Control of wing size and proportions by *Drosophila* Myc

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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.

INTRODUCTION

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, initiating the expansion of the WP region (COUSO et al. 1993; DIAZ-BENJUMEA and COHEN 1995; KIM et al. 1995; KIM et al. 1997; NEUMANN and COHEN 1996; NEUMANN and COHEN 1997; WILLIAMS et al. 1993; 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; NEUMANN and COHEN 1996; WILLIAMS et al. 1993). 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 (ROGULJA et al. 2008; ZECCA and STRUHL 2007a; ZECCA and STRUHL 2007b). Fat/Hippo signaling regulates the transcription of several genes required for cell survival, cell division, and growth (CHO et al. 2006; HARVEY et al. 2003; HUANG et al. 2005; PANTALACCI et al. 2003; UDAN et al. 2003; WU et al. 2003). 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 Drosophila, 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 non-proliferating cells (ZNC) that surrounds the D/V boundary, to enforce a cell cycle arrest of these cells (DUMAN-SCHEEL et al. 2004; HERRANZ et al. 2008; JOHNSTON et al. 1999). 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 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: dmP0 (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 dmP0 FRT19A/FM7c, w dm4 FRT19A/FM7, and FRT82B Tub-dmycWT 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 hour periods and ≤ 50 first instar larvae were transferred to freshly yeasted, molasses food vials and raised at 25°C.

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°C incubator. Heat shocks were carried out for 40 minutes at 48h after egg laying (AEL) or for 30 minutes at 72h AEL.
Clones induced at 48h AEL were allowed to grow until either 81h AEL or 112h AEL. Clones induced at 72h AEL were allowed to grow until 112h AEL. Discs were stained for either Wg or Hth protein to define the hinge, pouch and notum. Clonal area was measured using Axiovision software (Zeiss) as described (De La Co VA et al. 2004).

Minute experiments
Tub-dmycWT (STEIGER et al. 2008) was recombined onto FRT82B M(3)96C, arm-lacZ. Experiments were done with dm4 mutant males rescued with Tub-dmycWT. Animals were heatshocked mid 2nd instar (72h AEL for dm4; FRT82B Tub-dmyc/+ and 76h AEL for dm4; FRT82B M Tub-dmyc/+ and dissected 64 hours later (136h and 140h). dm4 mutant clones were marked with two copies of pi-myc or lack of arm-lacZ. FRT82B control clones were induced at 48 h AEL and dissected at 112 h AEL.

Wing disc size measurements
The inner ring (IR) and outer ring (OR) of Wg expression was used to demarcate the wing pouch (WP) and hinge (Figure 2A). Hinge size was measured at 82, 96 and 110h 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 96h and 110h 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 non-parametric 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 30-40 late L3 wing discs of each genotype (Table 1). RNA was isolated using TRIZol (Invitrogen) and single stranded cDNA was produced from 1 µg RNA using SuperScript First-Strand Synthesis kit (Invitrogen). PCR reactions were performed using LightCycler FastStart DNA MasterPlus 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 versus ubiquitous dmyc expression is 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 as described (JOHNSTON and EDGAR 1998). The following antibodies and dilutions were used: mouse anti-Digoxenin, 1:2000 (Roche); rabbit anti-GFP, 1:2000 (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; VgGal4, UAS-flp, Tub>CD2>Gal4/+, UAS-GFP; Tub>dmyc>Gal4/+ Cells that express VgGal4 at any point during development will express UAS-Flp recombinase and excise 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 (Figure S5A-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 1C-1F). 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.
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 dmP0 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 (de la Cova et al. 2004; Johnston et al. 1999; Neufeld et al. 1998).

