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

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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 G_2 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 mitogens 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, pattern 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 *Cdc2*$^{E1\text{-}24}$ was used (Stern et al., 1993). The enhancer trap lines *odd-skipped* (Cohen, 1993), *dpp*-lacZ (Blackman et al., 1991) and *omb*-lacZ (Lecuit 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 only the posterior compartment of *Cdc2*$^{E1\text{-}24}$ wings. Therefore *Cdc2*$^{E1\text{-}24}$, P[w^{+}, *en*-Gal4]/*T(2;3)SM6-TM6B, Cy Tb Hu* flies (bearing a P[GawB] insertion at the *engrailed* locus; Brand and Perrimon, 1993; Yoffe et al., 1995) were crossed to *Cdc2*$^{E1\text{-}24}$, P[w^{+}, UAS-*Cdc2*]/*T(2;3)SM6-TM6B, Cy Tb Hu* 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). *Cdc2*$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$ were generated in *Cdc2*$^{E1\text{-}24}$ heterozygotes by FLP recombinase-induced mitotic recombination (Xu and Rubin, 1993). *Cdc2*$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$, P[ry^{+}, *Hs-neo*, *FRT*]40A/*CyO*. P[ry^{+}, *Hs-FLP*], P[w^{+}, *Hs-NM*]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-deoxiuridine (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-BrdU immunolabeling) or in 4% paraformaldehyde in PBS with 0.2% Triton X-100 (for labeling with anti-MYC or double labeling with anti-BrdU and anti-MYC antibodies) for 30 minutes. After fixation, pupal wings were removed from their pupal membranes. Prior to immunolabeling with anti-BrdU, 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 BrdU 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 Axiophot 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 *Cdc2*$^{E1\text{-}24}$ 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 *engrailed*-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 G_2 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). *Cdc2*$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$ discs processed in parallel (Fig. 1A,B), confirm-

ing that cell division is effectively inhibited at 30°C in *Cdc2*$^{E1\text{-}24}$ pupal wing discs.

Cdc2$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$, while cells in the posterior compartment were rescued by expressing a UAS-*Cdc2*$^{+}$ transgene under control of *en*-Gal4 (in a *Cdc2*$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$ 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, *Cdc2*$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$ clones showed that the clustered trichomes are produced by the large *Cdc2*$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$ 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-

