

## A Cdc2 dependent checkpoint maintains diploidy in *Drosophila*

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### SUMMARY

DNA replication in G2 does not normally occur due to the checkpoint control. To elucidate its mechanism, the functions of the *escargot* and *Dmcdc2* genes of *Drosophila* were studied. When *escargot* function was eliminated, diploid imaginal cells that were arrested in G2 lost Cyclin A, a regulatory subunit of G2/M cdk, and entered an endocycle. *escargot* genetically interacted with *Dmcdc2* which encodes a catalytic subunit of G2/M cdk. The mutant phenotypes of *Dmcdc2* itself was similar to those of *escargot*: many diploid cells in imaginal discs, salivary glands and the central nervous system entered an

endocycle and sometimes formed polytene chromosomes. Since mitotically quiescent abdominal histoblasts still required *Dmcdc2* to remain diploid, the inhibitory activity of G2/M cdk on DNA replication appeared to be separable from its activity as the mitosis promoting factor. These results suggest that in G2, *escargot* is required to maintain a high level of G2/M cdk that actively inhibits the entry into S phase.

Key words: *Drosophila*, Cdc2, *escargot*, endoreplication, checkpoint, cell cycle

### INTRODUCTION

Eucaryotic cell cycle is regulated by an oscillation of two cyclin-dependent kinases G1/S cdk and G2/M cdk, responsible respectively for execution of S phase and M phase (Nurse, 1994). Activation of the two kinases take place alternately so that diploid genome size is maintained. The checkpoint controls ensure that the M phase entry depends on the completion of S phase, and the S phase entry depends on the completion of M phase (reviewed by Hartwell and Weinert, 1989; Nurse, 1994; Su et al., 1995). In the former case, the M phase entry is allowed only if DNA is fully replicated and is not damaged. Its presence can be recognized when DNA replication is blocked or the DNA repair system is activated by DNA damage. In such a situation, mitosis is also blocked. The presence of replication forks is thought to be the source of a signal which inhibits the activation of G2/M cdk, a complex including a regulatory subunit G2 cyclin and a catalytic subunit Cdc2. This inhibition involves phosphorylations at conserved Thr14 and Tyr15 which overlap the ATP binding site of Cdc2 (Gould and Nurse, 1989; Krek and Nigg, 1991a,b; Norbury et al., 1991). Dephosphorylation of these phosphates by String/Cdc25 phosphatase converts the G2/M cdk complex to the MPF (mitosis promoting factor) kinase which promotes mitosis (Edgar and O'Farrell, 1990; Gautier et al., 1991; Kumagai and Dumphy, 1991). Constitutively active Cdc2 mutant forms, which have amino acid substitutions at these phosphorylation sites, induce M phase entry before S phase completion (Gould and Nurse, 1989; Krek and Nigg, 1991b; Norbury et al., 1991). Thus the dependence of M phase on S phase appears to be maintained by the inhibitory phosphorylations of Cdc2. If this checkpoint control fails, cells enter M phase before S phase to reduce ploidy, as in meiosis, or attempt abortive mitosis while DNA is still replicating.

The S phase entry is controlled by another checkpoint which

restrains DNA replication before M phase. Various drugs such as colcemid block mitosis and simultaneously inhibit DNA replication. In *Drosophila*, a mutation of *string* (*stg*) arrests cells at the G2/M boundary and such cells do not enter S phase (Edgar and O'Farrell, 1989; Smith and Orr-Weaver, 1991). These observations suggest that cells in G2 and M phase are unable to activate replication machinery. The failure to enter S phase from G2 and M phase could be due to a lack of an essential replication licensing factor (Blow and Laskey, 1988). Another possibility is that S phase entry is actively repressed in G2 and M phase. Failure of such inhibition is expected to result in repeated S phase without M phase; a cell cycle called an endocycle. The focus of this paper is a negative control of the endocycle.

A well known example of an endocycle is found in *Drosophila* larval cells. Starting in late embryogenesis, larval cells undergo a temporally and spatially ordered series of endocycles to form polytene chromosomes (Smith and Orr-Weaver, 1991). The entire larval growth is accomplished without mitosis. During metamorphosis, the polyploid larval cells die and are replaced by a set of diploid imaginal cells that express a zinc finger transcription factor encoded by *escargot* (*esg*, Whiteley et al., 1992; Hayashi et al., 1993; Younossi-Hartenstein et al., 1993; Fuse et al., 1994). In *esg* hypomorphic mutants, diploid abdominal histoblasts, the precursor for the adult abdomen, entered an endocycle; a phenotype that can be rescued by the heat inducible *esg* transgene. Furthermore, ectopic *esg* expression in the salivary gland cells inhibited the endocycle. From these results, we proposed that the role of *esg* is to inhibit the endocycle to maintain diploidy so that imaginal cells retain an ability to proliferate and differentiate upon metamorphosis (Hayashi et al., 1993; Fuse et al., 1994).

Although the role of *esg* in maintaining diploidy of the abdominal histoblasts was clearly demonstrated, not all cells expressing *esg* entered an endocycle in *esg* mutants. Imaginal

disc cells in hypomorphic *esg<sup>P3</sup>/esg<sup>VS8</sup>* mutants grow and differentiate normally (Hayashi et al., 1993). Humeral imaginal disc cells of such larvae, however, become polyploid when the cell cycle is arrested by an additional *D-raf<sup>fl</sup>* mutation (Hayashi et al., 1993, Fig. 1G). This result suggests that cells need to be arrested in a certain stage of the cell cycle to be sensitive to the *esg* mutations (Hayashi et al., 1993). This also implies that *esg* is not a direct inhibitor of S phase entry, but rather acts as a regulator of the checkpoint control. In this study, more direct inhibitors of the endocycle were sought and Cdc2 was identified as an essential component of the checkpoint control.

