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decapentaplegic is required for arrest in G_1 phase during *Drosophila* eye development

Julie Horsfield[1,*], Andrea Penton[*,‡], Julie Secombe[1], F. Michael Hoffman[2] and Helena Richardson[1]

[1]Department of Genetics, Adelaide University, South Australia, 5005, Australia
[2]McArdle Laboratory for Cancer Research and Laboratory of Genetics, University of Wisconsin Medical School, Madison, WI 53706, USA
*These authors contributed equally to this work
‡Present address: Department of Developmental Biology, Howard Hughes Medical Institute, Stanford University, Beckman Centre, Stanford, CA 94305-5428, USA
Author for correspondence (e-mail: herichardson@gina.science.adelaide.edu.au)

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SUMMARY

During eye development in *Drosophila*, cell cycle progression is coordinated with differentiation. Prior to differentiation, cells arrest in G_1 phase anterior to and within the morphogenetic furrow. We show that Decapentaplegic (Dpp), a TGF-β family member, is required to establish this G_1 arrest, since Dpp-unresponsive cells located in the anterior half of the morphogenetic furrow show ectopic S phases and ectopic expression of the cell cycle regulators Cyclins A, E and B. Conversely, ubiquitous over-expression of Dpp in the eye imaginal disc transiently inhibits S phase without affecting Cyclin E or Cyclin A abundance. This Dpp-mediated inhibition of S phase occurs independently of the Cyclin A inhibitor Roughex and of the expression of Dacapo, a Cyclin E-Cdk2 inhibitor. Furthermore, Dpp-signaling genes interact genetically with a hypomorphic *cyclin E* allele. Taken together our results suggest that Dpp acts to induce G_1 arrest in the anterior part of the morphogenetic furrow by a novel inhibitory mechanism. In addition, our results provide evidence for a Dpp-independent mechanism that acts in the posterior part of the morphogenetic furrow to maintain G_1 arrest.

Key words: *dpp*, TGF-β, G_1 arrest, Cell cycle, Eye development, *Drosophila melanogaster*

INTRODUCTION

As multicellular organisms develop, growth signals regulating cell proliferation must be coordinated with developmental signals controling tissue patterning. Recent research in this area has revealed the existence of strong connections between the cell cycle and developmental decisions (reviewed by Follette and O'Farrell, 1997a; Gao and Zelenka, 1997; Lehner and Lane, 1997). Cell cycle regulation is one function of the developmental regulator Transforming Growth Factor-β (TGF-β), a member of the TGF-β superfamily of signaling molecules (reviewed by Massague and Polyak, 1995).

The TGF-β superfamily is involved in a wide range of developmental functions in both vertebrates and invertebrates (reviewed by Wall and Hogan, 1994; Alevizopoulos and Mermod, 1997). In *Drosophila melanogaster*, there are three TGF-β homologs, of which *decapentaplegic* (*dpp*) is the best understood. Dpp is most closely related to the mammalian BMP2/BMP4 subgroup within the TGF-β superfamily and acts as a morphogen, mediating cell proliferation, differentiation and patterning in many *Drosophila* tissues (Capdevila and Guerrero, 1994; Campbell and Tomlinson, 1995). Dpp signaling in *Drosophila* is similar to TGF-β signaling in vertebrates, which occurs via type I and type II ser/thr protein kinase receptors (reviewed by Massague, 1996). Cells respond to Dpp through two type I receptors, Thick-veins (Tkv) or Saxophone (Sax) and a type II receptor, Punt. The Mad family members, Mothers-against-Dpp (Mad) and Medea (Med), and the transcription factor Schnurri (Shn) are positive effectors of Dpp signaling that function downstream of the receptors (reviewed by Massague, 1996). Daughters-against-Dpp (Dad), a Mad-like protein, acts as an antagonist of the Dpp-signaling pathway (Tsuneizumi et al., 1997).

Our study concerns the role of Dpp in eye development. The adult eye develops from an epithelial monolayer known as the eye imaginal disc. In third instar larvae, differentiation begins in the posterior end of the disc, progresses anteriorly and is marked by an indentation in the disc epithelium, termed the morphogenetic furrow (MF). Within the MF, unpatterned cells are induced to differentiate into the highly ordered array of retinal cells and non-neural accessory cells that produce the approx. 750 ommatidia of the adult eye (reviewed by Wolff and Ready, 1993). Differentiated cells posterior to the MF express the signaling molecule Hedgehog (Hh) that directs the anterior advancement of the MF (reviewed by Heberlein and Moses, 1995). Hh induces *dpp* and genes involved in neuronal

development, such as *atonal* (Heberlein et al., 1995). It has been postulated that Hh and Dpp coordinate MF initiation and propagation across the eye disc (Ma et al., 1993; Chanut and Heberlein, 1997; Pignoni and Zipursky, 1997; Borod and Heberlein, 1998). In addition, a role for Dpp in cell cycle control in the MF has recently been described (Penton et al, 1997).