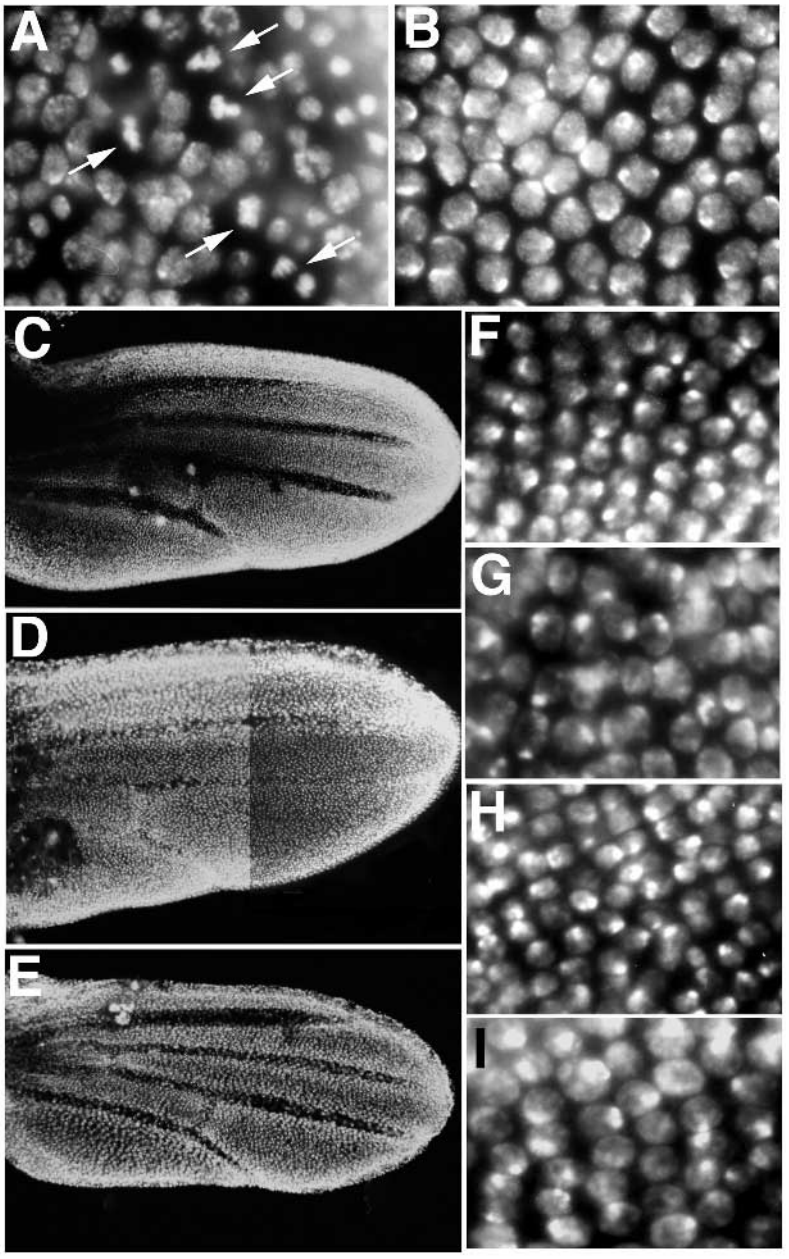

Fig. 1. Inhibition of pupal cell division does not perturb growth. Wings from pupae shifted to 30°C at the start of pupariation are shown. (A,B) Detail of wild-type (A) and *Cdc2*$^{E1\text{-}24}$ (B) pupal wings after a 16 hours incubation at 30°C. At this stage, many mitotic figures are present in wild-type (arrows in A), but absent from *Cdc2*$^{E1\text{-}24}$ wings. (C) Wild-type, (D) *Cdc2*$^{E1\text{-}24}$ expressing UAS-*Cdc2*$^{+}$ under control of *en*-Gal4 in the posterior compartment (*Cdc2*$^{E1\text{-}24}$, *en*-Gal4; UAS-*Cdc2*$^{+}$) and (E) *Cdc2*$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$, *en*-Gal4; UAS-*Cdc2*$^{+}$ (G), the posterior region of *Cdc2*$^{E1\text{-}24}$, *en*-Gal4; UAS-*Cdc2*$^{+}$ (H) and *Cdc2*$^{E1\text{-}24}$ (I) wings are shown. *Cdc2*$^{E1\text{-}24}$ 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 *Cdc2*$^{E1\text{-}24}$, *en*-Gal4; UAS-*Cdc2*$^{+}$ and *Cdc2*$^{E1\text{-}24}$ wings is not affected.

