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Compartments and the control of growth in the *Drosophila* wing imaginal disc

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The mechanisms that control organ growth are among the least known in development. This is particularly the case for the process in which growth is arrested once final size is reached. We have studied this problem in the wing disc of *Drosophila*, the developmental and growth parameters of which are well known. We have devised a method to generate entire fast-growing Minute$^+$ (M$^+$) discs or compartments in slow developing Minute/+ (M/+) larvae. Under these conditions, a M$^+$ wing disc gains at least 20 hours of additional development time. Yet it grows to the same size of Minute/+ discs developing in M/+ larvae. We have also generated wing discs in which all the cells in either the anterior (A) or the posterior (P) compartment are transformed from M/+ to M$^+$. We find that the difference in the cell division rate of their cells is reflected in autonomous differences in the developmental progression of these compartments: each grows at its own rate and manifests autonomous regulation in the expression of the developmental genes *wingless* and *vestigial*. In spite of these differences, 'mosaic' discs comprising fast and slow compartments differentiate into adult wings of the correct size and shape. Our results demonstrate that imaginal discs possess an autonomous mechanism with which to arrest growth in anterior and posterior compartments, which behave as independent developmental units. We propose that this mechanism does not act by preventing cell divisions, but by lengthening the division cycle.

KEY WORDS: *Drosophila*, Wing disc, Compartments, Growth control

INTRODUCTION

The mechanisms that regulate the growth and size of different organs and tissues during development are among the least known biological processes (Leevers and McNeill, 2005). The *Drosophila* wing may be a useful model system with which to analyse growth control. It is formed by the cells of the wing imaginal disc, which initially contains about 40 cells (Lawrence and Morata, 1977) that proliferate during the larval period to reach ~50,000. There are about 10-11 divisions in the progeny of each initial cell, each of which takes ~10 hours (García-Bellido and Merriam, 1971). Shortly after the beginning of pupariation, cell divisions cease and the differentiation of adult structures begins (Milán et al., 1996). The disc is subdivided at the beginning of embryogenesis into anterior (A) and posterior (P) compartments (Lawrence and Morata, 1977), which appear to grow similarly.

Thus, during most of the larval period there is a stimulus for cell division, which is responsible for the growth of the disc. This stimulus is the result of two mechanisms: one is triggered by extrinsic signals and depends on the insulin/insulin-like growth factor (insulin/IGF) system; the other is intrinsic to the disc and appears to depend principally on the activity of the Dpp signalling pathway (Day and Lawrence, 2000).

The insulin/IGF system exerts a general control over cell size and cell proliferation in response to nutritional conditions (reviewed by Oldham and Hafen, 2003). In addition, the absence of Dpp activity prevents growth of the disc (Spencer et al., 1982; Zecca et al., 1995), whereas its excess produces abnormally large wings (Martín-Castellanos and Edgar, 2002; Martin et al., 2004). There is also evidence of a functional connection between the insulin/IGF and Dpp pathways, as the functional levels of Dpp appear to respond to the levels of insulin/IGF activity (Teleman and Cohen, 2000).

Thus, there appears to be a general stimulus, activation of the insulin/IGF pathway, that affects all body parts. In the wing disc cells, the insulin/IGF levels are translated into Dpp activity levels, which control directly the growth of the disc. As the final aim of the process is to form a wing of a stereotyped size, cell division has to be closely regulated in order to achieve a wing of the correct dimensions.

A particularly intriguing phenomenon is the process of arresting growth when the disc reaches its final size. Experiments involving transplantation of wing discs into female hosts (Bryant and Levinson, 1985; Bryant and Simpson, 1984) have shown that mature discs do not continue to grow after several days in culture, suggesting that they may contain an endogenous growth control mechanism. However, the behaviour of discs after larval dissection and transplantation into heterologous hosts may not reflect their normal properties in situ.

In this report, we examine some aspects of growth control in the wing disc. We demonstrate that the disc contains an endogenous mechanism to arrest growth once it has reached final size. This mechanism does not operate in the disc as a whole but functions independently in A and P compartments. We also show that other key developmental functions, such as *wg* and *vg* activities, are independently regulated in A and P compartments.

