Cell cycle progression, growth and patterning in imaginal discs despite inhibition of cell division after inactivation of *Drosophila* Cdc2 kinase

Katrin Weigmann1,2, Stephen M. Cohen2 and Christian F. Lehner1,*

1Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Spemannstrasse 37/39, 72076 Tübingen, Germany
2European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

*Author for correspondence at present address: Department of Genetics, University of Bayreuth, 95440 Bayreuth, Germany (e-mail: chle@uni-bayreuth.de)

SUMMARY

During larval development, *Drosophila* imaginal discs increase in size about 1000-fold and cells are instructed to acquire distinct fates as a function of their position. The secreted signaling molecules Wingless and Decapentaplegic have been implicated as sources of positional information that globally control growth and patterning. Evidence has also been presented that local cell interactions play an important role in controlling cell proliferation in imaginal discs. As a first step to understanding how patterning cues influence growth we investigated the effects of blocking cell division at different times and in spatially controlled manner by inactivation of the mitotic kinase Cdc2 in developing imaginal discs. We find that cell growth continues after inactivation of Cdc2, with little effect on overall patterning. The mechanisms that regulate size of the disc therefore do not function by regulating cell division, but appear to act primarily by regulating size in terms of physical distance or tissue volume.

Key words: Cdc2, *Drosophila*, imaginal disc, growth, pattern formation

INTRODUCTION

In early stages of development of the *Drosophila* embryo and the larva, cell numbers increase exponentially. In the embryo, the division cycles are essentially growth-independent because of the abundant maternal stores deposited during oogenesis in the egg. By contrast, the imaginal disc cell division cycles are coupled to an extensive increase in the size of the disc by growth. In the embryo, cell fates are assigned in part during the nuclear division cycles. Cells with a common developmental fate can be recognized as mitotic domains after cellularization because they divide almost synchronously at defined developmental stages (Foe, 1989). A similar correlation between cell fate and time of cell division exists in the pupal wing disc. At pupariation, cells of the wing blade arrest in G2 before progressing through two programmed cell divisions that are separated by an intervening S-phase. Cells in vein regions divide in a reproducible pattern before the cells in the adjacent intervein regions (Schubiger and Palka, 1987). By contrast, no correlation is observed between cell fate and proliferation during larval stages of imaginal disc development when most of the growth of the appendages takes place. Cells continue to proliferate while being instructed about their prospective fate (reviewed by Edgar and Lehner, 1996; Serrano and O’Farrell, 1997). With the exception of the subdivision of the discs into anterior-posterior (AP) and dorsal-ventral (DV) compartments, cell lineage relationships are for the most part unfixed, and clonal analysis has shown that the progeny of single marked cells can give rise to different cell fates, even if clones are induced late in larval development (González-Gaitán et al., 1994).

The AP and DV compartment boundaries play an important role in controlling growth and patterning of the imaginal discs (Diaz-Benjumea and Cohen, 1993; Basler and Struhl, 1994; Williams et al., 1994). The organizing activities of the compartment boundaries are mediated by localized expression of Wingless (WG) or Decapentaplegic (DPP), secreted signaling molecules that have long range effects on patterning and proliferation (Posakony et al., 1991; Struhl and Basler, 1993; Capdevilla and Guererro, 1994; Diaz-Benjumea and Cohen, 1995; Tabata et al., 1995; Zecca et al., 1995, 1996; Neumann and Cohen, 1996, 1997). Removal of WG or DPP leads to a failure to organize spatial pattern and to a reduction in growth of the discs. DPP is thought to have a direct effect on proliferation because clones of wing cells unable to transduce the DPP signal grow poorly (Burke and Basler, 1996). Likewise, clones of cells unable to transduce the WG signal grow poorly in the wing blade (Peifer et al., 1991; Neumann and Cohen, 1997), perhaps because they cannot express vestigial (Kim et al., 1996). Misexpression of WG or DPP can lead to respecification of cell fates and to a concomitant increase in proliferation, suggesting that these global patterning cues are important in regulating proliferation (reviewed by Lawrence and Struhl, 1996; Edgar and Lehner, 1996; Serrano and O’Farrell, 1997).