## MATERIALS AND METHODS

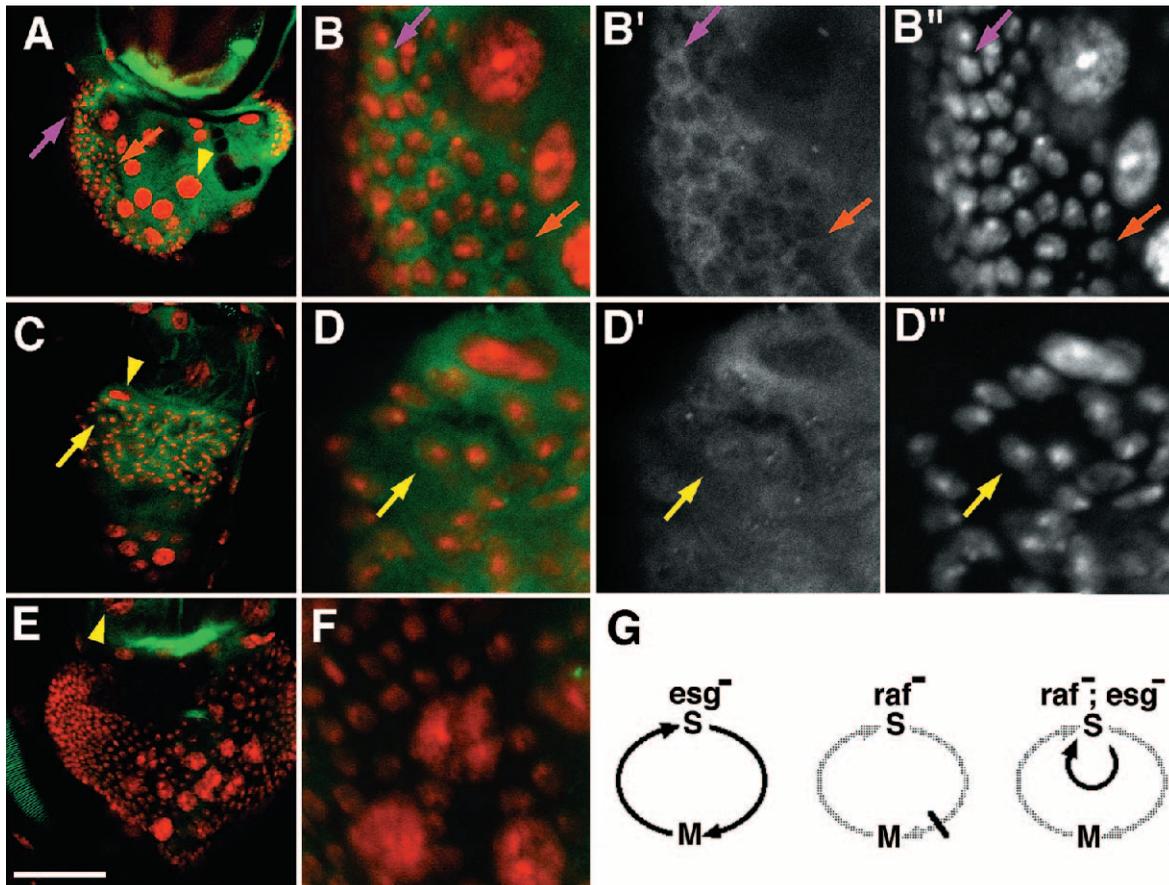
### *Drosophila* stocks

*y D-raf<sup>fl</sup>/Binsc* stock was obtained from Yasuyoshi Nishida, *Dmcdc2<sup>B47</sup>* and *Dmcdc2<sup>E1-24</sup>* stocks were obtained from Chris Lehner,

and the second chromosome insertion of *hs-stg* (RK2, Edgar and O'Farrell, 1990) was obtained from Ken-ichi Kimura. *Dmcdc2<sup>B47</sup> esg<sup>VS8</sup>* and *Dmcdc2<sup>E1-24</sup> esg<sup>P3</sup>* stocks were made by recombination. *Df(2L)J27* was obtained from Claire Cronmiller. *esg<sup>P3</sup>*, *esg<sup>VS8</sup>* and *esg<sup>G66B</sup>* were described by Whiteley et al. (1992); Hayashi et al. (1993). The mutant second chromosomes were balanced over *In(2LR)Gla*, *Bc Elp* (obtained from the Bloomington Stock Center) was used to help identify mutants. The *Dmcdc2* amorphic combination *Dmcdc2<sup>B47</sup>/Df(2L)J27* was cultured at 18°C. The temperature sensitive combination *Dmcdc2<sup>B47</sup>/Dmcdc2<sup>E1-24</sup>* was cultured at 29°C. *Dmcdc2<sup>B47</sup>/Df(2L)J27* showed stronger phenotypes.

### Chromosome analyses

Fig. 6A and B are images reconstructed from 15 serial optical sections (2 μm thick) of a propidium iodide (PI) stained specimen, taken using a Zeiss LSM 410-UV microscope. For squash preparations, a modified version of protocol 2 of Ashburner (1989) was used. Larval brains were incubated in 1% trisodium citrate for 5 minutes and fixed in a 3:1 mixture of glacial acetic acid and ethanol for 15 minutes,