MATERIAL AND METHODS

Fly stocks and antibodies

Fly stocks used were: *UAS-Flp* and *UAS-p35* (FlyBase), *esg-Gal4* (Goto and Hayashi, 1999), *en-Gal4* (Calleja et al., 1996), *ci-Gal4* (a gift from Robert Holmgren), *mwh jv P(w$^+$ FRT2A)* and *Sc^{J4} M(3)67C P(w$^+$ FRT2A)* (Erkner et al., 2002), and *brk^{X47}* (Campbell and Tomlinson, 1999).

Fixation and inmunohistochemistry were carried out as described (Aldaz et al., 2003). The following antibodies and dilutions were used: rabbit anti-Phospho-histone 3, 1:400 (Cell Signalling Technologies); mouse anti-Wg,

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1:50 (Hybridoma Center); rabbit anti-phosphorylated Mad, 1:5000; guinea pig anti-Vg, 1:5000; rabbit anti-cleaved Caspase 3, 1:50 (Cell Signalling Technology); To-Pro-3, 1:300 (InVitroGen); rabbit anti-p35, 1:5000 (StrataGene); and rabbit anti-β-Gal, 1:2000 (Cappel).

The TdT-mediated dUTP nick end-labelling (TUNEL) assay and BrdU staining were performed as described (Wang et al., 1999; Udan et al., 2003).

Generating M$^+$ discs and compartments in Minute/+ larvae

For the esg-M$^+$ experiment, we dissected discs from larvae of genotype esg-Gal4/UAS-Flp; *Sc^{J4} M(3)67C Ubi-GFP P(w$^+$ FRT2A)/mwh jv P(w$^+$ FRT2A)*.

For the ci-M$^+$ and en-M$^+$ experiments, the larvae were ci-*Gal4*/UAS-Flp; *Sc^{J4} M(3)67C Ubi-GFP P(w$^+$ FRT2A)/mwh jv P(w$^+$ FRT2A)* and *en-Gal4*/UAS-Flp; *Sc^{J4} M(3)67C Ubi-GFP P(w$^+$ FRT2A)/mwh jv P(w$^+$ FRT2A)*, respectively. For the experiments to prevent cell death, we used the *UAS-Flp, UAS-p35* instead *of UAS-Flp.*

Mitotic index

We labelled mitotic cells with the Phospho-histone 3 antibody. Images were taken and subsequently processed using Zeiss LSM Image Browser, ImageJ 1.36b and Adobe Photoshop 7.0. The mitotic index was calculated as the number of mitotic cells/area in μm $\times 10^3$. Statistical analyses were made by using Microsoft Excel.

RESULTS AND DISCUSSION

Experimental design

We have used the property of the *Minute* mutations to prolong the larval period of heterozygous animals without affecting the overall size of larvae or adults (reviewed by Lambertsson, 1998; Morata and Ripoll, 1975). In the case of *M(3)67C* allele, the larval period of *M(3)67C/+* individuals lasts 165 hours in standard culture conditions, 45 hours more than in wild-type strains (Ferrus, 1975). In larvae of this genotype, we have generated entire discs or compartments that have lost the retarding *M(3)67C/+* condition and that are therefore allowed additional time for development.

Wild-type discs in M(3)67C/+ larvae: the fast disc experiment

We addressed the issue of whether the wing disc contains an endogenous mechanism to control its own size. The rationale was to compare the growth of wild-type wing discs developing in normal larvae with that of equally wild-type discs developing in *M(3)67C/+* larvae.

The experiment is based on a combination of the Gal4/UAS, FRT/FLP and the Minute methods (Brand and Perrimon, 1993; Morata and Ripoll, 1975; Xu and Rubin, 1993) and consists of coupling a Gal4 line with a UAS-Flp construct so that all the cells of the Gal4 domain undergo FRT-mediated mitotic recombination. We have used the *esg*-Gal4 line, which is expressed in the precursor cells of imaginal discs (Fuse et al., 1994). In larvae of genotype *esg-Gal4/UAS-Flp; M(3)67C Ubi-GFP FRT2A/mwh jv FRT2A*, the presence of Flipase in the *esg*-expressing cells will induce high levels of FRT-mediated recombination. This results in the production of large number of *M*$^+$ clones. The growth advantage of the *M*$^+$ cells and the lethality of *M(3)67C* homozygous clones (Morata and Ripoll, 1975) should result in the colonisation of the entire disc by the progeny of the *M*$^+$ cells. As *esg* is expressed during embryogenesis in the imaginal disc precursors (Fuse et al., 1994), it is expected that the transition from *M(3)67C/+* to *M*$^+$ should occur early in development. The majority of the larval tissues do not express *esg* and will remain *M(3)67C/+*. Therefore, the experiment would allow the generation of *M*$^+$ discs that develop in slow-growing *M(3)67C/+* larvae.