With regard to the effects of WG and DPP on cell proliferation, it is important to note that cell divisions are not restricted to a growth zone in the proximity to the sources of the organizing molecules. Analysis of the imaginal cell proliferation demonstrated that proliferation occurs essentially uniformly throughout the wing during most of larval development.
(González-Gaitán et al., 1994; Milan et al., 1996). Though clearly required for growth, the organizing molecules might not act directly as mitogen in the wing blade, as proposed for FGF in the case of the progress zone in vertebrate limbs (Niswander et al., 1993). By contrast, WG is thought to function primarily as a mitogen in the wing hinge (Neumann and Cohen, 1996). In addition to the long-range cues provided by WG and DPP, there is evidence that short-range cell interactions control local proliferation throughout larval development. Careful analysis of cell proliferation patterns in the developing wing disc suggests that local properties must influence how groups of cells respond to these long-range cues when deciding whether or not to divide (Milan et al., 1996). At present the relationship between long-range and short range patterning cues is not understood.

As a first step toward addressing how patterning cues coordinate proliferation we have investigated effects of inhibiting cell division either globally or in localized regions of the disc. We make use of a temperature-sensitive allele of Cdc2 (Stern et al., 1993). Cdc2 encodes a protein kinase that regulates entry into mitosis. Although mitosis is inhibited in this mutant, we find that cell growth and DNA replication continue. Inhibition of cell division in parts of the disc during pupal development leads to formation of normally sized wings containing larger cells. When mitosis is inhibited in larval development, patterning elements occupy the same area but consist of fewer cells. Changes in the expression of patterning genes occurs normally after inhibition of cell division. This suggests, that the mechanisms that determine the correct size of the disc do not function by promoting cell division per se. Rather, the size of the disc appears to be defined in terms of distance or tissue volume, and not in terms of cell number.

MATERIALS AND METHODS

Fly stocks
The temperature-sensitive allele Cdc2E1-24 was used (Stern et al., 1993). The enhancer trap lines odd-skipped (Cohen, 1993), dpp-lacZ (Blackman et al., 1991) and omb-lacZ (Leclerc et al., 1996) were used as markers.

Locally restricted expression of Cdc2+ in Cdc2 mutants
The Gal4/UAS system (Brand and Perrimon, 1993) was used to express the wild-type Cdc2 in both the posterior compartment of Cdc2E1-24 wings. Therefore Cdc2E1-24, P[w+, en-Gal4/T2;3]Sm6-TM6B, Cy Tb Ha flies (bearing a P[GawB] insertion at the engrailed locus; Brand and Perrimon, 1993; Yoffe et al., 1995) were crossed to Cdc2E1-24, P[w+, UAS-Cdc2/T2;3]Sm6-TM6B, Cy Tb Ha flies. The P[w+, UAS-Cdc2] transgene was obtained by germ line transformation after inserting a Cdc2 cDNA (Lehner and O’Farrell, 1990) into pUAST (Brand and Perrimon, 1993). Cdc2E1-24 was inactivated by shifting larvae or pupae to 30°C at different developmental stages. In some experiments (see Results), incubation at 30°C was preceded by a brief heat shock (30 minutes, 37°C) for a more rapid inactivation.

Cdc2 mutant clones
Clones of cells homozygous for Cdc2E1-24 were generated in Cdc2E1-24 heterozygotes by FLP recombinase-induced mitotic recombination (Xu and Rubin, 1993). Cdc2E1-24 clones were identified by the lack of Hs-NM expression in pupae obtained from the cross of w, P[ry+, Hs-FLP], P[w+, Hs-NM]31E, P[ry+, Hs-neo, FRT]40A with Cdc2E1-24, P[ry+, Hs-neo, FRT]40A/CyO. P[ry+, Hs-FLP], P[w+, Hs-FLP]31E, and P[ry+, Hs-neo, FRT]40A, as well as induction and identification of clones have been described by Xu and Rubin (1993). Clones were induced in second instar, and larvae were kept at 18°C until pupariation followed by a shift to 30°C.

