> ;VgM/+;+	97	95	9.7	37
<i>yw</i> ;VgM/+ <i>Tub-Gal4</i> /+	94	94	9.5	30
<i>dm⁴</i> ;+ <i>Tub-dmyc</i> /+	72	69	9.9	28
<i>dm⁴</i> ;VgM/+; <i>Tub-Gal4</i> /+	45	55	7.8**	8 ^a

Wing specific loss of *Tub>dmyc* results in disproportionately smaller wing blades. ** $P = 9.3 \times 10^{-11}$ (vs. *yw*;VgM); $P = 2.0 \times 10^{-8}$ vs. *yw*); $P = 6.7 \times 10^{-13}$ (vs. *dm⁴*;Tub>dmyc>*Gal4*/+.)

^aSimilar trends were obtained in three independent experiments; $n = 15$ for *dm⁴*;VgM; *Tub-Gal4*/+.

wing disc growth proceeds and that this rate determines the size of each region at the end of larval development.

The complete absence of dMyc slows growth of wing discs and delays wing patterning: The *dm⁴* allele is lethal during late L1, primarily due to the requirement for dMyc in endoreplication of larval cells (PIERCE *et al.* 2008). The *Tub-dmyc* transgene in *dm⁴*;Tub-dmyc larvae rescues the entire animal, including the endoreplicating cells. To determine the role of dMyc in wing growth while avoiding animal lethality, we engineered animals in which larval cells express dMyc while in wing imaginal discs it is completely absent (see MATERIALS AND METHODS for details). We used the *dm⁴*;Tub-dmyc animal and selectively removed the *dmyc* transgene from wing discs with Flp/FRT-mediated recombination by taking advantage of FRT sites that flank the *dmyc* cDNA (DE LA COVA *et al.* 2004). Recombination is induced in wing discs upon expression of UAS-Flp recombinase under control of *Vestigial-Gal4* (*Vg-Gal4*), a wing driver (CRICKMORE and MANN 2006). This driver is expressed from ~50 hr AEL throughout the rest of disc development (Figure S5; Figure 5, A and B). As a wing disc cell expresses *Vg-Gal4*, UAS-Flp is expressed and excision of the *dmyc*-FRT cassette occurs. The progeny of every *Vg-Gal4*-expressing cell will heritably express UAS-GFP and create a permanent “memory” of *Vg-Gal4* expression in the disc (CRICKMORE and MANN 2006). For simplicity, we call this the *Vg*-memory experiment (*VgM*). In *dm⁴*;VgM;Tub-dmyc flies, WP and hinge cells excise the *dmyc* cDNA and therefore lose all *dmyc* expression, while the rest of the animal retains the intact *Tub-dmyc* cassette. Monitoring this process with expression of UAS-GFP indicates that *dmyc* is excised by 56 hr AEL (Figure S5B). The half-life of dMyc protein is ~30 min (GALLETTI *et al.* 2009); thus it is presumably lost soon after the excision.

We examined the development of these *dm⁴* mutant wings (*dm⁴*;VgM;Tub-Gal4 in Figure 5, C and D, and Table 2A) by dissecting them from L3 larvae at 96 and 137 hr AEL. By 96 hr, GFP fills the entire WP and hinge regions of both control and experimental discs, indicating that *VgGal4* had at one time been expressed in all of these cells (only a portion of the notum remains GFP-negative; Figure 5, A'' and C''). In wing discs from *dm⁴*;VgM;Tub-dmyc animals, dMyc is undetectable in all GFP-positive cells in the wing, although still present in some notum cells and in the metathoracic leg (Figure 5D'). Notably, the null mutant wing discs are smaller than both *yw*;VgM and *dm⁴*;+;Tub-dmyc controls (Table 2A), and their development is even more delayed. This delay is evident from the expression of Wingless, which is dynamic during wing development (Figure 5, A'', C'', and E'', and Figure S1). Null wing discs from *dm⁴*;VgM;Tub-dmyc animals express Wg in an immature pattern that consists of only the IR in the WP (Figure 5C''; compare to A'' and E''). Their pattern and small size is more typical of younger discs, for example, *yw* wing discs at 72 hr AEL (Figure S1). Since expression of dMyc in the larval cells of *dm⁴*;VgM;Tub-dmyc animals is identical to that of *dm⁴*;Tub-dmyc animals, we conclude that the specific loss of *dmyc* in the wing disc further slows disc growth and concomitantly delays its developmental patterning.

We considered the possibility that loss of dMyc in other tissues in which the *VgM* driver is transiently expressed, such as the haltere, and a few cells in the brain and leg discs (data not shown), might contribute nonautonomously to the delay in maturation of Wg expression in the wing disc. To control for this, we examined the patterning rate of eye discs, which never express *VgM*, by following the progression of the morphogenetic furrow. *dm⁴*;VgM;Tub-dmyc eye discs do not show a delay in size or pattern maturation compa-