To estimate the additional time allowed to the *M*$^+$ discs, we measured the duration of the larval period of *M(3)67C/+* and *M*$^+$ larvae in our experimental conditions. The results are shown in Fig. 1A; the average delay of *M(3)67C/+* with respect to *M*$^+$ is 40 hours, which coincides well with a published report of 45±6 hours (Ferrus, 1975).

To check that the experiment works as expected, we examined discs of different ages to determine at what stage all the cells are *M*$^+$. The earliest larvae examined were late second instar, which in our conditions corresponds to larvae collected 4 days after egg laying: six out of seven wing discs from *esg-Gal4/UAS-Flp; M(3)67C Ubi-GFP FRT2A/mwh jv FRT2A* studied in detail were completely transformed, as demonstrated by the loss of the GFP marker, whereas most of the larval tissues remained *M(3)67C/+*. These discs (referred to as esg-M$^+$) are already in a more advanced stage than discs from control *M(3)67C/+* larvae, as indicated by their *wg* expression (Fig. 1B,C).

Thus, the transition from *M(3)67C/+* to *M*$^+$ has already occurred by the beginning of the third instar. Assuming that the delay generated by the *M(3)67C* mutation is distributed uniformly throughout the larval period, a conservative estimate is that the *M*$^+$ discs are allowed 20 hours of additional growth time. Estimating an average cell division cycle of 10 hours, they could undergo two more division rounds, equivalent to about a fourfold increase in overall size.

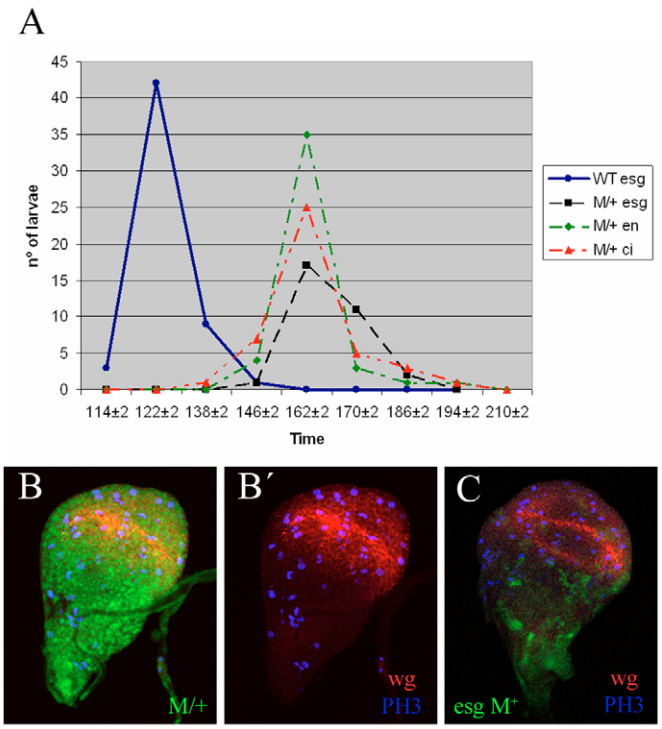

Fig. 1. Developmental delay of *M(3)67C/+* discs. (**A**) Duration of the larval period in hours in wild-type (blue line), *esg-Gal4*; *M(3)67C/+* (broken black line), *en-Gal4; M(3)67C/+* (broken green line) and *ci-Gal4*; *M(3)67C/+* (broken red line) flies. (**B**,**B′**) Second instar wing disc from a *esg-Gal4*; *M(3)67C Ubi-GFP FRT2A/+* larvae. The disc in B is triple labelled for GFP (green), wg (red) and PH3 (blue). All cells contain GFP expression, those in mitosis are scattered and *wg* expression is restricted to the region around the DV border. (**C**) esg-M$^+$ disc. All the cells have lost the GFP marker (the green label corresponds to adepithelial cells which are of mesodermal origin that do not express *esg*). *wg* expression is more advanced than in the disc in B.