Brdu injection and antibody staining
5-bromo-2-deoxyuridine (Brdu, Boehringer Mannheim) was injected between 12 and 18 hours after puparium formation (apf). Pupae were fixed to a slide with double stick tape, and a 10 mg/ml solution of Brdu in 5 mM KCl, 0.1 mM sodium phosphate buffer, pH 6.8 was injected into the abdomen with a glass capillary. As demonstrated by Schubiger and Palka (1987), this results in a 1-2 hours labeling pulse. Pupae were removed from their pupal cases at 30-34 hours apf and either fixed in methanol (for anti-BrduU immunolabeling) or in 4% paraformaldehyde in PBS with 0.2% Triton X-100 (for labeling with anti-MYC or double labeling with anti-BrduU and anti-MYC antibodies) for 30 minutes. After fixation, pupal wings were removed from their pupal membranes. Prior to immunolabeling with anti-Brdu-U, wings were incubated for 15 minutes in a 1:1 mixture of 0.4 N HCl and PBS containing 0.1% Triton X-100. Antibodies were obtained commercially or have been described previously: a mouse monoclonal antibody against BrduU from Becton Dickinson, a mouse monoclonal antibody recognizing a MYC-epitope from Oncogene Research, rabbit anti-β-galactosidase from Cappel, rabbit anti-Cyclin A by Lehner and O’Farrell (1989), mouse anti-WG by Brook and Cohen (1996), rabbit anti-WG (kindly provided by R. Nusse). For DNA labeling, imaginal discs were washed in PBS and incubated in 1 µg/ml Hoechst 33258 in PBS for 5 minutes. Discs were mounted in 80% glycerol/PBS and analyzed with a Zeiss Axiopt microscope equipped for epifluorescence.

RESULTS

To investigate the interdependence of patterning and proliferation, three aspects of disc patterning were investigated after inhibition of cell division: (1) the overall size and shape of the disc, (2) the pattern of pupal DNA replication as a ‘marker’ of cell identity, and (3) the expression of patterning genes during larval development. Two different techniques were used to inhibit cell division in populations of cells in larval and pupal discs. In one set of experiments clones of cells homozygous mutant for the temperature-sensitive allele Cdc2E1-24 were induced in Cdc2+ heterozygous discs by FLP-mediated mitotic recombination. In a second series of experiments, the two component UAS/Gal4 system was used to express the wild-type Cdc2 in the posterior compartment under the control of engraviled-Gal4 (en-Gal4) in an otherwise Cdc2 mutant background.

The final size and shape of the pupal wing is not affected by inhibiting pupal cell division
At pupariation, cells of the imaginal disc arrest in the G2 phase of the cell cycle. During pupal development, cells resume cell cycle progression, and most cells undergo two additional cell divisions between 10 and 24 hours after puparium formation (apf) before they enter terminal differentiation (Schubiger and Palka, 1987). Cdc2E1-24 homozygotes and wild-type controls were shifted to restrictive temperature (30°C) at pupariation to inhibit these two last cell divisions. While numerous mitotic figures were present in wild-type discs analyzed 16 hours after the temperature shift, we could not detect any mitotic figures in Cdc2E1-24 discs processed in parallel (Fig. 1A,B), confirm-
ing that cell division is effectively inhibited at 30°C in Cdc2E1-24 pupal wing discs.

Cdc2E1-24 pupal wings appeared to be of normal size 30 hours after the temperature shift but were composed of cells considerably larger than those seen in wild-type pupal wings raised under identical conditions (Fig. 1E, I compare with Fig. 1C, F). For a more accurate comparison of growth, we generated wing discs in which cells in the anterior compartment were mutant for Cdc2E1-24, while cells in the posterior compartment were rescued by expressing a UAS-Cdc2+ transgene under control of en-Gal4 (in a Cdc2E1-24 mutant background). As in the previous experiment, incubation at the restrictive temperature was started at pupariation and wing discs were analyzed 30 hours later. In the resulting discs, the anterior compartment was normal in size, but was apparently composed of fewer and larger cells, as indicated by the presence of fewer and larger nuclei compared to the posterior compartment (Fig. 1D, G, H). Adult flies did not eclose, but the pattern of vein and intervein regions analyzed 30 hours after the temperature shift was normal. The ratios of distances between the anterior margin and the various veins was not significantly different from wild type. This result demonstrates that the size of the pupal wing is not regulated by controlling cell numbers and that the formation of a normal vein pattern is not dependent on pupal cell divisions.