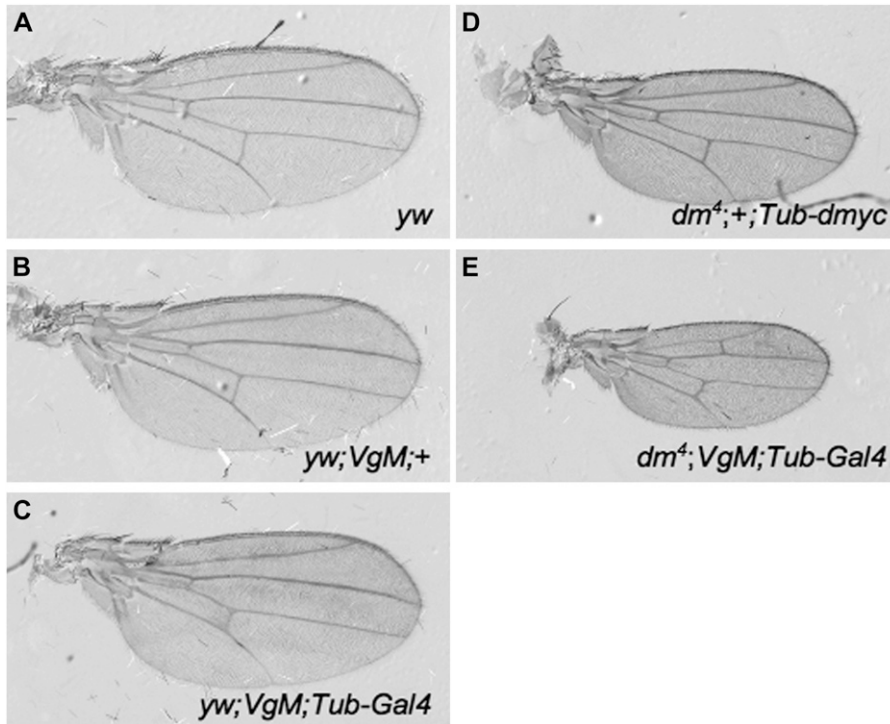


FIGURE 6.—In the complete absence of *dmyc*, wing size is severely reduced. (A–E) Adult wings (all to scale) from males of the following genotypes: (A) *yw*, (B) *yw;VgM;+*, and (C) *yw;VgM;Tub-Gal4/+*. The additional *dmyc* from the *Tub-dmyc* cassette is excised early in L2. (D) *dm⁴;+;Tub-dmyc/+*. These wings express *dmyc* solely from the *Tub-dmyc* transgene. (E) *dm⁴;VgM;Tub-Gal4/+*. *dmyc* expression is completely absent from these wings, which are significantly smaller than those in D (hinge, $P = 2.0 \times 10^{-4}$; blade, $P = 5.4 \times 10^{-9}$).

erable to wing discs from the same genotype (data not shown). These results indicate that the smaller size and delayed patterning are wing disc-autonomous responses to loss of dMyc.

Interestingly, although the *dm⁴; VgM; Tub-Gal4* null wing discs grow more slowly than wing discs from *dm⁴;+; Tub-dmyc* larvae, animals of both genotypes stop feeding and enter the wandering stage at the same time, 1 day later than *yw; VgM* controls. At this stage, null mutant wing discs are still 30% smaller than those from *dm⁴;+;Tub-dmyc* control larvae (Table 2A); however, the expression pattern of Wg has matured normally (compare Figure 5B'' with D''). This suggests that the additional day of larval development allows Wg expression to reach a mature pattern, but is not sufficient to overcome the growth defect.

Wing size is determined by the level of dMyc expression: Although our experiments suggest that the endogenous pattern of dMyc expression is non-essential, they indicate that the overall level of its expression is a critical regulator of wing disc size. The majority of wing growth occurs during larval development through cell proliferation, which ceases ~24 hr after puparium formation (SCHUBIGER and PALKA 1987). The amount of growth that occurs prior to this point largely determines the final size of the wing. However, in the pupa, the wing undergoes extensive morphologic changes that extend and flatten the blade (B) and cause compaction of the hinge (H) region, resulting in a 10-fold larger wing blade than hinge in adults (*yw* B:H size ratio = 9.6; Table 2B). To determine whether loss of dMyc expression affects these shape

changes, we examined the B:H ratio of null mutant adult wings.

Wings from control animals (*yw;VgM* and *yw;VgM;Tub-dmyc*) are similar in size to each other and to *yw*, indicating that the *VgM* condition *per se* does not alter growth and the early removal of *Tub-dmyc* in flies overexpressing dMyc leads to normal wing size (Table 2B, Figure 6, A–C). Consistent with the small wing disc size, complete loss of *dmyc* from *dm⁴;VgM;Tub-dmyc* wing discs results in significantly smaller adult wings that are only half the size of *yw* controls. However, only 1% of animals with *dmyc* mutant wings eclose (*dm⁴;VgM;Tub-dmyc*, 3 of 270 total; *yw;VgM;Tub-dmyc*, 104 of 223). The size of both blade and hinge is affected, but not equally: the blade is 55% smaller, and the hinge 45% smaller than *yw* controls. Loss of *dmyc* thus alters the B:H ratio significantly (Table 2B, Figure 6E). The reduction in blade and hinge size in adult wings is of similar magnitude to late L3 wing discs, suggesting that most of the effect of the loss of dMyc occurs during the larval stage. These results indicate that, although a rudimentary amount of wing growth can occur in the complete absence of dMyc, its loss during wing development compromises growth and alters both the size and proportions of the wing.

DISCUSSION

By examining the expression of dMyc over the course of wing development, we demonstrate that *dmyc* mRNA and protein are expressed in a temporally and spatially dynamic manner that corresponds to the subdivision of

the wing-blade primordium from the hinge primordium. This relationship raised the possibility that dMyc is specifically deployed, presumably by factors that specify regional fates, to control the growth of each region as it develops, thereby contributing to sculpting the adult wing shape. In this work, we make three major findings. First, our experiments indicate that the intricate pattern of dMyc expression in the wing disc helps cells proliferate at an appropriate rate at any given time during wing development. Second, an adult wing can form in the absence of this pattern, although it is misproportioned and rudimentary in size. Finally, the absolute level of dMyc expression determines the rate at which the developing wing grows and also the rate of pattern maturation. Each aspect of dMyc's role in wing development is discussed below.