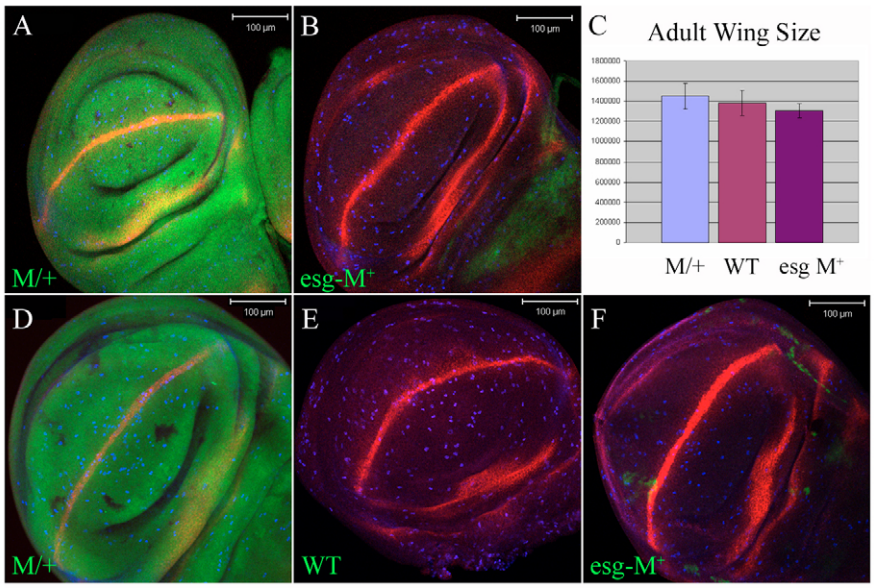

Fig. 2. Growth of esg-M$^+$ discs in *M(3)67C/+* larvae. (**A**,**B**) Six-day *M(3)67C/+* and esg-M$^+$ wing discs. The discs are triply stained for GFP, *wingless* (red) and PH3 (blue). The green label in the disc in B corresponds to the adepithelial cells. The pictures are taken at the same magnification. The disc in B is bigger. (**C**) Comparison of the size of the adult wings of *M(3)67C/+*, wild-type and esg-M$^+$ flies. There are no significant differences. (**D-F**) Wing discs from prepupal larvae of the same genotypes as in C, stained for GFP, PH3 and wg. All the discs are of the same size.

The M^+ discs growing in *M(3)67C/+* larvae are expected to attain mature size before the completion of larval development. Indeed, by day 6 of development, there is a clear difference in size between the esg-M$^+$ and the control esg-M discs; the former have already reached final size (Fig. 2A,B). A principal objective of the experiment was to determine whether the esg-M$^+$ discs continue growing during the additional time before the end of larval development. We measured their size when the larvae reached the prepupal stage (after the spiracles have been evaginated) and compared it with M^+ discs from prepupal M^+ larvae and with discs from equally prepupal *M(3)67C/+* larvae. There is no significant difference in the size of the three types of discs (Fig. 2D-F), indicating that M^+ discs in *M(3)67C/+* larvae do not grow during the extra-development time. However, we note that there still are cells in division in the esg-M$^+$ discs (Fig. 2F); their mitotic index (1.7±0.4, n=10) is not significantly lower than in *M(3)67C/+* controls (2.3±0.3, n=16).

The similarity in disc size is also corroborated by the comparison of the adult wings. The M^+ wings growing in *M(3)67C/+* larvae can be distinguished because they are homozygous for the marker mutants *mwh* and *jv*, in addition to being M^+. They are of the same size of M^+ wings growing in M^+ larvae (Fig. 2C).

Together, these results demonstrate that the wing disc does not grow beyond the standard final size, even if allowed additional time. Therefore, they strongly suggest that it contains an endogenous control mechanism to arrest growth. This conclusion can be extended to the rest of the imaginal discs, which also behave in the same way (see Fig. S1 in the supplementary material).

We have checked whether an increase of apoptosis could contribute to the arrest process. It has been suggested by the observation above that there are cells in mitosis in arrested prepupal esg-M$^+$ discs. A late round of apoptosis could eliminate the surplus of cells. However, the apoptotic levels found with the TUNEL method and anti-caspase staining are not increased (see Fig. S2 in the supplementary material). Additionally, we have constructed flies of genotype *esg-Gal4/UAS-FLP UAS-p35; M(3)67C Ubi-GFP FRT2A/mwh jv FRT2A*, in which all imaginal cells are protected from apoptosis by the caspase pan-inhibitor P35 (Hay et al., 1994). When these flies emerge, their imaginal structures are of normal size (see Fig. S2 in the supplementary material). These results demonstrate that apoptosis does not play a major controlling role.