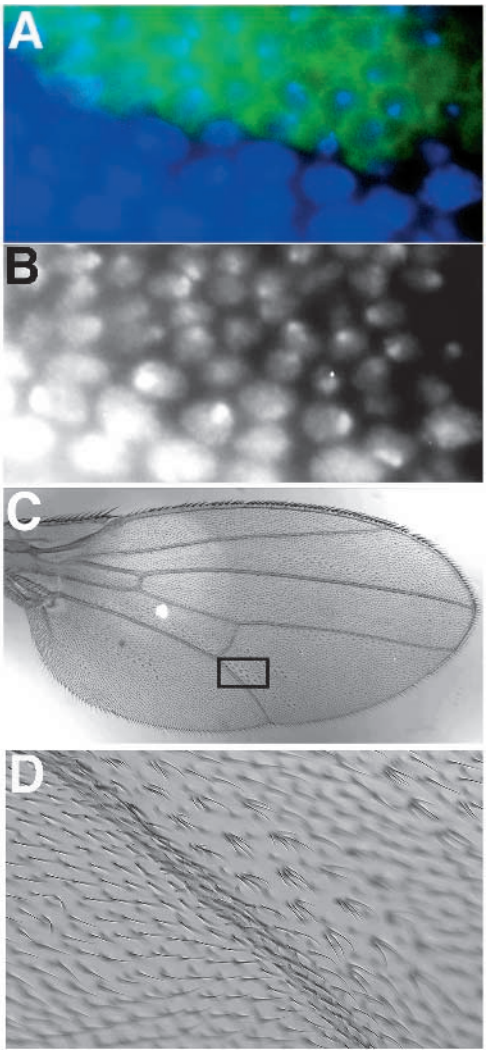

Fig. 2. Cells in*Cdc2*$^{E1\text{-}24}$ clones are increased in size and give rise to adult structures. The wings shown are from pupae which were shifted to 30°C at the start of pupariation. (A,B) Detail of a pupal wing containing a *Cdc2*$^{E1\text{-}24}$ clone fixed at 30 hours apf. This wing was stained with Hoechst (blue in A) and with an anti-Myc antibody (green in A), to identify wild-type cells (see Materials and methods). Wild-type cells are smaller than *Cdc2*$^{E1\text{-}24}$ cells. The abrupt transition from large to small cells coincides precisely with the clone border. The Hoechst single channel is shown in B. (C,D) An adult wing bearing *Cdc2*$^{E1\text{-}24}$ clones is shown. (D) A higher magnification of the region boxed in C. The clone can be identified by the presence of multiple clustered trichomes in mutant cells. Note that the cell density in the clone is reduced relative to the cell density outside the clone.

like pattern. The pattern of DNA replication was unchanged in clones of *Cdc2*$^{E1\text{-}24}$ 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

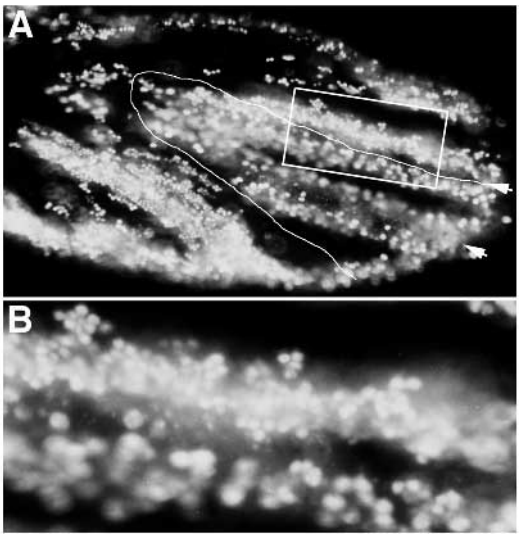

Fig. 3. Inactivation of Cdc2 inhibits cell division but DNA replication continues in the normal pattern in pupal wings. Pupal wing containing a *Cdc2*$^{E1\text{-}24}$ clone shifted to restrictive temperature at pupariation, injected with BrdU at 16 hours apf and analyzed at 30 hours apf is shown in A and the boxed region at higher magnification in B. The clone was identified based on the increased nuclear size revealed by the DNA labeling (not shown). The clone border is indicated by a line. The anti-BrdU staining shows that cells inside the clone replicate their DNA in the normal temporal and spatial pattern. The location of the 3rd and 4th vein are indicated by arrows. Nuclei of cells incorporating BrdU inside the clone are larger than nuclei outside the clone.

been described previously (Hayashi, 1996). However, we find that endoreduplication does not start immediately and synchronously in *Cdc2*$^{E1\text{-}24}$ mutant cells after shifting the G_2-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 *Cdc2*$^{E1\text{-}24}$ 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 G_2 at pupariation are partitioned into smaller cells by the two final divisions that they undergo. The large cell size observed after inactivation of *Cdc2*$^{E1\text{-}24}$ at pupariation, therefore, might not result from cell growth but could simply reflect the omission of such partitioning divisions. To establish that inactivation of *Cdc2*$^{E1\text{-}24}$ 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. *Cdc2*$^{E1\text{-}24}$ 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