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 after egg laying (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 hr 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 timepoint (Figure 2D). Throughout L2 and L3, dmP0 and dm4 mutant clones grew at substantially reduced rates compared to control clones, and were significantly smaller than controls at the end of the growth phase (Figure 2D). The amount of growth that occurred was dMyc dose-dependent, such that dmP0 clones grew more at each timepoint than dm4. 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 dmyc 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 \( dm^4 \) mutant clones were generated as above and scored based on 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 \( dmyc \) expression in cells fated to be hinge (Figure 1B-D; Figure S1) predicted that its loss at that time would compromise the growth of these cells. We therefore allowed \( dm^4 \) and control clones to grow from 48-81 hours 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). \( dm^4 \) hinge clones grew slowly and reached only 32% of control clone size at the end of the growth period (Figure 2G). \( dm^4 \) 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 \( dmyc \) during L2-early L3. This suggests that the increase in \( dmyc \) expression in hinge cells during this time contributes to their relatively faster rate of growth (Figure 2G).

Midway through L3, \( dmyc \) expression again changes: it increases in WP cells and is reduced in hinge cells (Figure 1E-G). These changes suggest that the requirement for dMyc in hinge cells is reduced as development 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 they grew more during 81-112 hours AEL (Figure 2E, 2F). Growth of \( dm^4 \) 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, \( dm^4 \) 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 \( dm^4 \) mutant clone that grew from 48 – 112 hours AEL was less than 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 non-autonomous cell death induced by cell competition (DE LA COVA et al. 2004; JOHNSTON et al. 1999). We assessed the contribution of cell competition in these
experiments by examining the size of wildtype sibling clones, marked by two copies of GFP (GFP++), that were generated along with the mutant clones by recombination. Sibling, GFP++ clones of \( dm^4 \) 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 wildtype sibling cells and \( dm^4 \) mutant cells (and also the non-clonal \( dm^4/+ \) 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 over-expression (DE LA COVA et al. 2004). \( dm^4 \) 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 increase the frequency of their recovery (16% of GFP++ sibling clones were not accompanied by \( dm^4 \) clones; this frequency was 14% in the \( H99/+ \) background) (Figure 2H). We also assessed the growth potential of \( dm^4 \) 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 \( dm^4 \) 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 \( dm^4 \) allele have a very limited growth potential. We therefore conclude that the slow growth of \( dm^4 \) 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 wildtype control (yw, Figure 3A, E, I), using dm mutants and strains containing a transgene that ubiquitously expresses dMyc at low levels under the Tubulin α-1 promoter (Tub>dmyc>Gal4, hereafter referred to as Tub-dmyc) (DE LA COVA et al. 2004), as follows:

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

**Condition 2 (dmP0)**: dmyc is expressed in the wildtype pattern but at 15% of control wing disc levels (Figure 3C, G, K; Table 1A).

**Condition 3 (dm4;Tub-dmyc/++; or dm4;+;Tub-dmyc/+)**: The endogenous pattern of expression is abolished due to the dm4 null mutation. This is replaced by ubiquitous expression of dmyc at approximately 77% of control wing disc levels (Figure 3D, H, L; Table 1A).

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 dm4 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 timepoints during L3 and in adult wings (see 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 S3).

In control wing discs, the hinge and WP are similar in size at 82 hours AEL (Figure 4A, C). During the subsequent 28 hours, 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 timepoint (Figure 4C). The late increase in WP:H ratio coincides with the upregulation of \textit{dmyc} expression in WP cells and the concomitant expression decrease in hinge cells (Figure 1).

\textbf{Condition 1:} Although the \textit{Tub-dmyc} transgene in \textit{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 hours 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 hours of growth in our experiments (Figure 4C).