**Fig. 1.** The cell cycle arrest phenotype of a *D-raf<sup>fl</sup>* humeral imaginal disc. Humeral discs with small diploid nuclei and associated anterior spiracles with large polyploid nuclei were stained with the monoclonal anti-Cyclin A antibody (green) and DAPI (red). (A,B) *D-raf<sup>fl</sup>* humeral disc. (C,D) *D-raf<sup>fl</sup>* humeral disc. Cell number was reduced due to the cell proliferation defect (Nishida et al., 1988). (E,F) *D-raf<sup>fl</sup>* control staining without incubation with the primary antibody. B', D' and B'', D'' are views of anti-Cyclin A and DAPI staining, respectively. *D-raf<sup>fl</sup>* cells accumulating Cyclin A in the cytoplasm (purple arrows) are presumably in G2. Cells with low Cyclin A levels are presumably in S or early G2 (orange arrows, Knoblich et al., 1994). In the *D-raf<sup>fl</sup>* humeral disc, Cyclin A was localized both in the cytoplasm and in the nucleus (yellow arrow). A similar pattern of Cyclin A accumulation was observed in cells in prometaphase in the embryo (Lehner and O'Farrell, 1989). The green signal in polyploid larval cells (yellow arrowheads) is at least in part, non-specific. Since it was occasionally detected in the control staining without the primary antibody (E) and staining with non-specific mouse IgG (not shown). These samples were stained simultaneously, scanned and processed under identical conditions, so that intensity of the signals can be directly compared. (G) Interpretation of *D-raf<sup>fl</sup>; esg<sup>-</sup>* double mutant phenotype. It was shown that the *esg* mutations do not affect the humeral disc cell cycle. However in *D-raf<sup>fl</sup>; esg<sup>-</sup>* double mutant, humeral disc cells entered an endocycle (Hayashi et al., 1993). This would suggest that in *D-raf<sup>fl</sup>* mutant humeral disc cells, DNA replication is inhibited by an *esg* dependent mechanism. Scale bar: 50 μm for A,C,E and 12.5 μm for B,D,F.

stained with 2% orcein in 33% acetic acid and 28% lactic acid for 15 minutes and squashed. It was not possible to assign the banding pattern to the cytological map of the salivary gland chromosome due to the diffuse appearance of the chromosomes. Salivary glands were processed similarly except that the trisodium citrate treatment was omitted.

### Histochemical staining

Larval tissues were immunostained as described by Hayashi et al. (1993), except that tissues were incubated in 0.5% NP40 in phosphate-buffered saline (PBS) for 15 minutes at room temperature prior to blocking. The samples were counter stained with 4'-diamidino-phenylindole dihydrochloride (DAPI) and/or PI to visualize nuclei. For anti-Cyclin A staining, monoclonal antibodies (gifts from Pat O'Farrell; two clones A2 and A19 were mixed) and FITC-labeled anti-mouse IgG (Jackson) were used. In some experiments, a rabbit polyclonal antibody (a gift from Ryu Ueda) was also used. The monoclonal anti-Engrailed antibody (Patel et al., 1989, a gift from Chihiro Hama) was used to stain imaginal discs. The monoclonal anti- $\beta$ -tubulin (Amersham) was used to stain the mitotic spindle. Dissected larval tissues were first treated with 1  $\mu$ M taxol in PBS for 60 seconds before fixation. For the bromodeoxyuridine (BrdU) labeling experiment, larvae were cultured at 29°C and were fed with 0.5 mg/ml BrdU for 18 hours during the late third instar and stained with anti-BrdU and FITC-conjugated secondary antibodies as described by Fuse et al. (1994). Mutant larvae were identified by the absence of the *Bc* marker. *Bc* larvae from the same vial were used as controls. Larval cuticle was peeled off the epidermis before staining. Anterior dorsal histoblast nests were identified by their position relative to the muscle insertion sites. The identity of these cells as abdominal histoblasts was confirmed by an experiment in which the *esg*<sup>P3</sup> enhancer trap was used as a marker for abdominal histoblasts (Hayashi et al., 1993). In most of the experiments, mutant and control larvae distinguishable with the *y* or *Bc* markers were placed and processed in the same tube to normalize staining efficiency.

### Heat shock experiment

Wandering third instar larvae in culture vials with fly food were heat shocked in an air incubator (38°C 30 minutes) then cultured at 25°C for the indicated time, fixed with 4% formaldehyde in PBS, stained with DAPI and observed with confocal microscopy. In some experiments, the samples were double stained with anti-Cyclin A and DAPI. There was some variability in the response of individual larvae to the heat treatment. Three out of four larvae, examined 30 minutes after the heat shock, and six out of seven larvae examined 60 minutes after the heat shock, exhibited the phenotype described in the Results. In contrast, all of the *y w* larva (*n*=8) showed normal morphology of abdominal histoblasts 60 minutes after heat shock. Both heat treated *hs-stg* and *y w* animals emerged with nearly normal adult morphology. The activity of *hs-stg* was confirmed by an induction of numerous mitotic figures in imaginal discs stained with an anti- $\beta$ -tubulin antibody.