Autonomy of compartment growth: the fast compartment experiments

The previous experiments demonstrate that the wing disc behaves as an autonomous unit of growth control. We then checked whether this autonomy is reflected in the A and P compartments.

We used same method described above. In these experiments, the *ci*-Gal4 and *en*-Gal4 lines drive Flipase and induce high levels of FRT-mediated recombination in the A and P compartments, respectively. This results in 'mosaic' discs in which either the A (*ci*-Gal4) or the P (*en*-Gal4) compartment is made M^+, whereas the other remains *M(3)67C/+* and serves as control. We refer to these as the ci-M$^+$ and the en-M$^+$ experiments, respectively. In both experiments, the fast compartment is already M^+ by late second instar (Fig. 3C,C′). Therefore, from that period onwards the discs are constituted by A and P compartments with different growth rates.

We have studied some developmental parameters of these mosaic discs: the relative size and growth rate of the A and P compartments and the expression of *wg* and *vestigial* (*vg*). These genes have expression patterns that evolve during imaginal development (Kim et al., 1996; Ng et al., 1996; Whitworth and Russell, 2003; Williams, 1994), thus allowing the comparison of the developmental progression of the A and P compartments. Discs from larvae 4, 5, 6 and 7 days after egg laying and prepupal stage were collected and fixed for examination.

The results are illustrated in Fig. 3. In control *M(3)67C/+* discs, the size of the P compartment is always smaller than that of the A compartment, although the P/A ratio increases during development from 0.37±0.03 (n=23) in the 4-day discs to 0.65±0.04 (n=10) (Fig. 3A). This reflects the original difference in the number of A and P precursor cells (Lawrence and Morata, 1977). The approximation in size of the A and P compartments in late development suggests that the P compartment grows faster than the A compartment.

In contrast to controls, the P/A ratio evolves differently in the ci-M$^+$ and en-M$^+$ discs (Fig. 3A). In the latter, the P/A ratio is 0.7±0.04 (n=10) in 4-day discs, significantly higher than in controls, indicating that the P compartment has grown bigger than in normal development. This difference is maintained thorough development, except at the end when the P/A size ratio becomes similar to controls (Fig. 3A). Comparable results are obtained with the ci-M$^+$ experiment, although the differences in size are less clear (Fig. 3A).