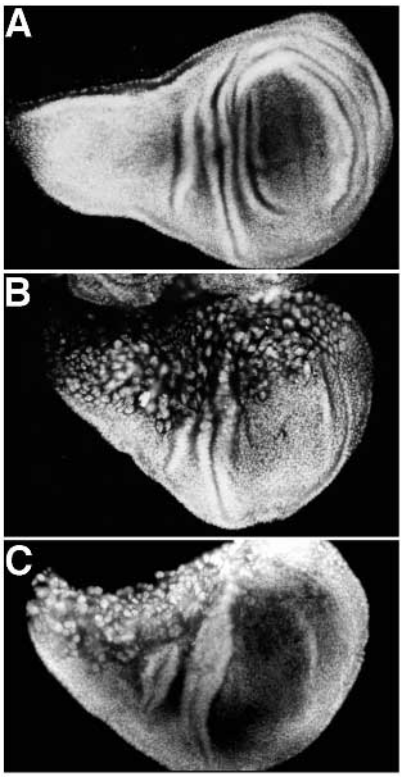

Fig. 4. Inactivation of Cdc2 function in larval development prevents cell division but not cell growth. DNA staining of wild-type (A) and *Cdc2*$^{E1-24}$, *en*-Gal4; UAS-*Cdc2*$^{+}$ wing imaginal discs (B,C); C was heat shocked at 37°C in early third instar and aged for 2 more days at 30°C. Anterior is to the top. Note the increased nuclear cell size in the cells of the anterior compartment of *Cdc2*$^{E1-24}$, *en*-Gal4; UAS-*Cdc2*+ (B,C). The proportion of cells that show increased nuclear size varies between discs.

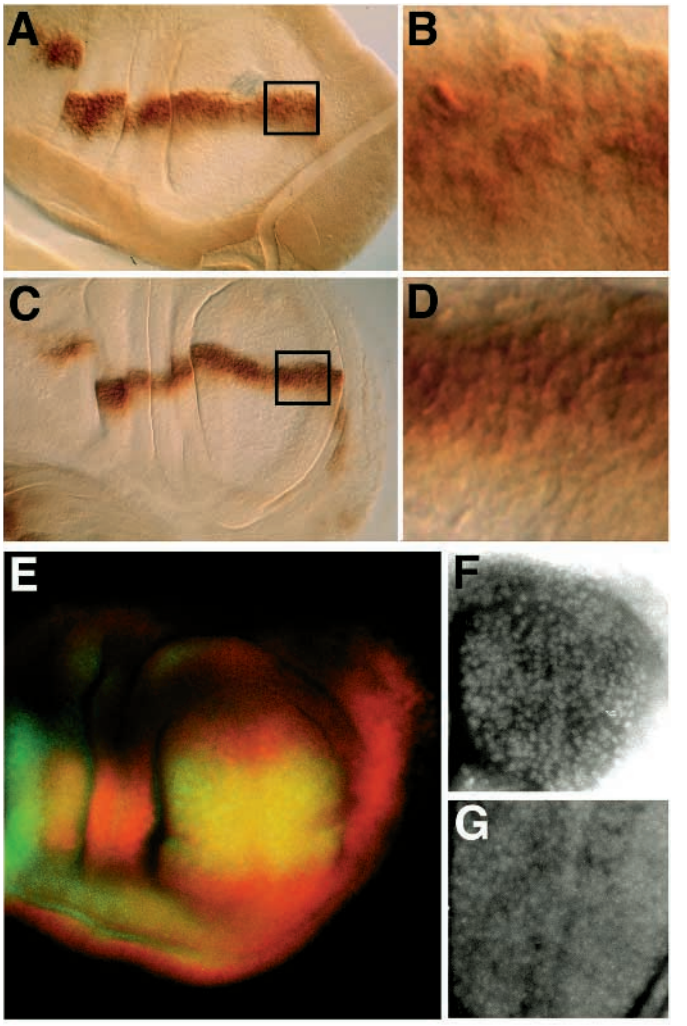

Fig. 5. Patterning along the anterior-posterior axis in *Cdc2*$^{E1-24}$ discs. (A-D) *dpp-lacZ* expression in *Cdc2*$^{E1-24}$ (A,B) and wild-type (C,D) discs. B and D show higher magnifications of A and C respectively. Cell size in B is increased relative to D. We note a slight relative increase in the width of the dpp-stripe in the *Cdc2*$^{E1-24}$ disc, which might reflect a slight developmental delay in *Cdc2* mutant discs at 30°C, equivalent to an earlier stage of development when the endogenous *dpp* stripe is broader. (E) Nested expression of Spalt protein (green) and *omb-lacZ* (red) visualized by anti-β-galactosidase staining, in a *Cdc2*$^{E1-24}$ disc. This nested pattern of *omb* and *spalt* expression reflects the wild-type situation (Lecuit et al., 1996; Nellen et al., 1996). (F) DNA staining of the same disc as in E shows fewer nuclei, which are less densely packed compared to a wild-type disc (G) treated in parallel.