Coordination of the cell cycle with differentiation is essential for correct patterning of the eye (Thomas et al., 1994; Richardson et al., 1995; Penton et al., 1997; Secombe et al., 1998). The eukaryotic cell cycle is regulated by the controled activity of cyclin-Cdk (cyclin-dependent protein kinase) complexes (reviewed by Nigg, 1995). In *Drosophila*, Cyclin E is necessary for the G_1 to S phase transition while Cyclins A and B are necessary for progression from G_2 into mitosis, although Cyclin A may also play a role in S phase (reviewed by Follette and O'Farrell, 1997b). Entry into mitosis also requires *Drosophila* String (Stg), a homolog of the Cdc25 phosphatase that activates Cyclin B-Cdk1 (Cdc2) and Cyclin A-Cdk1 protein kinases (reviewed by Lehner and Lane, 1997). The G_1 to S phase transition is negatively regulated by the Cyclin E-Cdk2 inhibitor Dacapo (Dap), a homolog of the p21/p27/p57 family of Cdk inhibitors, or by Roughex (Rux), a Cyclin A inhibitor (reviewed by Lehner and Lane, 1997). In the developing eye disc, 8-9 rows of cells become arrested in G_1 phase for about 14 hours within (and anterior to) the MF prior to differentiation (Wolff and Ready, 1993). For the sake of simplicity we are defining the MF as encompassing this zone of G_1-arrested cells. Immediately posterior to the MF, a subset of cells differentiate to form the precluster cells while the remaining cells enter a synchronous S phase (Wolff and Ready, 1993). Cyclin E is expressed in a subset of cells immediately posterior to the MF and its expression correlates with entry of these cells into S phase (Richardson et al., 1995). Correct temporal and positional expression of Cyclin E is critical for normal eye development. Ectopic Cyclin E expression causes precocious entry into S phase of most G_1-arrested cells within the MF and disrupts patterning in the adult eye (Richardson et al., 1995). Conversely, inhibition of Cyclin E function by expression of human p21 in cells posterior to the MF prevents entry into S phase and also disrupts eye patterning (de Nooij and Hariharan, 1995).

It has been suggested that Dpp may lead to G_1 arrest within the MF by regulating the G_2 to M phase transition in cells anterior to the MF (Penton et al., 1997). This study showed that *tkv*, *sax* or *shn* mutant clones, which cannot respond to Dpp, fail to arrest in G_1 and continue to express the G_2 marker Cyclin B within the MF. This implied that these cells are delayed in G_2 and it was proposed that Dpp has a role in inducing mitosis anterior to the MF (Penton et al., 1997). However, this conclusion contrasts with the established role of mammalian TGF-β in G_1 arrest, which acts by inducing G_1 cyclin-Cdk inhibitors (Massague and Polyak, 1995). Furthermore, the expression of other cell cycle markers in these Dpp-unresponsive clones was not examined, so it could not be concluded whether this apparent G_2 block was the only cell cycle defect caused by the absence of Dpp signaling. In the present study, we have examined G_1/S phase markers as well as G_2-M phase markers in Dpp-unresponsive clones and determined the effect of ectopic over-expression of Dpp on S phases in eye discs. Contrary to previous conclusions (Penton et al., 1997), our results provide evidence that Dpp induces G_1 arrest, rather than promoting the G_2 to M phase progression, in the MF during eye development.

MATERIALS AND METHODS

Fly strains and genetic interaction analysis

For examining *dpp*-pathway mutants for genetic interaction with *DmcycE*, the hypomorphic allele $DmcycE^{JP}$, which exhibits a rough eye phenotype (Secombe et al., 1998), was used. In order to test mutations in the *dpp*-signaling pathway for genetic interactions with $DmcycE^{JP}$, stocks were generated that contained $DmcycE^{JP}$ (either heterozygous over *CyO* or homozygous) together with the test allele over a balancer chromosome. For test alleles on the second chromosome (*dpp*, *tkv, mad*, *sax*), recombinants with $DmcycE^{JP}$ were generated using marked $DmcycE^{JP}$ stocks and the recombinant stock maintained over *CyO* or *CyO, Tb* second chromosome balancers. Test alleles on the third chromosome (*punt*, *med*, *dad*) were maintained over *TM6B, Tb*, *Hu* or *TM2, Ubx* balancer chromosomes. Stocks homozygous for $DmcycE^{JP}$ and heterozygous for 3rd chromosome alleles were generated. For analysis of genetic interactions with $DmcycE^{JP}$, stocks were outcrossed to $DmcycE^{JP}$ at 25ºC and progeny that were homozygous for $DmcycE^{JP}$ and heterozygous for the test allele (at least 50 progeny) were scored for their eye phenotype compared with $DmcycE^{JP}$. To minimize effects due to genetic background, stocks of $DmcycE^{JP}$ that were isogenic for the 2nd and 3rd chromosome were used, and for *dpp* pathway alleles on the second chromosome, at least two recombinant lines were examined for each allele. Various *dpp* or *dpp* pathway mutants were also examined for effects on the $DmcycE^{JP}$ rough eye phenotype when transheterozygous with $dpp^{d\text{-}ho}$ or other *dpp* alleles. To examine the effect of increasing the dosage of *dpp* on the $DmcycE^{JP}$ rough eye phenotype, leaky expression of *dpp* was obtained by crossing the $DmcycE^{JP}$; *UAS-*$dpp^{42B.4}$ flies to $DmcycE^{JP}$; *hsp70-GAL4* flies at 25ºC.