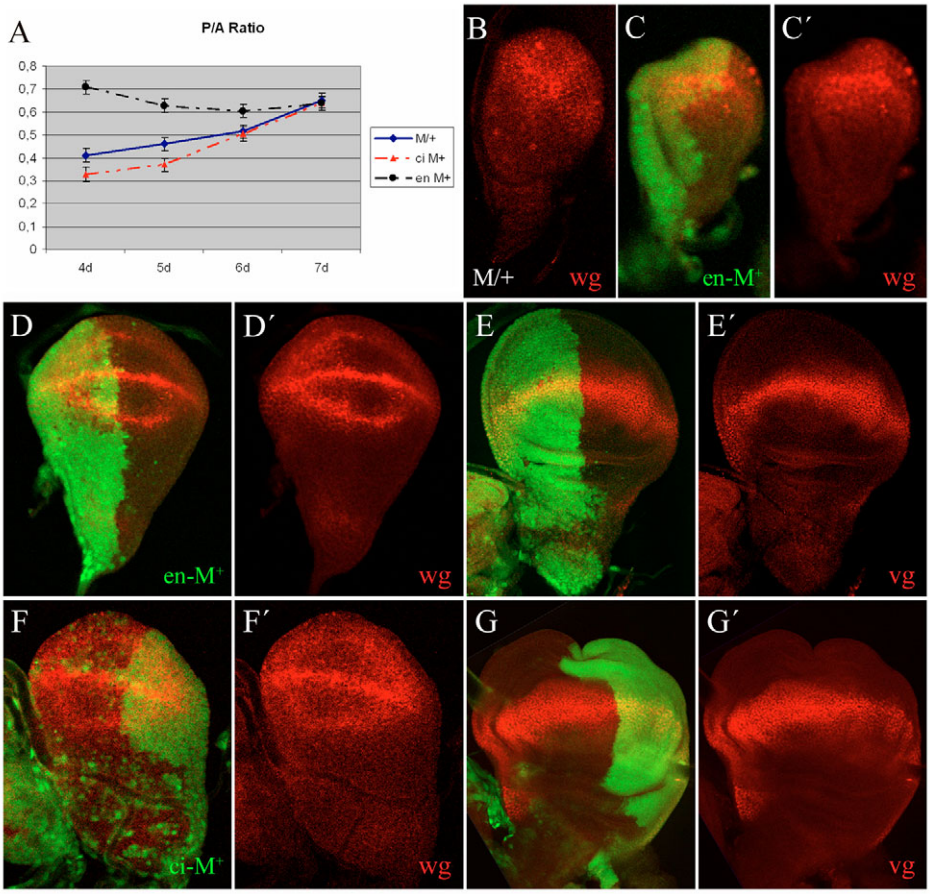

Fig. 3. Autonomous development of anterior and posterior compartments. (**A**) Evolution of the P/A ratio during the second half of the larval period in control *M(3)67C/+*, en-M$^+$ and ci-M$^+$ wing discs. In the control, the ratio increases during 3rd instar, whereas in en-M$^+$ discs the P/A ratio is approximately uniform. In ci-M$^+$, the ratio also increases, but it is significantly smaller than control until day 6. At the end of the larval period the P/A ratio of three classes of disc becomes the same. (**B**,**C**) A 2nd instar wild-type disc shows *wg* expression (red) in the presumptive wing pouch (B), and a similar aged en-M$^+$ disc has virtually all posterior cells transformed to M$^+$, as indicated by the loss of the GFP marker (C,**C′**). (**D-E′**) expression of *wg* and *vg* in en-M$^+$ early third instar discs. The expression of both genes is uncoupled in the anterior and posterior compartments, being more advanced in the posterior one. (D,D′) *wg* expression in the posterior compartment is restricted to a proximal ring and the DV region, whereas in the anterior compartment is more diffused in the wing pouch. (E,E′) *vg* expression is already expanding to the wing pouch in the posterior compartment, whereas it is still restricted to the DV region in the anterior compartment. (**F-G′**) Similar results observed in ci-M$^+$ discs: there is a slight but detectable difference in *wg* expression (F,F′), and more clear difference for *vg* (G,G′).

A significant observation is that the activities of *wg* and *vg* evolve independently in fast and slow compartments. During normal development, *wg* expression is first limited to the anteroventral region, later expands to most of the wing primordium and by early third instar it is further refined and becomes restricted to the DV border and hinge region (see Whitworth and Russell, 2003). At the time when the expression is becoming restricted to the DV boundary, the expression of *wg* is more advanced in the fast compartment (Fig. 3D,D′,F,F′); the ring expression in the hinge and the DV line are more sharply delineated.

The dynamics of *vg* expression also becomes different in fast and slow compartments (Fig. 3E,E′,G,G′). Normally, *vg* is first activated by the boundary enhancer at the DV boundary in response to Notch signalling (Williams et al., 1994), and is later expanded to most of the wing pouch under the control of the quadrant enhancer (Kim et al., 1996). In en-M$^+$ discs, *vg* expression is already expanding at early third instar in the P compartments, whereas it is still restricted to the DV boundary in the A compartment (Fig. 3E). In the ci-M$^+$ experiment, *vg* expression is more advanced in the A compartment (Fig. 3G,G′). These observations are of interest because they suggest that the implementation of the developmental program of imaginal discs occurs autonomously in compartments.

Although in these experiments the differences in growth rates of the A and P compartments of the wing disc cause size differences that are detectable in early discs, these differences disappear by the end of development. As shown in Fig. 3A, in the prepupal stage the P/A ratio is similar in en-M$^+$, ci-M$^+$ and controls discs. Furthermore, in the adult wings differentiated by the *en-M$^+$* and *ci-M$^+$* discs the A and P compartments are of normal proportional size (Fig. 4). In each case, the adult compartments match very well even though their growth rate was different (Fig. 4C,D).