Patterned expression of dMyc permits, but does not instruct, morphological growth of the wing: The expression pattern of dMyc in the wing is strikingly dynamic. Prior to the subdivision of the distal wing into hinge and blade, dMyc is expressed fairly uniformly, but as these regions are specified its expression undergoes transient up- and downregulation before stabilizing in a WP predominant pattern that prevails until the end of L3. Our clonal experiments indicate that the level of dMyc expression in a wing disc cell at any given time determines its rate of proliferation, and the changes in the dMyc expression pattern correlate well with changes in relative functional need. We detected clear region-specific differences in the functional requirement for dMyc that corresponded to the specification of proximal and distal wing fates. Moreover, we found that once the wing blade and hinge primordia are specified, they grow with distinct kinetics, such that midway through L2 these regions of the disc switch from isometric to allometric growth, resulting in a considerably larger WP than hinge by late L3.

Despite these correlations, however, modification of the endogenous expression pattern in whole animals demonstrated that the spatial and temporal components are less important than the absolute level of dMyc expressed. The conservative interpretation of our data is that dMyc's role in wing growth is permissive rather than instructive and that it augments a growth rate set by other mechanisms. However, it is puzzling why dMyc is expressed in an extravagant pattern that is not necessary. This pattern could be merely a remnant of evolution. Alternatively, compensatory post-transcriptional control of dMyc could occur. dMyc protein is highly regulated (GALLETTI *et al.* 2009) and is notably increased in the absence of Archipelago, a homolog of the vertebrate Fbw7 F-box protein (MOBERG *et al.* 2004). However, within our limits of detection, we observed no difference between the expression patterns of dMyc mRNA and protein at any time during wing development in our experiments. Given the high degree of flexibility during wing growth, it is possible that re-

dundancy among growth regulatory factors that function in the wing allows formation of a small but correctly shaped wing when dMyc is expressed ubiquitously or not at all. Indeed, as a whole, our results illustrate the inherent robustness of wing development.

dMyc levels determine wing scale and proportions and allow larval and imaginal growth to keep pace: The permissive role of dMyc ensures that cells proliferate at stage-appropriate rates, determines overall size, and allows the development of a wing of correct proximal and distal proportions. Our results complement those of PIERCE *et al.* (2008), who reported that wing discs carrying null mutations of both *dmyc* and *dmnt*, the dMyc antagonist, reach a size comparable to wild type after an extended L3 (3–7 days longer than wild type). In that case, loss of dMnt derepressed a subset of genes that rescued the *dm⁴* mutant phenotype. The fact that wings grow reasonably well under those conditions supports our hypothesis that the growth program of the disc is augmented rather than determined by dMyc. The larval delay in those and in our experiments is due to reduced endoreplication of larval cells, which is dMyc dependent (PIERCE *et al.* 2004, 2008; data not shown). In the *VgM* experiments, we maintained dMyc expression in most tissues while selectively removing it from the wing. Under these conditions, larval development progressed at the same rate as *Tub-dmyc*-rescued *dm⁴* mutants (condition 3), but wing disc growth was significantly slowed. The uncoupling of larval and disc growth rates resulted in an altered size relationship between the wing and hinge, implying that coordination between larval growth and imaginal growth is important for wing size and shape. Growth regulators such as dMyc thus contribute to body and organ proportionality by promoting a rate of wing disc growth that is compatible with the rate of endoreplication and growth of larval cells. Moreover, control of wing size by dMyc is dose dependent. Together, the data suggest that the *dm* gene could be an evolutionary target that contributes to the wide variability of wing size among *Drosophila* species (GARCIA-BELLIDO *et al.* 1994). Consistent with this possibility, evidence of strong selection at the *dm* locus has been documented (JENSEN *et al.* 2007).

How is dMyc expression connected to pattern formation?: Although the pattern of *dmyc* expression does not appear to instruct overall wing shape, wing cell requirements for dMyc change throughout development, possibly reflecting region-specific responsiveness to dMyc function or expression. What predisposes hinge or WP cells to respond to dMyc differently during wing disc development? Understanding how growth is governed in the different regions of the wing disc should help answer this question. Region-specific cues may be provided by the hinge selector Hth and the wing selector Vg and by Fat/Hippo signaling. The cadherins Fat and Dachshous regulate proximal (hinge) wing growth via Hippo signaling, whereas a feed-forward auto-regulatory

loop Vg brings about expansion of distal wing (blade) fates (CHO and IRVINE 2004; CHO *et al.* 2006; ZECCHA and STRUHL 2007b; ROGULJA *et al.* 2008). Regulation in both cases appears to be in response to signaling from Wg and Dpp. Wing growth appears therefore to be controlled quite indirectly. One possibility is that the amplitude of dMyc expression or activity is changed in response to modulation of Hippo and/or Vg activity by signals such as Wg and Dpp. This idea is supported by results showing that *dmyc* transcripts are significantly upregulated in *fat* mutant eye discs, in which Hippo activity is deregulated (GAROIA *et al.* 2005). Experiments to address how dMyc expression is directly regulated in the wing disc are an important goal for the future.

A growth delay in wing discs affects pattern maturation: A striking finding of our experiments is that the rate of wing disc patterning is directly influenced by the rate of its growth: complete loss of *dmyc* in the wing disc dramatically slows its growth and also slows the rate at which pattern formation matures. It is generally assumed that growth occurs downstream of patterning. This assumption is based on a variety of experimental models in which reorganization of pattern is always accompanied by growth (FRENCH *et al.* 1976). Consistent with this idea, Myc expression is regulated by several conserved factors that control pattern formation (HE *et al.* 1998; HERRANZ *et al.* 2008; JOHNSTON *et al.* 1999; PROBER and EDGAR 2002), whereas Myc itself controls the growth and proliferation of cells by regulating numerous genes required for ribosome biogenesis and protein synthesis (GREWAL *et al.* 2005; HULF *et al.* 2005). However, our experiments suggest that the hierarchy between pattern and growth is not absolute. Impaired cellular biosynthesis when dMyc is limiting may affect a cell's ability to produce proteins required for pattern specification as well as those required for cell division, cell survival, and mass accumulation. Our studies reveal an unappreciated relationship between patterning and growth that influences their coordination and is worthy of further study.