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 in cells lacking Cdc2 activity.

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.,

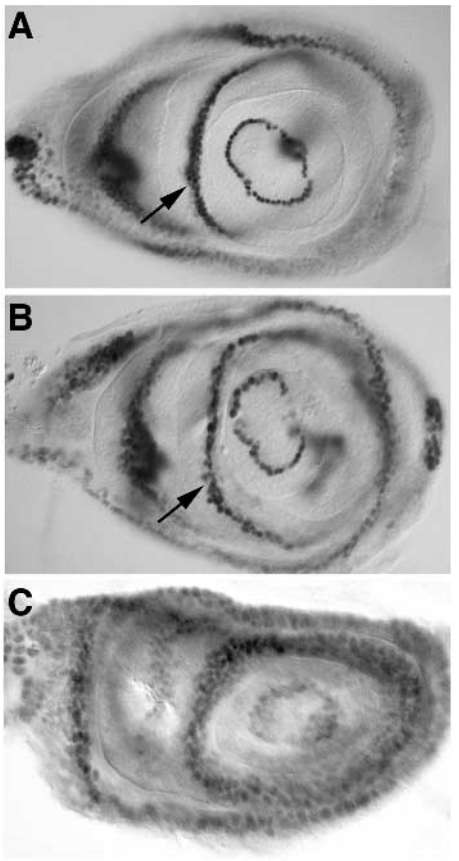

Fig. 6. *odd* expression is resolved to narrow stripes in *Cdc2*$^{E1-24}$ discs. *odd-lacZ* expression was detected by anti-β-galactosidase antibody staining. (A,B) late third instar discs from wild-type (A) and *Cdc2*$^{E1-24}$ (B) larvae, which were shifted to 30°C 20 hours after hatching to third instar. (C) wild-type disc fixed and stained 20 hours after hatching to third instar. Although *odd* is still expressed in broad rings at this time point, arresting cell division at this time point 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 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 G_2 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 G_2 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* Cdc2 kinase results in a transformation of mitotic division cycles into endoreduplication cycles. Hayashi (1996) has reported that imaginal discs cells 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

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*$^{E1\text{-}24}$ 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*$^{E1\text{-}24}$ 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 hyperactivation 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 (Santamaria, 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*$^{E1\text{-}24}$ 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 proliferate 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 information 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 patterning 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 accurately 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 developmental progam of cell divisions is inhibited (Hartenstein and Posakony, 1990). Similarly, although cell division is tightly regulated in the *Drosophila* retina, cell fate is assigned independent of cell lineage or the precise timing of division (de Nooij and Hariharan, 1995; Penton et al., 1997). Blocking cell divisions in *Xenopus* embryos does also not interfer with determination 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 Herskovitz, 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 patterning of trichomes.

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REFERENCES

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of *Drosophila* limb pattern by *hedgehog* protein. *Nature* **368**, 208-214.

Blackman, R. K., Sanicola, M., Raftery, L. A., Gillevet, T. and Gelbart, W. M. (1991). An extensive 3′ *cis*-regulatory region directs the imaginal disc expression of *decapentaplegic*, a member of the TGF-β family in *Drosophila*. *Development* **111**, 657-665.

Brand, A. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brook, W. J. and Cohen, S. M. (1996). Antagonistic interactions between *wingless* and *decapentaplegic* responsible for dorsal-ventral pattern in the Drosophila leg. *Science* **273**, 1373-1377.

Burke, R. and Basler, K. (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. *Development* **122**, 2261-2269.

Bryant, P. J. and Fraser, S. E. (1988). Wound healing, cell communication, and DNA synthesis during imaginal disc regeneration in Drosophila. *Dev. Biol.* **127**, 197-208.

Bryant, S. V., French, V. and Bryant, P. J. (1981). Distal regeneration and symmetry. *Science* **212**, 993-1002.