\textbf{Condition 2:} Wing discs from the hypomorphic mutant \textit{dmP0} express \textit{dmyc} in the endogenous pattern but at substantially reduced levels (Figure 3C, G, K; Table 1). The reduced \textit{dmyc} expression significantly impairs disc growth and leads to a smaller overall disc size at each timepoint (Figure 4D). We noticed several differences in the kinetics of growth of these discs. In contrast to controls, hinge size is significantly larger than WP size at 82 hours AEL (p = 0.007), and parity of size between the two regions is reached sometime after 82 hours but prior to 96 hours AEL (Figure 4D). Moreover, WP size does not exceed hinge size until 110 hours AEL, approximately 12 hours later than controls (Figure 4D). Although the WP:H size ratio of \textit{dmP0} discs increases incrementally (0.8 at 81 hr, 1.2 at 110 hr), it is consistently smaller than controls (Figure 4C and 4F). In general, the hinge region grows more than the WP at all timepoints, but both regions are approximately 50\% smaller than \textit{yw} controls at 110 hr. Larvae of this genotype are significantly delayed in their development and grow for an additional 10 hours beyond our last measuring point (data not shown). These observations suggest that the 85\% reduction in \textit{dmyc} expression in \textit{dmP0} wing discs leads to a growth program whose trend is virtually identical to controls, but which occurs at a significantly slower rate.

\textbf{Condition 3:} Wing discs in which endogenous expression of \textit{dmyc} is completely replaced by a low, ubiquitous level of \textit{dmyc} expression (\textit{dm^d}; \textit{Tub-dmyc}; Figure 3D, H, L) are severely reduced in size at all stages (Figure 4E). Like 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 hours and also at 96 hours AEL. Regional growth rates only diverge after 96 hours AEL, and by 110 hours AEL the WP is significantly larger than the hinge (p=2x10^{-7}). The increase in WP:H ratio of these discs is similar to \textit{dmP0} 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
timepoints (Figure 4F and 4C; Table 1).

Taken together, these experiments demonstrate that wing development can occur in the
absence of the endogenous pattern of \textit{dmyc} expression. In wildtype, 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 \textit{dmyc} expression is altered. This switch occurs even when
the endogenous pattern of \textit{dmyc} expression is completely replaced by ubiquitous expression,
although reduced levels of \textit{dmyc} expression significantly delay its onset. These data suggest that
the dynamic pattern of \textit{dmyc} expression does not instruct the regional growth changes in the
wing disc. Instead they imply that the absolute level of \textit{dmyc} expression is critical to set the rate
at which 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 \textit{dm}^4 allele is lethal during late L1, primarily due to the requirement for dMyc in
endoreplication of larval cells (Pierce \textit{et al.} 2008). The \textit{Tub-dmyc} transgene in \textit{dm}^4;\textit{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 Methods for
details). We used the \textit{dm}^4;\textit{Tub-dmyc} animal and selectively removed the \textit{dmyc} transgene from
wing discs with Flp/FRT-mediated recombination by taking advantage of FRT sites that flank
the \textit{dmyc} cDNA (Delacova \textit{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 approximately 50hr AEL
throughout the rest of disc development (Figure S5; Figure 5A-B). As a wing disc cell expresses
\textit{Vg-Gal4}, UAS-Flp is expressed and excision of the \textit{dmyc}-FRT cassette occurs. The progeny of
every \textit{Vg-Gal4}-expressing cell will heritably express UAS-GFP and create a permanent
“memory” of \textit{Vg-Gal4} expression in the disc (Crickmore and Mann 2006). For simplicity we
call this the \textit{Vg-memory} experiment (\textit{VgM}). In \textit{dm}^4;\textit{VgM};\textit{Tub-dmyc} flies, WP and hinge cells
excise the \textit{dmyc} cDNA and therefore lose all \textit{dmyc} expression, while the rest of the animal
retains the intact \textit{Tub-dmyc} cassette. Monitoring this process with expression of UAS-GFP
indicates that dmyc is excised by 56hr AEL (Figure S4B). The half-life of dMyc protein is approximately 30 minutes (Galletti et al. 2009), thus it is presumably lost soon after the excision.