This finding was confirmed by analyzing Cdc2E1-24 clones induced during second instar and allowed to proliferate at the permissive temperature (18°C) before shifting to the restrictive temperature at pupariation. Clones were identified by the lack of Hs-NM expression (Xu and Rubin, 1993). After 30 hours at 30°C, homozygous Cdc2E1-24 clones appear to consist of cells with larger nuclei than those of the surrounding Cdc2+ cells (Fig. 2A, B), suggesting increased cell size. The transition from low to high nuclear density coincided precisely with the clone borders (Fig. 2A, B). In spite of their increased size, Cdc2E1-24 mutant cells appear to contribute normally to the adult wing. We observed groups of large cells with multiple, clustered trichomes that are integrated normally into the local pattern of the surrounding wing tissue (Fig. 2C, D). Although the clones were not marked in the adult, double labeling pupal wings with phalloidin to visualize trichomes and a DNA stain to localize Cdc2E1-24 clones showed that the clustered trichomes are produced by the large Cdc2E1-24 mutant cells (data not shown). Despite the presence of the clonal regions with reduced cell number and altered differentiation (clustered trichomes), wings were of normal size and shape and had a regular vein pattern. This demonstrates that adult dimensions are reached normally with reduced cell number.

**Blocking cell division does not affect the spatial and temporal patterning of DNA replication in pupal wings**

To investigate whether inhibition of cell division has an effect on the cells’ developmental program, we analyzed the pattern of DNA replication in Cdc2E1-24 pupal discs. In wild-type discs, DNA replication in the vein regions occurs before DNA replication in the intervein regions. Replication spreads from the veins to the intervein region in a wave-

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**Fig. 1. Inhibition of pupal cell division does not perturb growth.** Wings from pupae shifted to 30°C at the start of pupariation are shown. (A, B) Detail of wild-type (A) and Cdc2E1-24 (B) pupal wings after a 16 hours incubation at 30°C. At this stage, many mitotic figures are present in wild-type (arrows in A), but absent from Cdc2E1-24 wings. (C) Wild-type, (D) Cdc2E1-24 expressing UAS-Cdc2+ under control of en-Gal4 in the posterior compartment (Cdc2E1-24, en-Gal4; UAS-Cdc2+) and (E) Cdc2E1-24 pupal wings were kept at 30°C during pupal development, fixed at 30 hours apf and stained with Hoechst. For comparison of cell size, magnifications of wild-type (F), the anterior region of Cdc2E1-24, en-Gal4; UAS-Cdc2+ (G), the posterior region of Cdc2E1-24, en-Gal4; UAS-Cdc2+ (H) and Cdc2E1-24 (I) wings are shown. Cdc2E1-24 cells which have not undergone pupal divisions (G, I) are enlarged relative to wild-type cells that have divided normally (F, H). The pattern of vein and intervein regions in Cdc2E1-24, en-Gal4; UAS-Cdc2+ and Cdc2E1-24 wings is not affected.
like pattern. The pattern of DNA replication was unchanged in clones of Cdc2E1-24 mutant cells (Fig. 3). BrdU was injected 16 hours apf, a time when the proliferation wave has spread into the intervein regions. The same DNA replication pattern is observed in mutant cells as in Cdc2+ cells outside the clone. These observations indicate that Cdc2 mutant cells duplicate their DNA according to a developmental program that they share with wild-type cells, in spite of the fact that they cannot divide. The finding that mitotic cell cycle progression is converted into endoreduplication cycles in Cdc2 mutants has been described previously (Hayashi, 1996). However, we find that endoreduplication does not start immediately and synchronously in Cdc2E1-24 mutant cells after shifting the G2-arrested cells to the restrictive temperature at pupariation. Rather entry into S phase occurs in the normal intricate pattern shared with wild-type cells. These observations suggest that inhibition of cell division in pupal stages has no discernible effect on the cells’ developmental program, although cell differentiation, as reflected by trichome number, is perturbed.