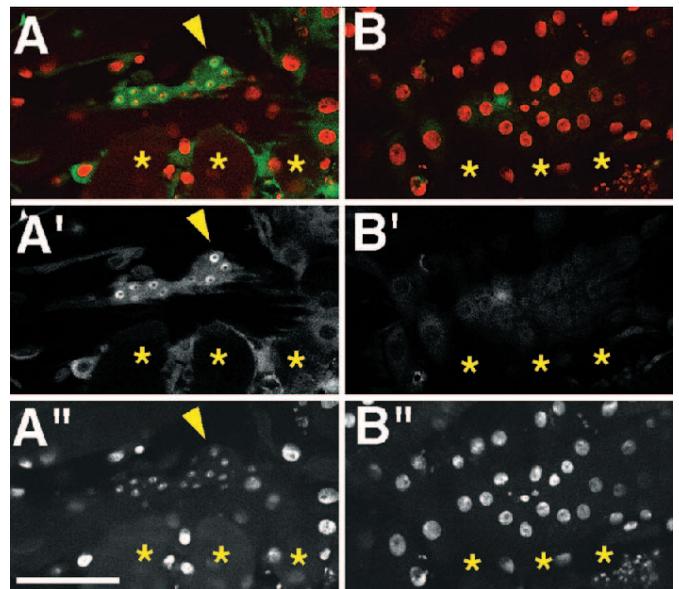
## RESULTS

### Cell cycle arrest promotes entry into an endocycle in *esg* mutants

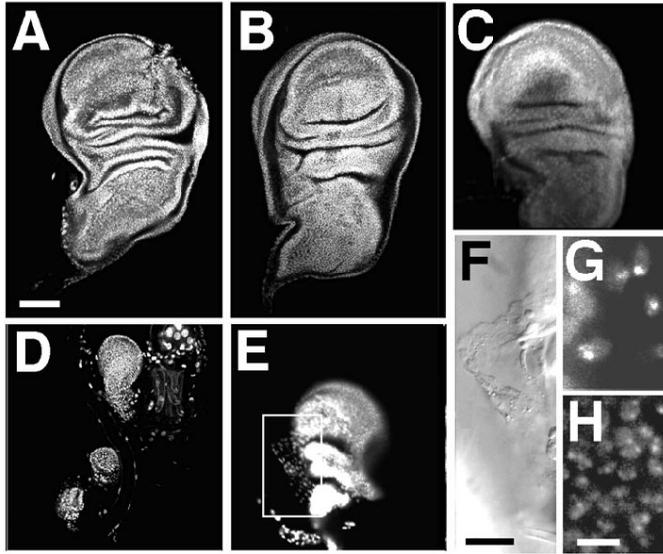
It was first determined whether entry into an endocycle in *esg* mutants is correlated to any particular stage of the cell cycle. The cell cycle phase of the *D-raf*<sup>l</sup> mutant humeral disc was assessed by using Cyclin A expression as a marker. It was shown that in the *Drosophila* embryo, accumulation and sub-cellular localization of Cyclin A change during the cell cycle (Lehner and O'Farrell, 1989). In G2, Cyclin A accumulates in the cytoplasm and then moves to the nucleus at the onset of

prophase. Cyclin A is then redistributed to both the cytoplasm and the nucleus and finally degraded at metaphase (Lehner and O'Farrell, 1989). Anti-Cyclin A staining identified two cell populations in the control disc, one is presumably in G2, accumulating high levels of Cyclin A in the cytoplasm, and the other is presumably in S or early G2 with low Cyclin A accumulation (Fig. 1B). In the *D-raf*<sup>l</sup> mutant, the number of humeral disc cells was reduced compared to the controls (Fig. 1C). Many of the *D-raf*<sup>l</sup> mutant humeral disc cells accumulated Cyclin A throughout the cell body (Fig. 1D), and in some cases, nuclear localization was also observed (Fig. 1D, yellow arrows). This observation suggests that *D-raf*<sup>l</sup> humeral disc cells were arrested at the G2/M boundary (Fig. 1G).

Although the localization of Cyclin A is suggestive of G2/M arrest, such an unusual localization could be due to pleiotropic effects of *D-raf*<sup>l</sup> mutation. Therefore arrested cells in wild-type larva were examined. Another example of *esg* mutant cells which enter an endocycle is the abdominal histoblasts (Roseland and Schneiderman, 1979). Their number remain constant in the larva. Since sister chromatid exchange is inducible in abdominal histoblasts throughout the larval stages, it was suggested that the abdominal histoblasts must be in G2 (Postlethwait, 1978). This idea was tested by anti-Cyclin A staining. Abdominal histoblasts were found to accumulate high level of Cyclin A, more preferentially in the nucleus (Fig. 2A); confirming that abdominal histoblasts are in G2/M boundary. Taken together, these observations would suggest that in *esg*



**Fig. 2.** Cyclin A is accumulated in abdominal histoblasts, and is lost when *esg* was mutated. The body wall of control *esg*<sup>V58/+</sup> (A) and *esg*<sup>V58/esg</sup><sup>P3</sup> (B) third instar larvae, stained with anti-Cyclin A (green, A',B') and DAPI (red, A'',B'') are shown. The anterior dorsal histoblast nest is located at the dorsal side of the insertion site of lateral transverse muscle 1,2 and 3 (asterisks; Roseland and Schneiderman, 1979). In the *esg*<sup>V58/+</sup> larva (A) abdominal histoblasts accumulated Cyclin A in the nucleus (arrowheads). In the *esg*<sup>V58/esg</sup><sup>P3</sup> larva (B) abdominal histoblasts that have become polyploid (Hayashi et al., 1993; Fuse et al., 1994) have lost Cyclin A expression. Scale bar: 100  $\mu$ m.



**Fig. 3.** Genetic interaction between *esg* and *Dmcdc2*. Imaginal discs stained with DAPI are shown. Sizes and overall morphologies of *esg*<sup>P3/+</sup> (A), *esg*<sup>P3/*esg*<sup>VS8</sup></sup> (B) and *esg*<sup>G66/*esg*<sup>VS8</sup></sup> (C) were normal. (D) *Dmcdc2*<sup>B47</sup> *esg*<sup>VS8/*esg*<sup>P3</sup></sup> imaginal discs. From top to bottom, a wing, haltere and a third leg discs are seen. The size of each disc was much smaller than the control discs in A,B and C, suggesting the 50% reduction of zygotic *Dmcdc2* dose prevented cell proliferation in the *esg* mutant discs. *Dmcdc2*<sup>B47</sup> heterozygotes developed normally (Clegg et al., 1993; Stern et al., 1993). (E) An example of a *Dmcdc2*<sup>B47</sup> *esg*<sup>VS8/*esg*<sup>G66</sup></sup> wing disc. The disc was reduced in size and a sac of tissue protrudes from wing pouch (boxed and enlarged in F). In addition, malformed discs were frequently observed. (F) Nomarski view of the boxed region of E. The sac-like tissue was continuous with the disc epithelium. (G) DAPI staining of F. Nuclei in the protrusion were about two-fold larger than nuclei in the control wing disc shown in H. Scale bars: (A-E) 100  $\mu$ m, (F) 50  $\mu$ m, (H) 10  $\mu$ m.

mutants, both *D-raf*<sup>l</sup> humeral disc cells and wild-type abdominal histoblasts enter the endocycle from G2.