We thank the Bloomington Stock Center, the Developmental Studies Hybridoma Bank, Peter Gallant, Gines Morata, Michael Crickmore, Richard Mann, Erika Bach, and Gary Struhl for reagents; Cleo Tsanis for technical support; and Andrew Tomlinson and members of the Johnston lab for comments on the manuscript. This work was supported by grants from the National Institutes of Health (RO1HD042770 and RO1GM078464).

LITERATURE CITED

- AZPIAZU, N., and G. MORATA, 2000 Function and regulation of homothorax in the wing imaginal disc of *Drosophila*. *Development* **127**: 2685–2693.
- CASARES, F., and R. S. MANN, 2000 A dual role for homothorax in inhibiting wing blade development and specifying proximal wing identities in *Drosophila*. *Development* **127**: 1499–1508.
- CHO, E., and K. D. IRVINE, 2004 Action of fat, four-jointed, dachsous and dachs in distal-to-proximal wing signaling. *Development* **131**: 4489–4500.
- CHO, E., Y. FENG, C. RAUSKOLB, S. MAITRA, R. FEHON *et al.*, 2006 Delineation of a Fat tumor suppressor pathway. *Nat. Genet.* **38**: 1142–1150.
- COUSO, J. P., M. BATE and A. MARTINEZ-ARIAS, 1993 A wingless-dependent polar coordinate system in *Drosophila* imaginal discs. *Science* **259**: 484–489.
- CRICKMORE, M. A., and R. S. MANN, 2006 Hox control of organ size by regulation of morphogen production and mobility. *Science* **313**: 63–68.
- DE LA COVA, C., M. ABRIL, P. BELLOSTA, P. GALLANT and L. A. JOHNSTON, 2004 *Drosophila myc* regulates organ size by inducing cell competition. *Cell* **117**: 107–116.
- DIAZ-BENJUMEA, F. J., and S. M. COHEN, 1995 Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the *Drosophila* wing. *Development* **121**: 4215–4225.
- DUMAN-SCHEEL, M., L. A. JOHNSTON and W. DU, 2004 Repression of dMyc expression by Wingless promotes Rbf-induced G1 arrest in the presumptive *Drosophila* wing margin. *Proc. Natl. Acad. Sci. USA* **101**: 3857–3862.
- FRENCH, V., P. J. BRYANT and S. V. BRYANT, 1976 Pattern regulation in epimorphic fields. *Science* **193**: 969–981.
- GALLETI, M., S. RICCARDO, F. PARISI, C. LORA, M. K. SAQCENA *et al.*, 2009 Identification of domains responsible for ubiquitin-dependent degradation of dMyc by glycogen synthase kinase 3 β and casein kinase 1 kinases. *Mol. Cell. Biol.* **29**: 3424–3434.
- GARCIA-BELLIDO, A., F. CORTES and M. MILAN, 1994 Cell interactions in the control of size in *Drosophila* wings. *Proc. Natl. Acad. Sci. USA* **91**: 10222–10226.
- GAROIA, F., D. GRIFONI, V. TROTTA, D. GUERRA, M. C. PEZZOLI *et al.*, 2005 The tumor suppressor gene fat modulates the EGFR-mediated proliferation control in the imaginal tissues of *Drosophila melanogaster*. *Mech. Dev.* **122**: 175–187.
- GREWAL, S. S., L. LI, A. ORIAN, R. N. EISENMAN and B. A. EDGAR, 2005 Myc-dependent regulation of ribosomal RNA synthesis during *Drosophila* development. *Nat. Cell Biol.* **7**: 295–302.
- HARVEY, K. F., C. M. PFLEGER and I. K. HARIHARAN, 2003 The *Drosophila* Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**: 457–467.
- HE, T. C., A. B. SPARKS, C. RAGO, H. HERMEKING, L. ZAWEL *et al.*, 1998 Identification of c-MYC as a target of the APC pathway. *Science* **281**: 1509–1512.
- HERRANZ, H., L. PEREZ, F. A. MARTIN and M. MILAN, 2008 A Wingless and Notch double-repression mechanism regulates G1-S transition in the *Drosophila* wing. *EMBO J.* **27**: 1633–1645.
- HUANG, J., S. WU, J. BARRERA, K. MATTHEWS and D. PAN, 2005 The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the *Drosophila* homolog of YAP. *Cell* **122**: 421–434.
- HULF, T., P. BELLOSTA, M. FURRER, D. STEIGER, D. SVENSSON *et al.*, 2005 Whole-genome analysis reveals a strong positional bias of conserved dMyc-dependent E-boxes. *Mol. Cell. Biol.* **25**: 3401–3410.
- JENSEN, J. D., V. L. BAUER DUMONT, A. B. ASHMORE, A. GUTIERREZ and C. F. AQUADRO, 2007 Patterns of sequence variability and divergence at the diminutive gene region of *Drosophila melanogaster*: complex patterns suggest an ancestral selective sweep. *Genetics* **177**: 1071–1085.
- JOHNSTON, L. A., 2009 Competitive interactions between cells: death, growth and geography. *Science* **324**: 1679–1682.
- JOHNSTON, L. A., and B. A. EDGAR, 1998 Wingless and Notch regulate cell-cycle arrest in the developing *Drosophila* wing. *Nature* **394**: 82–84.
- JOHNSTON, L. A., and A. L. SANDERS, 2003 Wingless promotes cell survival but constrains growth during *Drosophila* wing development. *Nat. Cell Biol.* **5**: 827–833.
- JOHNSTON, L. A., D. A. PROBER, B. A. EDGAR, R. N. EISENMAN and P. GALLANT, 1999 *Drosophila myc* regulates cellular growth during development. *Cell* **98**: 779–790.
- KIM, J., K. D. IRVINE and S. B. CARROLL, 1995 Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing *Drosophila* wing. *Cell* **82**: 795–802.
- KIM, J., K. JOHNSON, H. J. CHEN, S. CARROLL and A. LAUGHON, 1997 *Drosophila* Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. *Nature* **388**: 304–308.