**Growth continues in the absence of cell division during larval stages**

The presence of large cells after inactivation of Cdc2E1-24 suggested that Cdc2 kinase is not required for cell growth. However, as a caveat to this interpretation, it has not been demonstrated that individual cells grow in size during pupal stages of wild-type development. Therefore we cannot exclude the possibility that the cells arrested in G2 at pupariation are partitioned into smaller cells by the two final divisions that they undergo. The large cell size observed after inactivation of Cdc2E1-24 at pupariation, therefore, might not result from cell growth but could simply reflect the omission of such partitioning divisions. To establish that inactivation of Cdc2E1-24 does not inhibit cell growth, we shifted discs to the restrictive temperature during larval stages when it is clear that extensive growth is taking place. Cdc2E1-24 larvae expressing UAS-Cdc2+ directed by en-Gal4 were heat shocked for 30 minutes at 37°C shortly after the molt to third instar, followed by incubation at 30°C until wandering stage. Some of the resulting
wing discs had fewer but very much larger cells in the anterior compartment than in the posterior compartment (Fig. 4B). Surprisingly, the size of the anterior compartment was almost comparable to wild-type controls in these cases (compare Fig. 4A and B). Although such a dramatic increase in cell size in the anterior compartment combined with relatively normal overall disc shape was observed rarely, it demonstrates that cell growth can continue to a large extent in the absence of cell division.

Most of the discs in this experiment, as well as in experiments where discs were shifted to the restrictive temperature without a prior heat shock, displayed a smaller number of very large cells in the most anterior dorsal region of the wing disc and a distorted overall disc shape (Fig. 4C). However, in these cases mitotic figures were observed in the anterior compartment and many anterior cells were normal in size. Clonal analysis involving marked lineages showed that the small cells in the anterior compartments of such discs were of posterior compartment origin and therefore that cells were able to cross the AP compartment boundary under these conditions (data not shown). This indicates that even though these cells lose en expression and thereby the expression of the UAS-Cdc2 transgene as well, the perdurance of UAS-Cdc2 gene products appears to be sufficient to allow a few more cell divisions.

Inhibition of cell division in mid-larval stages does not block patterning processes

Growth of the discs depends on global patterning cues coming from the AP compartment boundary. In the wing disc, Hedgehog (HH) secreted by cells of the posterior compartment induces dpp expression in anterior cells adjacent to the compartment boundary. Secreted DPP in turn controls growth of the A and P compartments and directs symmetric expression of the target genes optomotor blind (omb) and spalt in domains of different width centered on the dpp stripe (Lecuit et al., 1996).
cells at the end of third instar. In Cdc2^{E1-24} discs shifted to restrictive temperature in early third instar, the number of cells in the corresponding ring was reduced to 20-40. Likewise the second ring is 2-3 cells wide in wild type (arrow in Fig. 6A) but is only 1-2 cells wide in the mutant discs (Fig. 6B). In younger discs (20 hours after the third instar molt) the rings are broader than at later stages (compare Fig. 6A and C). This suggests that the mature pattern is formed by refinement of an initially crude pattern into narrower rings. By counting cells in a defined region of the second ring (1/4 of the circumference) at the time of the shift to the restrictive temperature (20 hours after the third instar molt), we find that the cell number in the immature ring is higher than in an equivalent region of the mature ring in the mutant disc (about 40 and 30 cells, respectively). The fact that there are fewer odd-expressing cells in the mature ring indicates that the refinement process occurs normally in spite of the absence of cell division in Cdc2^{E1-24} mutant discs.

DISCUSSION

To ensure the correct formation of adult structures, patterning and growth must be well co-ordinated. As a first step toward studying this process, we have examined the requirement for cell division in patterning during imaginal disc development. Blocking cell division, either globally or in a spatially restricted manner using the temperature-sensitive allele Cdc2^{E1-24} was found to have consequences at the cellular level but surprisingly little effect on disc patterning. We found that growth and several aspects of pattern formation can proceed relatively normally in the absence of cell division. Thus we suggest that long-range growth and patterning cues are interpreted by measuring tissue volume or linear distance, in a manner that is largely independent of cell number.