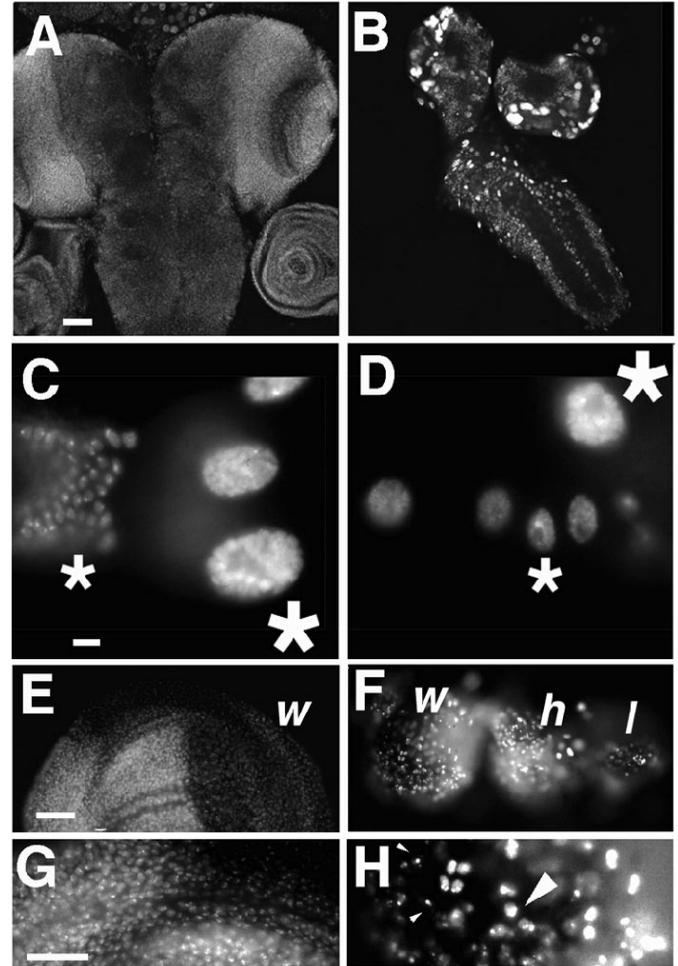
### Genetic interaction between *esg*, Cyclin A and Cdc2

If *esg* is required in G2 to prevent re-replication, an interaction between *esg* and a gene required for G2/M transition may be expected. Therefore the effect of a mutation in *esg* on G2-specific accumulation of Cyclin A was examined in abdominal histoblasts. In *esg*<sup>VS8/*esg*<sup>P3</sup></sup> larvae, abdominal histoblasts become polyploid (Hayashi et al., 1993). Such cells were found to no longer express high level of Cyclin A (Fig. 2B); suggesting that the Cyclin A accumulation is dependent upon *esg* activity.

Next, genetic interaction between *esg* and *Dmcdc2* was examined to determine whether *Esg* and G2/M cdk functionally interact. A single copy of the null *Dmcdc2*<sup>B47</sup> mutation was combined with hypomorphic *esg* mutations that do not affect the overall growth and pattern of imaginal discs (Fig. 3). In such larvae, imaginal discs were reduced in size (Fig. 3D,E), and were sometimes associated with cells with large nuclei (Fig. 3E, F, G). These results suggest that *esg* and *Dmcdc2* act in a common process to support cell proliferation. It also raised a possibility that Cdc2 may be involved in the maintenance of diploidy.

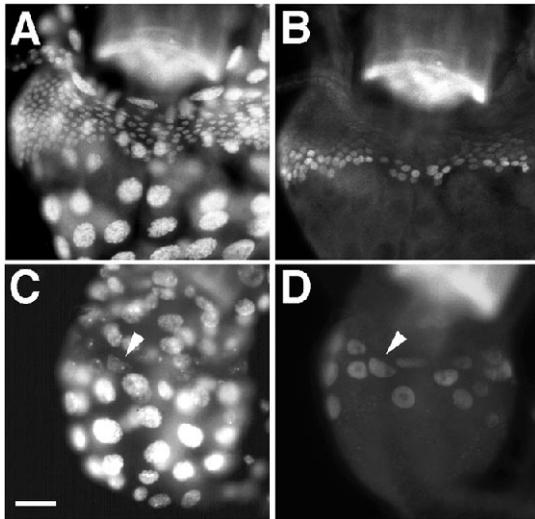
### Imaginal cells enter endocycle in *Dmcdc2* mutants

Phenotypes of larvae mutant for *Dmcdc2* were studied next.



**Fig. 4.** Mitotic cells enter an endocycle in *Dmcdc2* mutants. All samples were stained with PI or DAPI. (A) *Dmcdc2*<sup>B47/+</sup> control CNS. (B) A *Dmcdc2*<sup>B47/Df(2L)J27</sup> CNS. A and B are the same magnification. Note reduction of the size of CNS and presence of large nuclei in the outer optic lobe in B. (C) A control (*Dmcdc2*<sup>B47/+</sup>) salivary gland. The small asterisk indicates diploid cells in the imaginal ring. The large asterisk indicates the larval salivary gland cells. (D) A *Dmcdc2*<sup>B47/Df(2L)J27</sup> salivary gland. Note reduction of the number of imaginal ring cell (Stern et al., 1993) and an increase of nuclear size. (E) A control (*Dmcdc2*<sup>B47/+</sup>) wing imaginal disc. (F) *Dmcdc2*<sup>B47/Dmcdc2</sup><sup>E1-24</sup> wing (w), haltere (h) and third leg (l) imaginal discs. To allow partial growth of discs, animals were cultured at 18°C for 7 days and shifted to 29°C for 2 days to inactivate Cdc2 before fixation. (G) Enlargement of E. (H) Enlargement of the wing disc in F. Note enlarged nuclei (large arrowhead). Dying cells with small fragmented chromatin (small arrowheads) suggests that upon Cdc2 inactivation, imaginal disc cells take one of two alternative fates, either to die or to enter an endocycle. Samples in E-H were additionally stained with anti-engrailed antibody and HRP immunohistochemistry. Scale bars: (A,E) 100  $\mu$ m, (C) 10  $\mu$ m, (G) 20  $\mu$ m.