- MOBERG, K. H., A. MUKHERJEE, A. VERAKSA, S. ARTAVANIS-TSAKONAS and I. K. HARIHARAN, 2004 The Drosophila F box protein archipelago regulates dMyc protein levels in vivo. *Curr. Biol.* **14**: 965–974.
- NEUFELD, T. P., A. F. DE LA CRUZ, L. A. JOHNSTON and B. A. EDGAR, 1998 Coordination of growth and cell division in the Drosophila wing. *Cell* **93**: 1183–1193.
- NEUMANN, C. J., and S. M. COHEN, 1996 A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the Drosophila wing. *Development* **122**: 3477–3485.
- NEUMANN, C. J., and S. M. COHEN, 1997 Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. *Development* **124**: 871–880.
- PANTALACCI, S., N. TAPON and P. LEOPOLD, 2003 The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. *Nat. Cell Biol.* **5**: 921–927.
- PIERCE, S. B., C. YOST, J. S. BRITTON, L. W. LOO, E. M. FLYNN *et al.*, 2004 dMyc is required for larval growth and endoreplication in Drosophila. *Development* **131**: 2317–2327.
- PIERCE, S. B., C. YOST, S. A. ANDERSON, E. M. FLYNN, J. DELROW *et al.*, 2008 Drosophila growth and development in the absence of dMyc and dMnt. *Dev. Biol.* **315**: 303–316.
- PROBER, D. A., and B. A. EDGAR, 2002 Interactions between Ras1, dMyc, and dPI3K signaling in the developing Drosophila wing. *Genes Dev.* **16**: 2286–2299.
- ROGULJA, D., C. RAUSKOLB and K. D. IRVINE, 2008 Morphogen control of wing growth through the Fat signaling pathway. *Dev. Cell* **15**: 309–321.
- SCHUBIGER, M., and J. PALKA, 1987 Changing spatial patterns of DNA replication in the developing wing of Drosophila. *Dev. Biol.* **123**: 145–153.
- STEIGER, D., M. FURRER, D. SCHWINKENDORF and P. GALLANT, 2008 Max-independent functions of Myc in Drosophila melanogaster. *Nat. Genet.* **40**: 1084–1091.
- TRUMPP, A., Y. REFAELI, T. OSKARSSON, S. GASSER, M. MURPHY *et al.*, 2001 c-Myc regulates mammalian body size by controlling cell number but not cell size. *Nature* **414**: 768–773.
- UDAN, R. S., M. KANGO-SINGH, R. NOLO, C. TAO and G. HALDER, 2003 Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat. Cell Biol.* **5**: 914–920.
- WANG, S. H., A. SIMCOX and G. CAMPBELL, 2000 Dual role for Drosophila epidermal growth factor receptor signaling in early wing disc development. *Genes Dev.* **14**: 2271–2276.
- WILLIAMS, J. A., S. W. PADDOCK and S. B. CARROLL, 1993 Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing Drosophila wing disc into discrete subregions. *Development* **117**: 571–584.
- WU, S., J. HUANG, J. DONG and D. PAN, 2003 hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114**: 445–456.
- XU, T., and G. M. RUBIN, 1993 Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**: 1223–1237.
- ZECCA, M., and G. STRUHL, 2002 Subdivision of the Drosophila wing imaginal disc by EGFR-mediated signaling. *Development* **129**: 1357–1368.
- ZECCA, M., and G. STRUHL, 2007a Control of Drosophila wing growth by the vestigial quadrant enhancer. *Development* **134**: 3011–3020.
- ZECCA, M., and G. STRUHL, 2007b Recruitment of cells into the Drosophila wing primordium by a feed-forward circuit of vestigial autoregulation. *Development* **134**: 3001–3010.
- ZECCA, M., K. BASLER and G. STRUHL, 1996 Direct and long-range action of a wingless morphogen gradient. *Cell* **87**: 833–844.