Blocking cell division does not alter the developmental program of DNA replication

In wild-type development the majority of wing disc cells arrest in the G2 phase of the cell cycle before pupariation. After pupariation, they divide twice and exit from the cell cycle (Schubiger and Palka, 1987) When shifted to the restrictive temperature during the G2 arrest at pupariation, cells homozygous for Cdc2^{E1-24} no longer divide but progress normally through S phase in synchrony with nearby wild-type cells. Therefore, inactivation of Drosophila Cdk2 kinase results in a transformation of mitotic division cycles into endoreduplication cycles. Hayashi (1996) has reported that imaginal discs and abdominal histoblasts undergo endoreduplication cycles in Drosophila Cdc2 mutants. While the histoblasts apparently replicate prematurely, we find that endoreduplication in the wing disc does not start immediately and synchronously after inactivation of Cdc2. In contrast, the cells in Cdc2^{E1-24} discs progress through DNA replication in the same temporal and spatial pattern as wild-type cells.

These observations indicate that the timing of entry into S phase must be regulated by a distinct process in addition to the inactivation of Cdc2 kinase activity, which normally occurs during each mitosis because of the proteolytic degradation of the regulatory cyclin subunits (Cyclin A, B and B3). Since Cyclin E/cdk2 activity is known to be required for entry into

Fig. 6. odd expression is resolved to narrow stripes in Cdc2^{E1-24} discs. odd-lacZ expression was detected by anti-β-galactosidase antibody staining. (A,B) late third instar discs from wild-type (A) and Cdc2^{E1-24} (B) larvae, which were shifted to 30°C 20 hours after hatching to third instar. (C) wild-type disc fixed and stained 20 hours after hatching to third instar. (A) Arrows in A and B point to the second reduced cell number, these rings are of fewer and larger cells allows the odd expression to resolve into narrow rings (B). Due to reduced cell number, these rings are of fewer and larger cells compared to wild-type. (A) Arrows in A and B point to the second ring, which is 2-3 cell wide in wild-type (A), but only 1-2 cells wide in Cdc2^{E1-24}. A and B are shown at the same magnification, C is shown at a 1.5-fold higher magnification.

1996; Nellen et al., 1996). We examined the expression of dpp-lacZ, spalt and omb-lacZ in Cdc2^{E1-24} discs shifted to the restrictive temperature in early third instar to assess the effects of cell division and cell size on patterning. The width of the dpp stripe is thought to reflect the range of action of HH. In Cdc2^{E1-24} mutant discs the dpp-lacZ stripe was of essentially normal width (Fig. 5A,C), although the disc was slightly smaller (see Discussion). Similarly, the patterns of omb and spalt expression were essentially normal (Fig. 5E). These results suggest that the effective range of the HH and DPP signals were not significantly affected by inhibiting cell division; the concomitant increase in cell size appears to be sufficient to support proper patterning.

To ask whether patterning is similarly independent of cell size and number in the leg, we investigated the expression of odd-skipped (odd). In mature third instar leg discs, odd is expressed in a pattern of concentric rings; one per leg segment (Fig. 6A). In wild type the distal-most ring consists of 40-50
S phase (Knoblich et al., 1994), it might be the developmental regulation of this activity which controls the pattern of S phase in wild type and endoreduplication in Cdc2<sup>E1-24</sup> pupal discs at the restrictive temperature. This idea is supported by results obtained with mutant embryos unable to express Cyclin A (i.e. one of the cyclins that activate Cdc2 kinase). In these embryos, a transformation of mitotic cycles into endoreduplication cycles is observed analogous to that in Cdc2 mutant discs, and in these Cyclin A mutants the endoreduplication is known to start just after a developmentally regulated pulse of Cyclin E expression (Knoblich et al., 1994; Sauer et al., 1995). All these observations are consistent with the idea that licensing of a new round of DNA replication in mitotic cycles is dependent on the inactivation of Cdc2 kinase activity (and perhaps in particular on the inactivation of Cyclin A/Cdc2 complexes), while the actual onset of DNA replication is dependent on the developmentally regulated activation of Cyclin E/Cdk2 activity (Duronio and O’Farrell, 1994; Knoblich et al., 1994; Edgar and Lehner, 1996).