We examined the development of these dm^4 mutant wings (dm^4; VgM; Tub-Gal4 in Figure 5C,D and Table 2B) by dissecting them from L3 larvae at 96hr and 137hr AEL. By 96hr, 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 5A'''', C''''). In wing discs from dm^4; 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 dm^4;+; 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 5A’’, C’’, E” and Figure S1). Null wing discs from dm^4; 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 dm^4; VgM; Tub-dmyc animals is identical to that of dm^4; 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 non-autonomously 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. dm^4; VgM; Tub-dmyc eye discs do not show a delay in size or pattern maturation comparable 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 dm^4; VgM; Tub-Gal4 null wing discs grow slower than wing discs from dm^4;+; 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 dm^4;+; Tub-dmyc control larvae (Table 2),
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 nonessential, 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 approximately 24 hours 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 over-expressing dMyc leads to normal wing size (Table 2B, Figure 6A-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 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 \textit{dmyc} mRNA and protein are expressed in a temporally and spatially dynamic manner that corresponds to the subdivision of the wing blade primordium from 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 ensures that 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 mis-proportioned 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 down-regulation 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 merely be a remnant of evolution. Alternatively, compensatory post-transcriptional control of dMyc could occur. dMyc protein is highly regulated (Galletti et al. 2009), and is noticeably 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 redundancy 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. (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 wildtype after an extended L3 (3-7 days longer than wildtype). In that case, loss of dMnt de-repressed 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 (data not shown) (Pierce et al. 2008; Pierce et al. 2004). 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 Dachsous 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 et al. 2006; CHO and IRVINE 2004; ROGULJA et al. 2008; ZECCA and STRUHL 2007b). 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 up-regulated in fat mutant eye discs, in which Hippo activity is de-regulated (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 their 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.

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References


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-C) Early in the third instar dmyc expression increases in hinge cells (arrow 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.
Scale bars = 50 µm.

Figure 2. Regional and temporal growth requirements for dMyc reflect its expression pattern during wing disc growth.
(A) Analysis of control and dmyc mutant clones in female larvae to determine growth requirements for dMyc. Clones were induced at specific times and scored based on 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 dmyc mutant clones (dmP0, hypomorphic allele, or dm4, null allele) (C) were compared to GFP negative control clones (B).
(D) Clonal growth of dmP0 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).
(E) Hinge cell clones grow significantly larger than WP clones early (p = 0.04).
(F) WP clones grow more later to reach the same size as hinge clones by 112 hours 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 dmyc early in development, whereas WP cells are more sensitive to loss of dmyc 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}). Late, 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 less than 20% of corresponding control clone size.
(H) Wildtype sibling clones (GFP++) of dm4 mutant clones that have grown from 48h AEL to 112h AEL are significantly bigger than corresponding control GFP++ clones (p <10^{-9}). In a H99/+ background, wildtype 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).

Figure 3. dMyc expression after experimental manipulation. 
dmyc mRNA in wing discs at 96h AEL (A-D) and 112h AEL (E-H) of male larvae. 
(A,E) yw, wildtype level and patterned dmyc expression. 
(B,F) yw; Tub-dmyc/+; +, wildtype pattern plus additional ubiquitous expression driven by Tub-dmyc (Condition 1). 
(C,G) dmP0, low level, but wildtype pattern dMyc expression (Condition 2). 
(D,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 96h when the disc is less folded. 
(I-L) dMyc protein in wing discs at 114h AEL. 
(I) yw. 
(J) yw;Tub-dmyc/+; +. 
(K) dmP0. 
(L) dm4;Tub-dmyc/+; +.