Communicating editor: T. SCHÜPBACH

GENETICS

Supporting Information

<http://www.genetics.org/cgi/content/full/genetics.109.110379/DC1>

Control of Wing Size and Proportions by *Drosophila Myc*

D. Christine Wu and Laura A. Johnston

Copyright © 2009 by the Genetics Society of America

DOI: 10.1534/genetics.109.110379

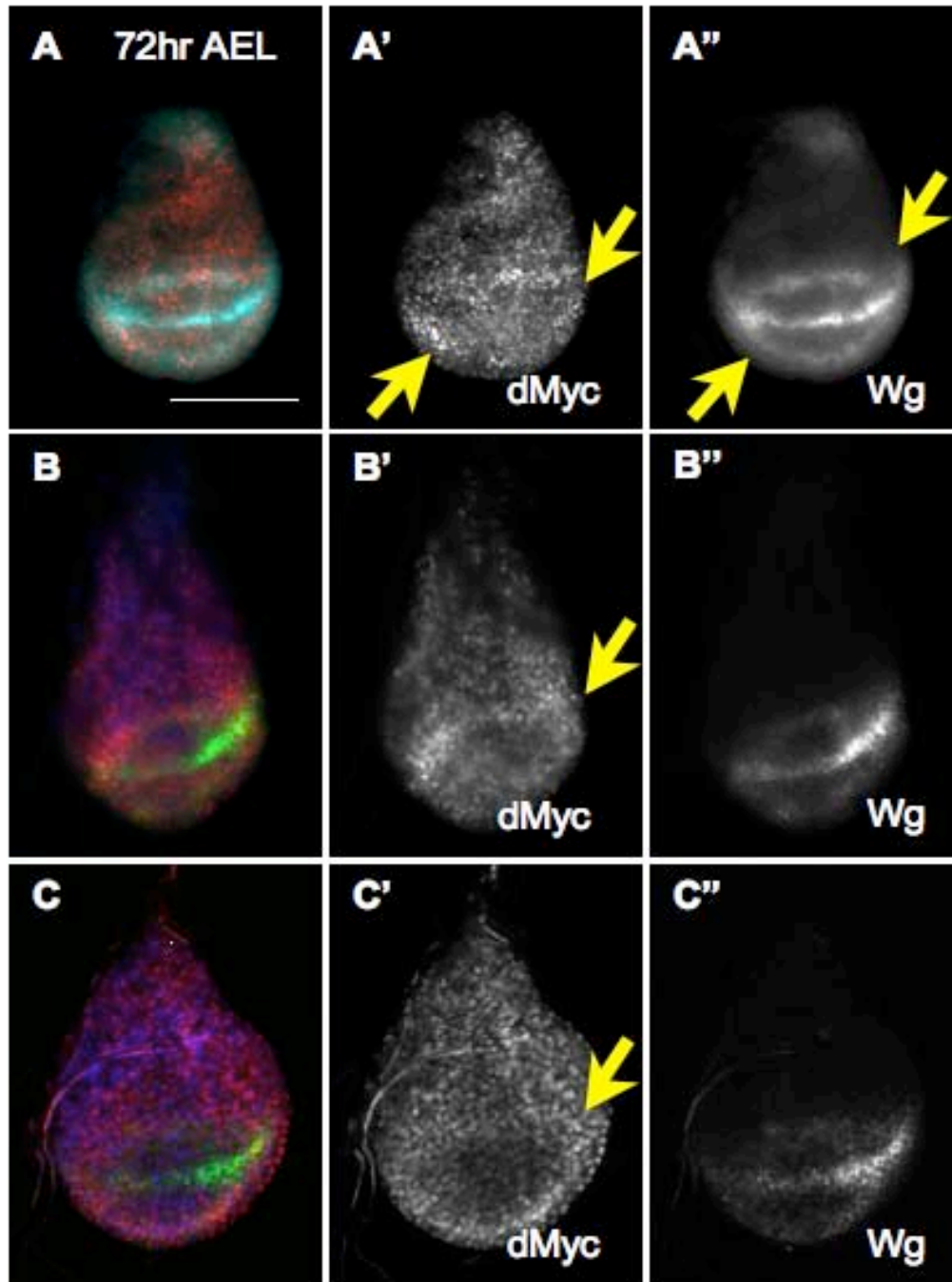


FIGURE S1.—dMyc expression transiently increases in hinge cells early in 3L. (A-C) 72h AEL *yw* wing discs, showing that dMyc antibody staining overlaps with the IR expression of Wg in control discs (arrows). (A', B', C') Single channel showing dMyc expression. (A'', B'', C'') Single channel showing Wg expression. Scale bar is representative for all images and equals 50 μm .

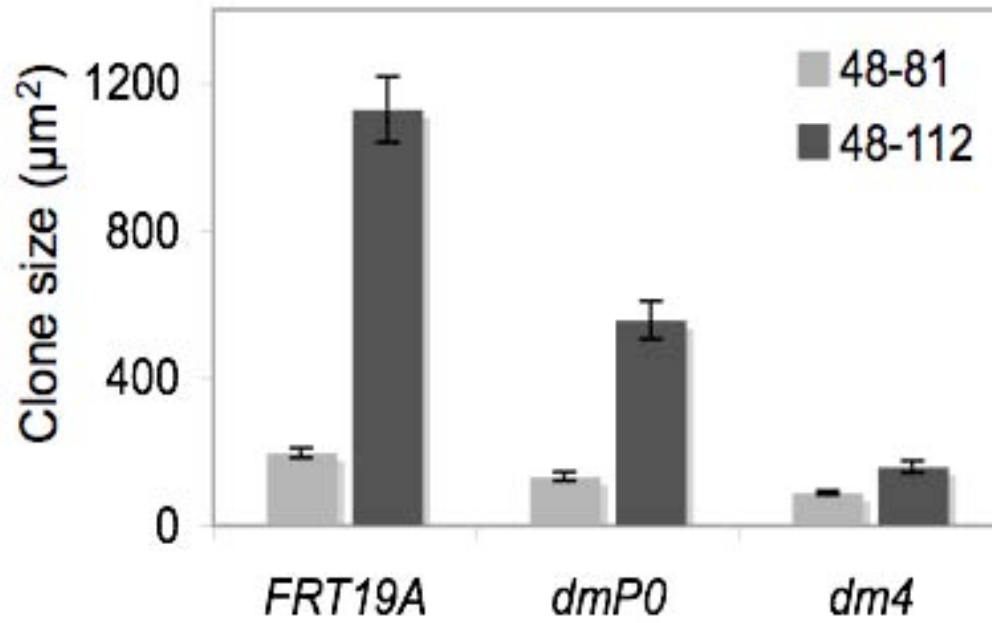


FIGURE S2.—Growth regulation by dMyc is dose-dependent. Clonal growth of *dm^{P0}* mutant cells and *dm⁴* mutant cells show a dose-dependent requirement for *dmyc* in cell proliferation and growth. Clones were induced at 48 hours AEL and dissected at either 81 hours AEL or 112 hours AEL to assess growth over time (where clone growth is the product of cellular growth, cell division and cell survival).