Our findings in Drosophila where the cell cycle is controlled (as in vertebrates) by multiple cdk’s and cyclins are similar but not identical to those described in budding and fission yeast where the cell cycle is governed by a single cdk and multiple cyclins. This single cdk in yeast triggers entry into mitosis. It must then be inactivated during mitosis to allow licensing of DNA replication and re-activated subsequently to start DNA replication (Wuarin and Nurse, 1996; Stillman, 1996). Inactivation of temperature-sensitive alleles encoding this single yeast cdk blocks cell cycle progression completely. In contrast, inactivation of Cdc2<sup>E1-24</sup> in Drosophila still allows endoreduplication cycles, presumably as long as periodic activation of Cyclin E/Cdk2 continues.

**Cell growth in the absence of cell division**

Although inactivation of yeast cdk completely blocks cell cycle progression, it does not inhibit cell growth, suggesting that cell growth is independent of cell cycle control (reviewed by Nasmyth, 1996). In Drosophila imaginal discs, inactivation of Cdc2 also does not block cell growth. However, because inactivation of Drosophila Cdc2 is followed by endoreduplication, it remains possible that the observed cell growth is a consequence of this endoreduplication. In principle, cell growth and cell cycle progression might be related in either of two fundamentally different ways. Cell growth might dictate cell cycle progression by triggering the activation of cell cycle regulators once a critical size has been reached. Alternatively, cell cycle progression might result in cell growth by increasing gene copy number. In yeast, the former model is favored, because hyper-activation of yeast cdk does not cause cells to grow faster; instead, they divide at a smaller size (reviewed by Nasmyth, 1996). The relationship between growth and cell cycle progression in different animal cell types might be more complex. At present, we are not able to determine whether the long-range patterning cues required for disc growth regulate cell growth per se or whether they work through regulation of S phase.

**Pattern formation is relatively independent of cell number**

Our results suggest that regulation of growth and pattern formation are largely independent of cell number during imaginal disc development. Inhibition of pupal cell divisions does not affect the size or shape of the wing: the pattern of vein and intervein regions is normal after global or local inhibition of pupal cell division. Also, analysis of Cdc2 mutant clones show that local patterning of the adult wing is not affected by locally inhibiting cell division. This shows that size control is independent of the exact cell number. Independence of disc size from cell number has also been shown in experiments involving discs mosaic for diploid and haploid cells (Santa-maria, 1983). In these experiments, the overall size and shape of the mosaic discs was found to be normal, in spite of the fact that large territories were composed of many more, smaller haploid cells. This independence of overall size from cell number has also been found in vertebrates. In polyploid amphibia, all cells are greatly enlarged, but the organs are normal in shape and size. The increased volume of the polyploid cells therefore is compensated for by a decrease in cell numbers (Fankhauser, 1941, 1945a,b).

We also observed that the expression of patterning genes is relatively independent of cell number during larval stages. Expression of the patterning genes dpp, spalt, omb and odd is essentially normal in spite of quite striking changes in cell number and cell size. Thus the patterning processes that determine the sizes of these expression domains can continue in the absence of cell division. Because the decrease in cell number is balanced by increased cell sizes, the discs continue to grow at an apparently normal rate, at least over a limited time interval and the long range action of the patterning cues in defining the expression domains of the target genes appears to proceed normally.

There is clearly a limitation to disc growth in the absence of cell division. Cdc2<sup>E1-24</sup> larvae shifted to restrictive temperature at early stages of development did not grow to normal size (unpublished observations, but see also Fig. 5). In this case, where cell number and overall dimensions of the disc is greatly reduced, pattern elements (e.g. odd expressing stripes) do not resolve properly. This is consistent with results of experiments with growing amphibian limbs. Treatment with colchicine inhibits cell divisions, reduces the size of the limb and also reduces the number of digits that are formed (Albrech and Gale, 1983). Taken together, these results imply that patterning of a field with fewer and larger cells can proceed as in a field with many small cells, but only as long as the size of the field is maintained.

These observations are difficult to reconcile with models for pattern formation and growth control that are based on intercalation of positional values through local cell interactions. The polar coordinate model proposes that cells possess discrete positional values and that apposition of cells with disparate values stimulates local proliferation, which stops when a complete map of positional values is established in the limb field (French et al., 1976; Bryant et al., 1981; Bryant and Fraser, 1988). We observe that pattern specification and size control appear to be independent of cell number at least over a broad range, which seems incompatible with a model that requires cell division and comparison of discrete ‘cellular’ positional values to control growth and patterning.