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 wing pouch (WP) sizes throughout development in male larvae. 
(A) Our wildtype strain, yw. 
(B) yw plus Tub-dmyc/+ wing discs (Condition 1). 
(A and B) At 82h AEL, hinge and WP sizes do not differ in either yw or yw;Tub-dmyc/+ wing discs (hinge versus WP yw 82h: p = 0.18, yw;Tub-dmyc/+  p = 0.23), but are different at 96h and 110h for both genotypes (hinge versus WP 96h: yw p = 4.0 x 10^{-9}, yw;Tub-dmyc/+  p = 2.2 x 10^{-4}), hinge versus WP 110h: yw p = 2.2 x 10^{-7}, yw;Tub-dmyc/+  p = 2.2 x 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: 82h v 96h: p = 0.027, 96h v 110h: p = 5.0 x 10^{-4}, yw;Tub-dmyc/+: 82h v 96h: p = 4.8 x 10^{-3}, 96h v 110h: p = 8.0 x 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 versus yw;Tub-dmyc/+; 82h: p = 0.47, 96h: p = 0.91, 110h: p = 0.41).
(D) dmP0 (Condition 2). 
(E) dm4;Tub-dmyc/+ (Condition 3).
(D and E) At 82 hours, hinge and WP size are significantly different in dmP0 wing discs (p <10^{-3}) but are not different in dm4;Tub-dmyc/+ wing discs (p = 0.27). At 96h AEL, hinge and WP size are not significantly different in either dmP0 or dm4;Tub-dmyc/+ wing discs (hinge versus WP 96h: dmP0 p = 0.99, dm4;Tub-dmyc/+ p = 0.26) but are different at 110h in both genotypes (hinge versus WP 110h: dmP0 p = 8.0 x 10^{-4}, dm4; Tub-dmyc/+ p = 2.2 x 10^{-7}).
(F) Ratios of WP to hinge (WP:H) in dmP0 and dm4;Tub-dmyc/+ wing discs throughout development. WP:H ratios of dmP0 and dm4;Tub-dmyc/+ wing discs significantly increases over time (Mann-Whitney test dmP0: 82h v 96h, and 96h v 110h: p = 1.0 x 10^{-9}, dm4;Tub-dmyc+: 82h v 96h and 96h v 110h: p <10^{-4}), and were consistently smaller than wildtype at every time point (Mann-Whitney test yw v dmP0 82h, 96h, 110h: p<10^{-4}, yw v dm4;Tub-dmyc/+ 82h, 96h: p = 1.0 x 10^{-4}, 110h: p = 2.0 x 10^{-4}). WP:H ratios of dmP0 and dm4;Tub-dmyc/+ wing discs were not
significantly different at any timepoint (Mann-Whitney test \(dm^{P0}\) v \(dm^4\);\(Tub-dmyc/+\) 82: \(p = 0.44\), 96h: \(p = 0.91\), 110h: \(p = 0.41\)). Measurements were done with animals carrying a \(Tub-dmyc\) cassette on chromosome 2. All \(p\) values are derived from Students t-tests unless otherwise indicated. Error bars show standard deviation. Three additional experiments showed similar trends.

**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 96h and late L3 wing discs.  

(A) 96h \(yw;VgM;+\) wing disc. (A’) dMyc. (A”) Wg. (A”’) GFP.  

(B) 114h (late L3) \(yw;VgM;+\) wing disc. (B’) dMyc. (B”) Wg. (B”’) GFP.  

(C) 96h \(dm^4;VgM;Tub-Gal4/+\) wing disc. (C’) dMyc. (C”) Wg expression in 96h \(dm^4;VgM;Tub-Gal4/+\) wing discs is immature relative to \(dm^4;Tub-dmyc/+\) wing discs of the same chronological age (Compare to E”). The pattern in C” is instead comparable to control \(yw\) wing discs at 72h AEL (Figure S1A-C”). (C”’) GFP.  

(D) 137h (late L3) \(dm^4;VgM;Tub-Gal4/+\) wing disc. (D’) dMyc. (D”) By late L3 Wg expression has matured (compare to B”). (D”’) GFP.  

(E) 96h AEL \(dm^4;Tub-dmyc/+\) wing disc. (E’) dMyc. (E”) Wg expression for comparison to discs in C-C”.

**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) \(yw;VgM;+\).  

(C) \(yw;VgM;Tub-Gal4/+\). The additional dmyc from the \(Tub-dmyc\) cassette is excised early in L2.  

(D) \(dm^4;+;Tub-dmyc/+\). These wings express dmyc solely from the \(Tub-dmyc\) transgene.  