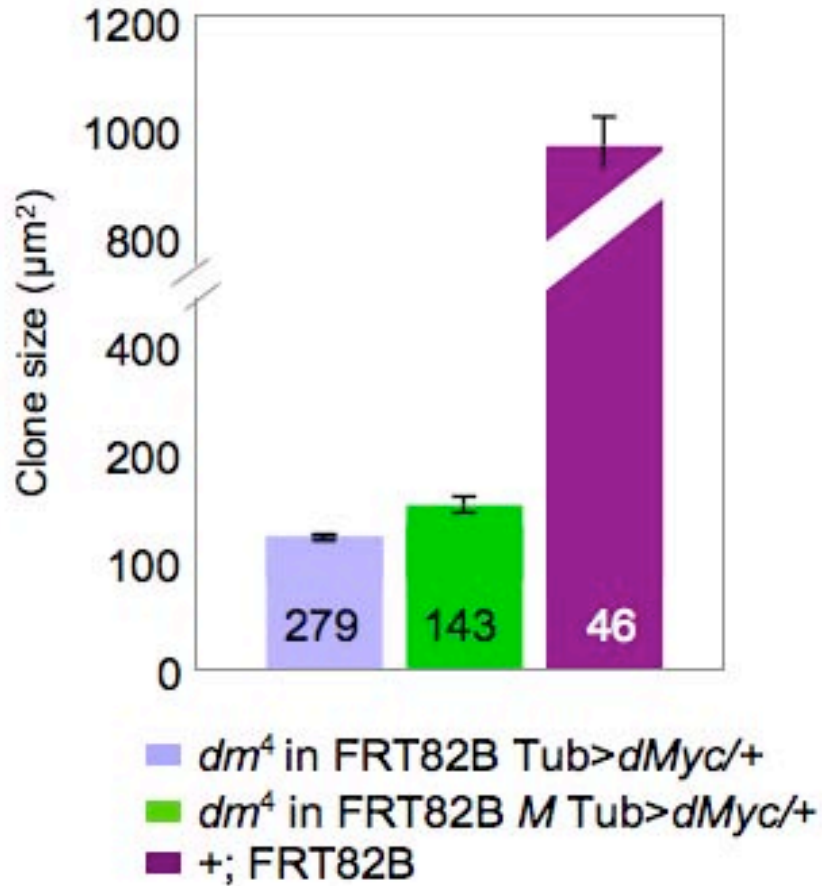


FIGURE S3.—*dm*⁴ mutant cells have limited ability to proliferate, even when given an advantage over *Minute*/+ cells. Clones were induced mid 2nd instar (72h AEL for *dm*⁴ mutant clones in a *Tub-dmyc*/+ background and 76h AEL for *dm*⁴ mutant clones in a *M Tub-dmyc*/+ background) and dissected 64h hours later. *dm*⁴ mutant clones in a *M Tub-dmyc*/+ background (131.23 µm²) are significantly bigger than *dm*⁴ mutant clones in a *Tub-dmyc*/+ background (105.04 µm²) (combined from all regions, $p = 1.0 \times 10^{-4}$); however, they are still vastly (and significantly, $p = 3.7 \times 10^{-17}$) smaller than wildtype control clones grown for the same time length (*dm*⁴ mutant clones in *M Tub-dmyc*/+ = 131.23 µm² vs. wildtype control clone = 1038.39 µm²).