Maternal store of Cdc2 allows strong *Dmcdc2* mutants to complete embryogenesis and develop until puparium formation (Clegg et al., 1993; Stern et al., 1993). In the third instar mutant larvae, sizes of the central nervous system (Fig. 4B) and imaginal discs (Fig. 4F) were severely reduced due to the cell division defect as reported previously (Clegg et al., 1993; Stern et al., 1993). In addition, many mutant cells in the



**Fig. 5.** *esg-lacZ* expression in humeral imaginal discs of a *Dmcdc2* mutant. Humeral disc-anterior spiracle complexes of *esg<sup>P3</sup>/+* (A,B) and *Dmcdc2<sup>E1-24</sup> esg<sup>P3</sup>/Dmcdc2<sup>B47</sup> +* (C,D) are double stained with DAPI (A,C) and anti- $\beta$ -galactosidase (B,D). The large nuclei in the *Dmcdc2* mutant humeral disc (C, arrowhead) express *lacZ* (D, arrowhead) as in the control (B), demonstrating their identity as imaginal cell. Scale bar: 20  $\mu$ m.

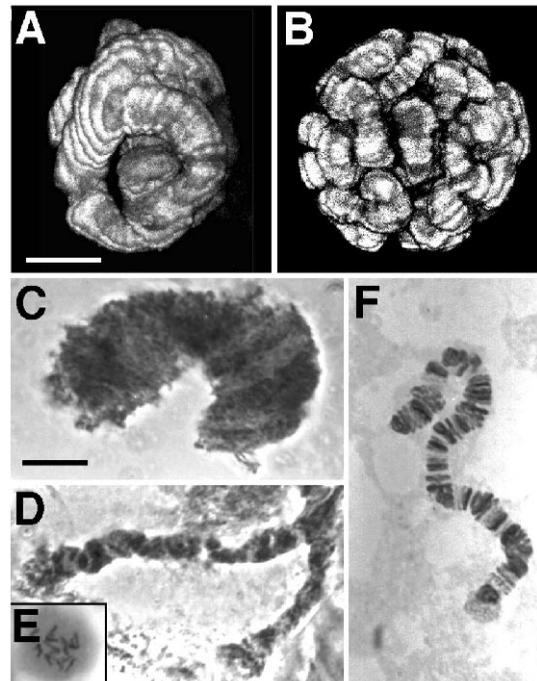
CNS, salivary gland and imaginal discs were found to be larger and stain more intensely with DAPI than normal diploid cells (Figs 4B,D,H, 5C). Although cell division was greatly reduced in *Dmcdc2<sup>B47</sup>/Dmcdc2<sup>E1-24</sup>* larvae, the cells in the CNS and the salivary gland imaginal ring incorporated BrdU during the third instar (data not shown). Identification of these large cells as imaginal cells was confirmed by expression of *lacZ* under control of the *esg* enhancer in the *esg<sup>P3</sup>* enhancer trap line (*esg-lacZ*) in humeral imaginal discs (Fig. 5) and abdominal histoblasts (data not shown).

The most striking phenotype was seen in the optic lobe. Many cells in the outer part of the lobe of *Dmcdc2<sup>B47</sup>/Df(2L)J27* larvae contained huge banded polytene chromosomes comparable in size and morphology to the polytene chromosomes in the salivary gland (Agard and Sedat, 1983, Fig. 6). Such cells expressed neuronal markers recognized by the monoclonal antibody 22C10 (Fujita et al., 1982) and by anti-horseradish peroxidase antibody (Jan and Jan, 1982, data not shown). These results demonstrate that diploid cells in *Dmcdc2* mutants enter an endocycle and form polytene chromosomes.

The next question to be answered was whether reduction of the *esg* dose has any effect on the *Dmcdc2* mutant phenotype. *Dmcdc2<sup>B47</sup>/Dmcdc2<sup>E1-24</sup>* was combined with either *esg<sup>P3</sup>*, *esg<sup>VS8</sup>* or both. No further enhancement of the polyploid phenotype was observed in the humeral imaginal disc, suggesting the loss of Cdc2 is sufficient to account for the polyploid phenotype (data not shown).

### Inhibition of endocycle is distinct from the mitotic function of Cdc2

The phenotype of the *Dmcdc2* mutants described above could be due to a direct consequence of the loss of Cdc2. Alternatively, the phenotype may be caused by a secondary effect of prolonged G2/M arrest. To distinguish these possibilities, the effect of *Dmcdc2* mutations on abdominal histoblasts that are