(E) \(dm^4;VgM;Tub-Gal4/+\). dmyc expression is completely absent from these wings, which are significantly smaller than those in D (hinge \(p = 2.0 \times 10^{-4}\), blade \(p = 5.4 \times 10^{-9}\)).

**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 \(\mu m\).

**Figure S2.** Growth regulation by dMyc is dose-dependent.  

Clonal growth of \(dm^{P0}\) mutant cells and \(dm^4\) 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 \(\mu m^2\)) are significantly bigger than \(dm^4\) mutant clones in a \(Tub-dmyc/+\) background (105.04 \(\mu m^2\)) (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 \(\mu m^2\) vs. wildtype control clone = 1038.39 \(\mu m^2\)).

**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) \(dm^4;Tub-dmyc/+;+\)

**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 \(\mu m\).

**Table S1.** Eclosion rates of \(dm^4\) mutants with one copy of \(Tub-dmyc\) vs. two copies.
Table 1. Modulation of the *dmyc* expression pattern alters wing disc size

<table>
<thead>
<tr>
<th>Condition</th>
<th>Genotype</th>
<th>% <em>yw</em> WP</th>
<th>% <em>yw</em> H</th>
<th>WP:H ratio</th>
<th>dmyc mRNA</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><em>yw</em></td>
<td>100</td>
<td>100</td>
<td>1.4</td>
<td>1.0</td>
<td>19</td>
</tr>
<tr>
<td>1</td>
<td><em>yw</em>; <em>Tub-dmyc/+</em></td>
<td>105</td>
<td>109</td>
<td>1.4</td>
<td>1.7</td>
<td>31</td>
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<tr>
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<td>56</td>
<td>1.2</td>
<td>0.2</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td><em>dm</em>&lt;sup&gt;4&lt;/sup&gt;; <em>Tub-dmyc/+</em></td>
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<td>74</td>
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<tr>
<td></td>
<td><em>dm</em>&lt;sup&gt;4&lt;/sup&gt;;+;*Tub-dmyc/+</td>
<td>62</td>
<td>77</td>
<td>1.2</td>
<td>nd</td>
<td>19</td>
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</table>

Wing disc size from male larvae at late L3 (110h time-point of Figure 4). Conditions 1-3 are as described in the text. *yw* is used as a wildtype (WT) control. Values are expressed as a percentage of the mean size of *yw* wing pouch (WP) and hinge (H). *dmyc* mRNA level per wing disc cell is relative to *yw*. *dm*<sup>4</sup>;+;*Tub-dmyc/+ measurements were measured in a separate experiment; this genotype is delayed by 23 hours compared to *yw* and *dm*<sup>4</sup>;*Tub-dmyc/+. 
Table 2. Rudimentary, disproportionate wings form in the absence of dMyc

**A. Wing discs**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% yw WP</th>
<th>% yw H</th>
<th>WP:H ratio</th>
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<tbody>
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<td>100</td>
<td>1.5</td>
<td>6</td>
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<tr>
<td>dm^4;+; Tub-dmyc/+</td>
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<td>dm^4; VgM/+; Tub-Gal4/+</td>
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<td>56</td>
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**B. Adult wings**

<table>
<thead>
<tr>
<th>Genotype</th>
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<th>% yw hinge</th>
<th>B:H ratio</th>
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<td>94</td>
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<td>69</td>
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<td>55</td>
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</table>

Measurements in A are of male wing discs, and in B are adult male wings. Wing-specific loss of Tub>dmyc results in disproportionately smaller wing blades.

** p=9.3 x 10^{-11} (v. yw; VgM); p=2.0 x 10^{-8} (v. yw); p=6.7 x 10^{-13} (v. dm^4; Tub>dmyc>Gal 4/+)

aSimilar trends were obtained in three independent experiments; n=15 for dm^4; VgM; Tub-Gal4/+.
Figure 6

(A) yw

(D) dm^{4};+;Tub-dmyc

(B) yw;VgM;+

(E) dm^{4};VgM;Tub-Gal4

(C) yw;VgM;Tub-Gal4