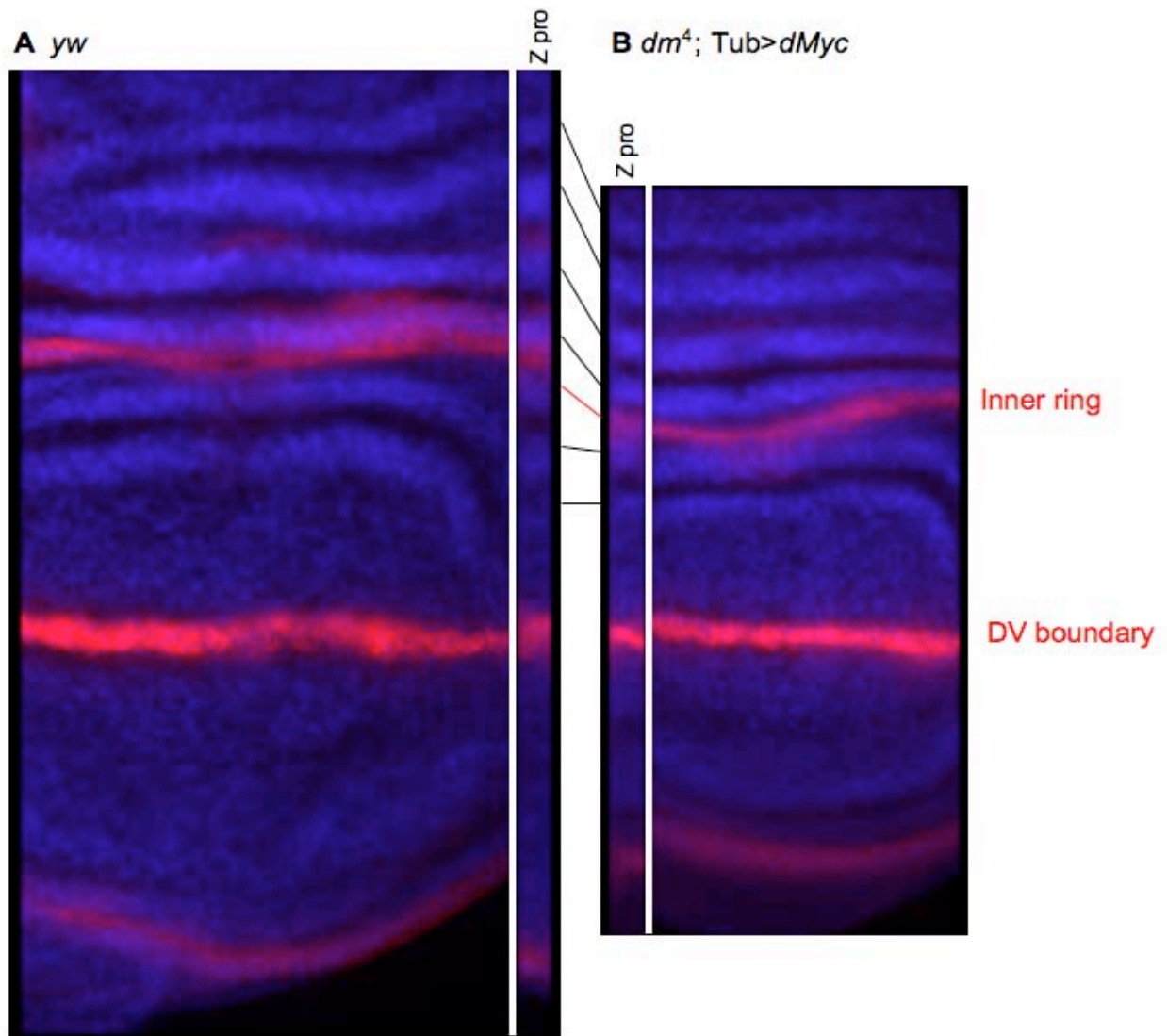


FIGURE S4.—The hinge region of discs with ubiquitous dMyc expression folds appropriately. (A, B) Cross-sections of hinge folds at 112h AEL. Discs are stained with antibodies against Wg and the folds of the dorsal hinge are aligned. Appropriate folding occurs in both genotypes. Images are to scale. (A) *yw* (B) *dm⁴; Tub-dmyc/+;+*

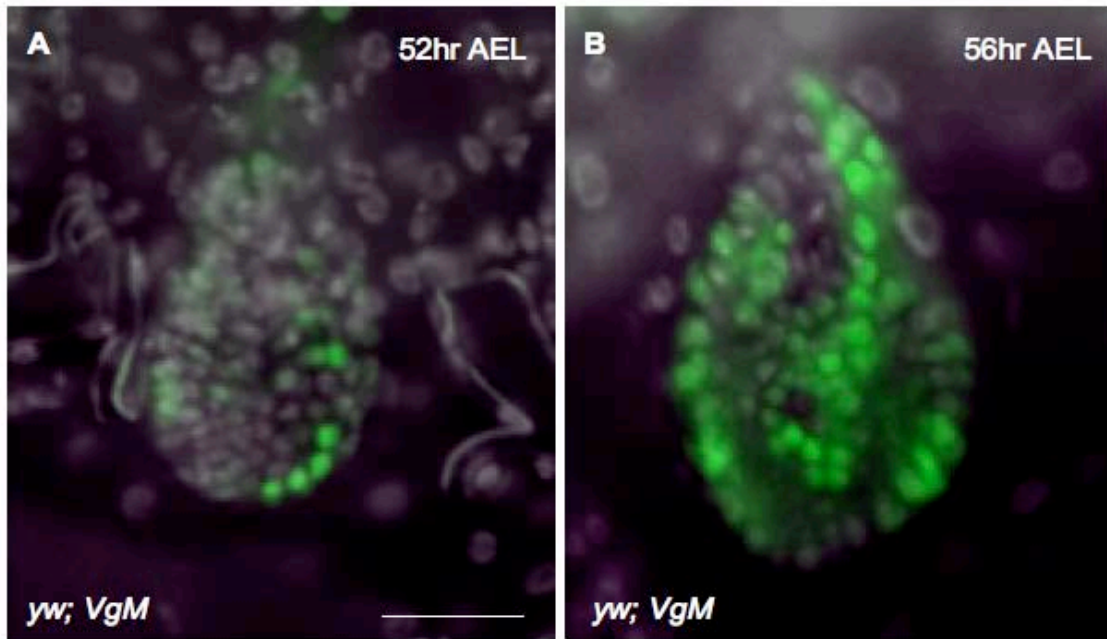


FIGURE S5.—Vg Gal4 expression initiates during early 2L. (A, B) *VgM*-GFP expression. *VgM* is activated as early as 52h AEL. Scale bar = 100 μ m.

TABLE S1

Eclosion rates of *dm⁴* mutants with one copy of *Tub-dmyc* vs. two copies

Genotype	n	% eclosed	sem
<i>yw</i> ;+;+	150	94.9	2.2
<i>dm^{P0}</i> ;+;+	42	81.2	9.9
<i>dm⁴</i> ; <i>Tub-dmyc</i> /+; +	534	1.7	0.7
<i>dm⁴</i> ; <i>Tub-dmyc</i> / <i>Tub-dmyc</i> ; +	176	16.6	5.6
<i>dm⁴</i> ; <i>Tub-dmyc</i> /+; <i>Tub-dmyc</i> /+	165	35.2	4.3