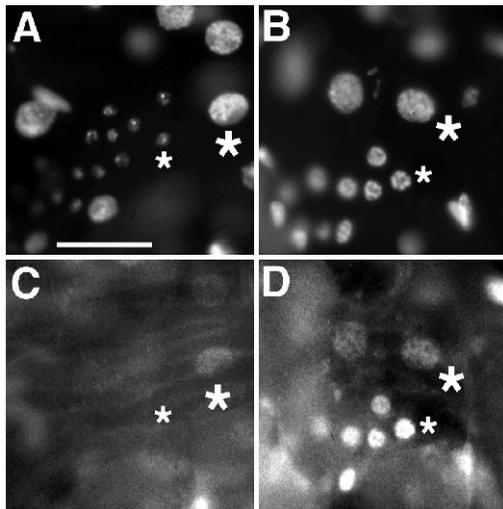


**Fig. 6.** Polytene chromosomes in the optic lobe of *Dmcdc2<sup>B47</sup>/Df(2L)J27* larvae (A,C,D). Polytene chromosomes of salivary glands are shown for comparison (B,F). (A,B) Views of polytene nuclei of *Dmcdc2<sup>B47</sup>/Df(2L)J27* reconstituted from a set of serial confocal optical sections. (A) One of the large optic lobe nuclei shown in Fig. 4B was enlarged. It clearly shows banded chromosome arms which are thicker and shorter than salivary gland chromosomes shown in B, which is identical in appearance to wild-type control (Stern et al., 1993). C-F. Squash preparations of chromosomes stained with orcein. Magnifications are the same for C-F so that the sizes are directly comparable. (C,D) Two examples of *Dmcdc2<sup>B47</sup>/Df(2L)J27* polytene chromosomes. The chromosomes were more fragile than salivary gland chromosomes and appeared diffuse. A mitotic figure of a wild-type brain cell is shown in E for a comparison. (F) An X chromosome from a salivary gland of a *Dmcdc2<sup>B47</sup>/+* larva. Scale bars: 10  $\mu$ m. A-B and C-F are in the same magnification respectively.

normally arrested in G2 in the larva was examined (Fig. 7). Abdominal histoblasts start proliferation only after puparium formation (Roseland and Schneiderman, 1979). Consequently, no BrdU incorporation was detectable in the control histoblasts labeled at the third instar (Hayashi et al., 1993; Fig. 7C). In contrast, abdominal histoblasts in *Dmcdc2* larvae incorporated BrdU without increasing the cell number, suggesting that they were in endocycle (Fig. 7B,D). This result indicates that the polyploid phenotype can be uncoupled from the mitotic arrest phenotype of *Dmcdc2* mutant.

### G2 form of Cdc2 inhibits endoreplication

The results presented above suggest that the Cdc2 protein inhibits endocycle in G2. Cdc2 exists as several isoforms with differential phosphorylation. To gain insight into which form of Cdc2 exists in abdominal histoblasts, its sensitivity to Stg was tested, which is a specific phosphatase that removes phosphate at Thr14 and Tyr15 and activates Cdc2 kinase. Stg was expressed in late third instar larvae by the heat inducible *hs-stg* transgene (Edgar and O'Farrell, 1990). 30 minutes after

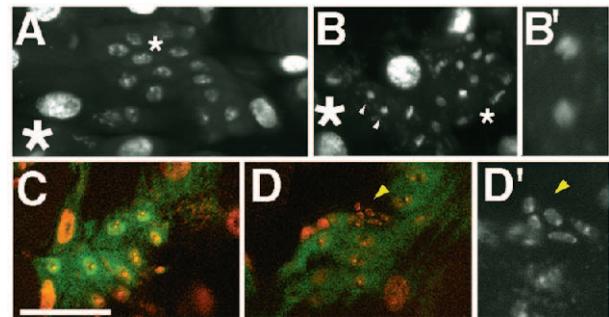


**Fig. 7.** *Cdc2* is required to inhibit the endocycle in abdominal histoblasts. Third instar larvae were fed with BrdU before fixation and double staining with DAPI (A,B) and anti-BrdU (C,D). Abdominal histoblasts are labeled with small asterisks and larval epidermal cells are labeled with large asterisks. While nuclei of control abdominal histoblasts were small (A) and did not incorporate BrdU (C), nuclei of *Dmcdc2<sup>B47</sup>/Dmcdc2<sup>E1-24</sup>* abdominal histoblasts were larger (B) and incorporated BrdU (D). The *Dmcdc2* mutations did not change the number of the histoblasts. BrdU incorporation by the larval epidermal cells (large asterisks) serves as an internal control. Scale bar: 50  $\mu$ m.

a 38°C heat shock, abdominal histoblast chromatin started to condense, and at 60 minutes after the heat shock, numerous condensed, chromatin was observed (Fig. 8B). Occasionally, pairs of nuclei which apparently have just completed division were observed (Fig. 8B'). Such cells were found to lose Cyclin A expression; suggesting they have finished mitosis. Most of these nuclei were irregular in size and shape. Condensation of chromatin was reported for mammalian cells expressing the constitutively active form of *Cdc2* (Krek and Nigg, 1991b). These phenotypes suggest that in abdominal histoblasts, MPF was activated by the ectopic expression of *Stg*. Such cells attempt premature mitosis which was completed in some cases. This result indicates that *Cdc2* in abdominal histoblasts exists as a form phosphorylated at Thr14 and Tyr15.

## DISCUSSION

The function of *Cdc2* in inhibiting endoreplication and its relationship to *esg* has been investigated and it has been found that many diploid cells lacking *Dmcdc2* entered an endocycle. This phenotype is similar to that of abdominal histoblasts in *esg* hypomorphic mutants, but is more widespread and pronounced. The cell cycle arrest phenotype of *D-raf<sup>l</sup>* mutant humeral disc (Fig. 1) and wild-type abdominal histoblasts (Fig. 2), and the genetic interaction between *esg* and *Dmcdc2* (Fig. 3) are consistent with an idea that *esg* is required in G2 to prevent endoreplication. A reduction of *Dmcdc2* dose enhanced *esg* mutant phenotype, but a reduction of *esg* activity in the absence of *Dmcdc2* activity had no further effect. Expression of Cyclin A, a regulatory subunit of G2/M cdk, in