The loss of the relative size difference between A and P compartments suggests that at a specific point in late development the growth of the fast compartment decelerates, whereas the slow one continues to grow. That is, the mechanism of growth arrest functions autonomously in the A and P compartments. Previous experiments have also reported that a fast-growing region does not overgrow, although cell division was not studied at that time (Simpson, 1976). We have tried to visualise this phenomenon by examining BrdU and PH3 staining in prepupal discs of the ci-M$^+$ and en-M$^+$ experiments and comparing fast and slow compartments. To our surprise, we found that there is no significant difference either in BrdU incorporation or in the mitotic index in the fast and slow compartments. In the en-M$^+$ experiment, the mitotic index in the P (fast) compartment was 2.5±0.6 (n=13), whereas in the A (slow) compartment it was 2.4±0.6. For comparison, the mitotic index in M(3)67C/+ control was 2.6±0.5 for the P and 2.0±0.3 for the A compartment (n=13). The presence numerous cells in mitosis in the arrested compartment was unexpected and may suggest that the mechanism to arrest growth does act not by blocking cell division, but by lengthening the duration of the division cycle. It has already been shown that the length of the cycle becomes longer as development progresses (Neufeld et al., 1998). At the end of development, the length of the cycle might become so long as to in effect suppress cell proliferation.

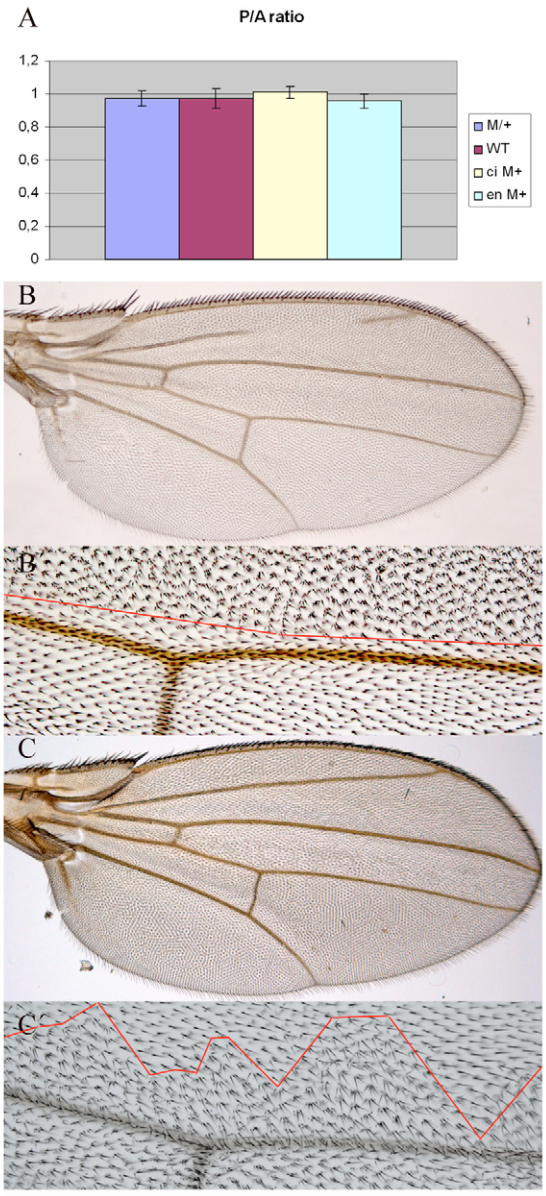

Fig. 4. Final size is not affected by differential growth rates. (**A**) P/A ratio in adult wings that are wild type or heterozygous for M(3)67C, (M/+), ci-M$^+$ and en-M$^+$. (**B**,**C**) Wings of ci-M$^+$ (B) and en-M$^+$ (C) genotypes showing that the A and P compartments match perfectly well in spite of having had different growth rates. In each case, the M$^+$ tissue is labelled with *mwh* and *jv*. (**B′**,**C′**) Are amplifications of the central regions of the wings. A sharp border exists between *mwh* tissue in the ci-M$^+$ wing (red line) compared with the 'wiggly' *mwh* border in the en-M$^+$ wing. This is due to the late tier of *en* expression induced by Hh (Blair, 1992).

The arrest of growth of the fast compartment in the experiments above could also be mediated by an increase in apoptosis. It would also be consistent with the persistence of cells in division in the arrested compartment. However, the levels of TUNEL or of caspase activity in the two compartments are similar (see Fig. S3 in the supplementary material). Furthermore, blocking cell death in fast compartments does not alter the size (see Fig. S3 in the supplementary material).

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Supplementary material

Supplementary material for this article is available at
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