**Local versus global patterning cues**

Our results suggest that the ability of cells to respond appropriately to the long range patterning cues provided by WG and DPP is relatively independent of cell number. We favor the
view outlined in a recent review (Serrano and O’Farrell, 1997),
that cells assess the local concentrations of organizing
molecules and respond by differential gene expression and by
cell proliferation. We would modify this model to include the
observation that cell size continues to increase if cell division
is blocked. This suggests that long-range patterning cues might
control growth through a mechanism that ultimately measures
tissue volume. While global patterning cues are clearly
important there is also evidence that local properties influence
how groups of cells respond to these cues when deciding
whether or not to divide. One indication that local properties
may be important comes from analyzing Minute mutants which
produce cells with inherently different growth rates. Minute
mutant cells divide more slowly than wild-type Minute+ cells
(Morata and Ripoll, 1975). Consequently Minute mutant cells
can be out-competed during larval development by relative
overgrowth of the surrounding Minute+ cells (Simpson, 1979).
To compare the growth of cells in different regions of Minute+
clones, Simpson and Morata (1981) produced small clones of
genetically marked, but otherwise wild-type cells in discs
carrying large clones of Minute+ cells (which had been induced
earlier). Clones of wild-type cells located near the edge of the
Minute+ clone grew larger than genetically equivalent clones
far from the clone border. These observations suggested that
cells near the Minute mutant territory are stimulated to prolif-
erate more strongly than cells surrounded by wild-type cells.

A second indication for the importance of local interactions
was provided by a careful examination of the patterns of cell
proliferation in the wing disc (Milan et al., 1996). Although
growth is on average uniform throughout the wing disc, cells
do not simply pass directly from one cell cycle to next as would
be expected if there were only long-range mitogenic stimuli.
Rather, small groups of cells divide in synchrony, while nearby
cells may or may not divide. The groupings at each round of
division appear to be random in that cells within a group are
not related by cell lineage or by whether they were part of a
particular proliferative cluster in the previous round of division.
These observations suggest that cells assess some local infor-
mation which influences whether or not they and their
immediate neighbors undergo a proliferative cycle.

At present the relationship between global cues and local
cell interactions is not understood. Our results are consistent
with the idea that global cues are important in promoting
growth (reflected in cell size increase if cell division is
blocked). One suggestion as to how this might work comes
from the recent finding that nitric oxide plays an important role
in controlling cell proliferation in the discs (Kurzin et al.,
1996). While it remains to be determined whether global pat-
terning cues exert any direct influence on nitric oxide levels, it
is worth noting that a small molecule like nitric oxide could in
principle provide a means of measuring tissue volume. It is
possible that the local cell interactions provide a means to fine-
tune the response of cells to the long-range cues, to more accu-
trately balance growth. Alternatively local cues might act in
some as yet undefined process.

Requirement of cell division for cell differentiation

In Drosophila embryos, normally differentiated cell types are
also formed when progression through the complete develop-
mental program of cell divisions is inhibited (Hartenstein and
Posakony, 1990). Similarly, although cell division is tightly
regulated in the Drosophila retina, cell fate is assigned inde-
pendent of cell lineage or the precise timing of division (de
Nooij and Hararihan, 1995; Penton et al., 1997). Blocking cell
divisions in Xenopus embryos does also not interfere with deter-
mination of the different cell types investigated (Harris and
Hartenstein, 1991). We show here that subdivision of imaginal
discs into different regions is not dependent on cell division.
Nevertheless, it is clear that cell division is required for the
correct determination of cell fates in specific lineages in
Drosophila and other organisms (Horvitz and Herskowitz,
1992; Edgar and McGhee, 1988; Weigmann and Lehner,
1995). Blocking cell division in these cases may result in
mixed cell fates or loss of specific cell fates (Cui and Doe,
1995; Hartenstein and Posakony, 1990; Weigmann and Lehner,
1995). In addition, our experiments presented here show that
blocking cell division in pupal wing discs affects the pattern-
ing of trichomes.

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