Capdevilla, J. and Guerro, I. (1994). Targeted expression of the signalling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. *EMBO J.* **13**, 4459-4468.

Cohen, S. M. (1993). Imaginal disc development. in *Drosophila Development.* (A. Martinez-Arias and M. Bate), pp. 747-841. Cold Spring Harbor: Cold Spring Harbor Press.

Cui, X. and Doe, C.Q. (1995). The role of cell cycle and cytokinesis in regulating neuroblast sublineage gene expression in the *Drosophila* CNS. *Development* **121**, 3233-3243.

de Nooi, J. C. and Hariharan, I. K. (1995). Uncoupling cell fate determination from patterned cell division in the *Drosophila* eye. *Science* **270**, 983-985.

Diaz-Benjumea, F. J. and Cohen, S. M. (1993) Interaction between dorsal and ventral cells in the imaginal disc directs wing development in Drosophila. *Cell* **75**, 741-752.

Diaz-Benjumea, F. J. and Cohen, S. M. (1995). Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. *Development* **121**, 4215-4225.

Duronio, R. J. and O'Farrell, P. H. (1994). Developmental control of a G1-S program in *Drosophila*. *Development* **120**, 1503-1515.

Edgar, B. A. and Lehner, C. F. (1996). Developmental control of cell cycle regulators: a fly's perspective. *Science* **274**, 1646-1652.

Edgar, L. G. and McGhee, J. D. (1988). DNA synthesis and the control of embryonic gene expression in *C. elegans*. *Cell* **53**, 589-599.

Fankhauser, G. (1941). Cell size, organ and body size in triploid newts (*Triturus viridescens*). *J. Morphol.* **68**, 161-177.

Fankhauser, G. (1945a). Maintainance of normal structure in heteroploid salamander larvae, through compensation of changes in cell size by adjustment of cell number and cell shape. *J. Exp. Zool*. **100**, 445-455.

Fankhauser, G. (1945b). The effects of changes in chromosome number on amphibian development. *Quart. Rev. Biol.* **20**, 20-78.

Foe, V.E. (1989). Mitotic domains reveal early commitment of cells in *Drosophila* embryogenesis. *Development* **107**, 1-25.

French, V., Bryant, P. J. and Bryant, S. V. (1976). Pattern regulation in epimorphic fields. *Science* **193**, 969-981.

González-Gaitán, M., Paz Capdevila, M. and García-Bellido, A. (1994). Cell proliferation patterns in the wing imaginal disc of *Drosophila*. *Mech. Dev.* **40**, 183-200.

Hartenstein, V. and Posakony, J. W. (1990). Sensillum development in the absence of cell division: The sensillum phenotype of the *Drosophila* mutant *string*. *Dev. Biol*. **138**, 147-158.

Harris, W. A. and Hartenstein, V. (1991). Neuronal determination without cell division in Xenopus embryos. *Neuron* **6**, 499-515.

Hayashi, S. (1996). A cdc2 dependent checkpoint maintains diploidy in *Drosophila*. *Development* **122**, 1051-1058.

Horvitz, H. R. and Herskovitz, I. (1992). Mechanisms of asymmetric cell division: Two Bs or not two Bs, that is the question. *Cell* **68**, 237-255.

Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation by *Drosophila vestigial* gene. *Nature* **382**, 133-138.

Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R. and Lehner, C. F. (1994). Cyclin E controls S phase progression and its downregulation during *Drosophila* embryogenesis is required for arrest of cell proliferation. *Cell* **77**, 107-120.

Kurzin, B., Roberts, I., Peunova, N. & Enikolopov, G. (1996). Nitric oxide regulates cell proliferation during Drosophila development. *Cell* **87**, 639-649.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments and pattern: lessons from Drosophila? *Cell* **85**, 951-961.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-393.

Lehner, C. F. and O'Farrell, P. H. (1989). Expression and function of *Drosophila* cyclin A during embryonic cell cycle progression. *Cell* **56**, 957-968.

Lehner, C. F. and O'Farrell, P. H. (1990). *Drosophila cdc2* homologs: a functional homolog is coexpressed with a cognate variant. *EMBO J.* **9**, 3573-3581.