**Fig. 8.** Ectopic *string* expression induces premature chromosome condensation and mitosis in abdominal histoblasts. Abdominal histoblasts were stained either with DAPI alone (A,B), or with anti-Cyclin A (green) and DAPI (red; C,D). Small and large asterisks indicate abdominal histoblasts and larval epidermal cells respectively. (A) Anterior dorsal histoblast nest of a control *y w* larva 60 minutes after a heat shock. The appearance of the histoblast nuclei was identical to non-heat shocked control larvae (data not shown). (B) A heat shocked heterozygous *hs-stg* larva. The histoblast chromatin appeared much more compact with an irregular shape. Occasionally, pairs of nuclei which apparently are undergoing mitosis were observed (arrowheads and enlarged in B'). (C) A *y w* larva 60 minutes after heat shock doubly stained with anti-Cyclin A and DAPI. (D) *hs-stg* larva 60 minutes after heat shock. In this example, pairs of cells that have lost Cyclin A are seen (arrowhead). (D') Enlarged view of DAPI staining of D. Bar: 50  $\mu$ m (A-D).

abdominal histoblasts was lost in *esg* mutants (Fig. 2), suggesting *Cdc2* activity depends upon *esg*. In contrast, *esg* transcription as revealed by *esg-lacZ* reporter expression was not affected by the *Dmcdc2* mutations (Fig. 5; data not shown). Given the function of *Esg* as a transcriptional regulator and the function of *Cdc2* as a master regulator of the cell cycle, it would seem likely that G2/M cdk is downstream of *esg* in a regulatory cascade inhibiting endoreplication. It appears that *esg* acts to maintain high levels of G2/M cdk complex in G2. G2/M cdk then inhibits entry into S phase. However, with the data currently available, alternative possibilities such as *Cdc2* regulating *Esg* at a post-transcriptional level, or each protein acting in parallel pathways to inhibit the endocycle cannot be ruled out.

### G2/M cdk is a source of an inhibitory signal of S phase entry

Results presented here suggest S phase is actively inhibited in G2 by an activity requiring *Cdc2*. This idea is supported by the study of Cyclin E regulation in the *Drosophila* embryo. Cyclin E is an essential regulator of G1/S transition (Knoblich et al., 1994). It was shown that the H1 kinase activity associated with Cyclin E and *Dmcdc2c*, a putative G1/S cdk catalytic subunit, is detectable in G2 extracts as well as in G1 extracts (Sauer et al., 1995). Such cells do not enter S phase from G2; suggesting that the kinase activity of Cyclin E/*Dmcdc2c* is dominantly masked in G2.

Since *Cdc2* is constitutively present throughout the cell cycle (Edgar et al., 1994), its G2-specific form must be responsible for the inhibition of endoreplication. Sauer et al. (1995) demonstrated that DNA replication without cell division occurred in *Dmcdc2c* mutants. Therefore, taken together these findings would suggest that *Cdc2* associated with Cyclin A is likely to

be an active form which inhibits S phase entry. Support for this idea came from the observations in yeast, *S. pombe*, that mutants deficient for either *cdc2* or *cdc13* (encode B-type cyclin) or a strain overproducing the *rum1*<sup>+</sup> product, a putative G2/M cdk inhibitor, entered an endocycle (Broek et al., 1991; Hayles et al., 1994; Moreno and Nurse, 1994). The phenotype was ascribed to the loss of G2/M cdk that resets the cell cycle state to G1. It would appear that in *esg*, *Dmcdc2*, or in *Dmcyca* mutant cells, the G2/M cdk complex is lost and the cells switch to G1. Once in G1, replication origins, or a putative replication licensing factor become reactivated and cells enter S phase when an appropriate growth signal is provided (Su et al., 1995).

### Is the kinase activity of Cdc2 essential for the inhibition of S phase initiation?

Abdominal histoblasts maintain a long period of G2 arrest throughout the entire larval stage, during which DNA replication is inhibited by Cdc2. The results of the *hs-stg* experiment suggests that Cdc2 in larval abdominal histoblasts is phosphorylated at Thr14 and Tyr15. Since these phosphorylation sites overlap the ATP binding site, such a form of Cdc2 is probably ineffective as a kinase (Gould and Nurse, 1989). This raises a possibility that G2/M cdk uses a mechanism other than a kinase activity to send an inhibitory signal of the S phase entry. This idea is consistent with the phenotype of *stg* mutants in which no DNA replication was detectable except in endoreplicating tissues (Smith and Orr-Weaver, 1991). Such embryos accumulated the phosphorylated form of Cdc2 (Edgar et al., 1994). This form of Cdc2 is likely to be responsible for the inhibition of endoreplication if observations in the larva also apply to the embryo. An alternative idea that the kinase activity of G2/M cdk is responsible for the inhibition of DNA replication has been postulated, based on the effect of a kinase inhibitor to induce endoreplication (Usui et al., 1991, reviewed by Su et al., 1995). Testing effects of mutant forms of Cdc2 on DNA replication should resolve this issue in the future.

### Transition from mitotic to endo cell cycle during embryogenesis

Does the regulation of G2/M cdk have any role in the onset of endocycle? It was shown that initially high and ubiquitous *Dmcdc2*, *Dmcyca* and *Dmcycb* transcripts in the early embryo decrease in the late stage and become restricted only to proliferating tissues (Lehner and O'Farrell, 1989; Whitfield et al., 1989; Jimenez et al., 1990; Lehner and O'Farrell, 1990a,b). It is possible that in the first round of the endocycle, cells lacking some G2/M cdk subunits enter S phase. Such cells find no G2/M cdk in G2 and reset to G1 by default; which is a completion of the endocycle. Thus the down regulation of G2/M cdk is likely to have a permissive role for the transition to an endocycle. Keeping a level of G2/M cdk sufficient to repress endoreplication is thus an essential requirement for imaginal cells to remain diploid.

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