Milan, M., Campuzano, S. and Garcia-Bellido, A. (1996). Cell-cycling and patterned cell proliferation in the wing primordium of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **93**, 640-645.

Morata, G. and Ripoll, P. (1975). Minutes: mutants of Drosophila autonomously affecting cell division rate. *Dev. Biol.* **42**, 211-221.

Nasmyth, K. (1996). Another role rolls in. *Nature* **382**, 28-29.

Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.

Neumann, C. J. and Cohen, S. M. (1996). Distinct mitogenic and cell fate specification functions of wingless in different regions of the wing. *Development* **122**, 1781-1789.

Neumann, C. J. and Cohen, S. M. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the *Drosophila* wing. *Development* **124**, 871-880.

Niswander, L., Tickle, C., Vogel, A., Booth, I. and Martin, G. R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75**, 579-587.

Peifer, M., Rauskolb, C., Williams, M., Riggleman, B. and Wieschaus, E. (1991). The segment polarity gene *armadillo* interacts with the *wingless* signaling pathway in both embryonic and adult pattern formation. *Development* **111**, 1029-1043.

Penton, A., Selleck, S. B. and Hoffmann, F. M. (1997). Regulation of cell cycle synchronization by *decapentaplegic* during *Drosophila* eye development. *Science* **275**, 203-205.

Posakony, L., Raftery, L. A. and Gelbart, W. M. (1991). Wing formation in *Drosophila melanogaster* requires *decapentaplegic* gene function along the anterior-posterior compartment boundary. *Mech. Dev* **33**, 68-82.

Santamaria, P. (1983). Analysis of haploid mosaics in *Drosophila*. *Dev. Biol.* **96**, 285-295.

Sauer, K., Knoblich, J., Richardson, H. and Lehner, C. F. (1995). Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic and endoreduplication cycles of Drosophila embryogenesis. *Genes Dev.* **9**, 1327-1339.

Schubiger, M. and Palka, J. (1987). Changing spatial patterns of DNA replication in the developing wing of Drosophila. *Dev. Biol.* **123**, 145-153.

Serrano, N. and O'Farrell, P. (1997). Limb morphogenesis: connections between patterning and growth. *Curr. Biol.* **7**, 186-195

Simpson, P. (1979). Parameters of cell competition in the compartments of the wing disc of Drosophila. *Dev. Biol.* **69**, 182-193.

Simpson, P. and Morata, G. (1981) Differential mitotic rates and patterns of growth in compartments in the Drosophila wing. *Dev. Biol.* **85**, 299-308.

Stern, B., Ried, G., Clegg, N. J., Grigliatti, T. A. and Lehner, C. F. (1993). Genetic analysis of the *Drosophila* cdc2 homolog. *Development* **117**, 219-232.

Stillman, B. (1996). Cell cycle control of DNA replication. *Science* **274,** 1659-1664**.**

Struhl, G. and Basler, K. (1993) Organizing activity of *wingless* protein in Drosophila. *Cell* **72**, 527-540.

Tabata, T., Schwartz, C., Gustavson, E., Ali, Z. and Kornberg, T. B. (1995). Creating a *Drosophila* wing de novo, the role of engrailed, and the compartment border hypotheses. *Development* **121**, 3359-3369.

Weigmann, K. and Lehner, C. F. (1995). Cell fate specification by *even-skipped* expression in the *Drosophila* nervous system is coupled to cell cycle progression. *Development* **121**, 3713-3721.

Williams, J. A., Paddock, S. W., Vorwerk, K. and Carroll, S. B. (1994). Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary. *Nature* **368**, 299-305.

Wuarin, J. and Nurse, P. (1996). Regulating S phase: CDKs, licensing and proteolysis. *Cell* **85**, 785-787.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

Yoffe, K. B., Manoukian, A. S., Wilder, E. L., Brand, A. H. and Perrimon, N. (1995). Evidence for *engrailed*-independent *wingless* autoregulation in *Drosophila*. *Dev. Biol.* **170**, 636-650.

Zecca, M., Basler, K. and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the *Drosophila* wing. *Development* **121**, 2265-2278.

Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a Wingless morphogen gradient. *Cell* **87**, 833-844.

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