The following *dpp* alleles were examined for an interaction with $DmcycE^{JP}$: the dpp^{disc} alleles *d-ho, d-blk, d5 (disc II), d6 (disc III), d12 (disc V), d14 (disc V)*; the weak alleles *e87*, *102* and *99*; the temperature sensitive alleles *hr56*, *e90* and *hr4;* and the strong embryonic lethal alleles *98* and *97*. Homozygous $dpp^{d\text{-}blk}$, dpp^{d5} and dpp^{d6} exhibit small rough eyes, whereas $dpp^{d\text{-}ho}$ has held-out wings, but no noticeable eye roughening. The following *dpp* pathway alleles were examined: sax^4 (null), sax^P (strong hypomorph), tkv^5 (null), tkv^7 (null), shn^{1B} (strong hypomorph), shn^{P4738} (strong hypomorph), $mad^{P[walter]}$ (hypomorph), $punt^{135}$ (null), $punt^{p62}$ (hypomorph), med^3 (null), and the Dpp pathway antagonist *dad* alleles: dad^{P1883} (*dad P* allele), $dad^{\Delta 1883\text{-}3a}$ (*dad* deletion generated from the *P1883 P* allele), $dad^{\Delta 1E427\text{-}68}$ (deletion generated from the *l(3)1E4 P* allele). Fly stocks were obtained from L. Raftery, T. Tabata and the Bloomington stock center.

Generation of clones, antibody staining and BrdU labeling

tkv^5 and sax^4 clones were generated as described by Penton et al. (1997), using the method of Xu and Rubin (1993). For clonal analysis, the Myc protein was induced by heat shocking third instar larvae for 75 minutes in a 37ºC water bath in glass vials, followed by 75 minutes recovery at 18ºC prior to dissection of eye discs. For antibody detection of Myc and Cyclins A, B or E, third instar larval eye discs were fixed and stained as described by Penton et al. (1997). The following antibodies and dilutions were used: mouse anti-Myc monoclonal (1:5); rabbit anti-Stg (1:200) mouse anti-Cyclin E polyclonal (Richardson et al., 1995; 1:1000); rabbit anti-Cyclin B (1:200); rabbit anti-Cyclin A (1:200); mouse anti-Dacapo monoclonal (1:50), and mouse anti-MPM-2 monoclonal (DAKO Inc.; 1:10). Antibodies were visualized using either fluorescein, cy-5 or

rhodamine-conjugated secondary antibody or tertiary streptavidin complexes (Jackson Immunochemicals).

Bromodeoxyuridine (BrdU) labeling of S phases in third instar eye discs was carried out as described by Secombe et al. (1998), except where triple staining for BrdU, Myc and Cyclin A or B was done. In these cases, the BrdU incorporation step was performed first followed by antibody detection of Myc, and Cyclin A or B, and then samples were hydrolyzed and stained using an anti-BrdU antibody (Becton-Dickinson; 1:50) followed by a fluorophor-tagged secondary antibody.

For experiments examining the effect of rux^8 on S phases, y^2, cho^2, $rux^8/FM7C$ females (obtained from B. Thomas) were crossed to *hsp70-GAL4*, *UAS-dpp*$^{42B.4}$*/TM6B* males and larvae hemizygous for rux^8 and heterozygous for *hsp70-GAL4*, *UAS-dpp*$^{42B.4}$ were identified as *cho* (chocolate-colored Malphigian tubules) non-*TM6B* (non-tubby) larvae. As a control, y^2, cho^2, $rux^8/FM7C$ females were also crossed to w^{1118} males.

Heat shock induction of *dpp*

For over-expression of *dpp*, flies recombinant for *UAS-dpp*$^{42B.4}$ (3rd chromosome) and *hsp70-GAL4* (3rd chromosome; obtained from A. Brand) were generated and maintained over *TM6B* at 18ºC. Heat-shock induction of *dpp* in wandering third instar larvae was carried out by heat shocking at 38ºC for 1 hour. The samples were subsequently returned to 20ºC for 30 minutes or more before dissection.

Microscopy

Flies were prepared for scanning electron microscopy as described by Secombe et al. (1998) and viewed at 200× magnification. Imaginal disc samples were stained and mounted as described by Secombe et al. (1998) and viewed using either a Ziess Axioplan microscope with a 20× objective, a MRC1000 Confocal microscope with a 60× oil immersion objective or a Olympus Provis AX70 microscope mounted with a Photometrics Nu200 CCD camera, with a 40× oil immersion objective.

RESULTS

Relative expression of cell cycle markers shows that synchronization occurs anterior and posterior to the MF

During eye development of the third instar larval stage, cells become arrested in G_1 within the MF prior to differentiation (Wolff and Ready, 1993; Thomas et al., 1994; Richardson et al., 1995). It has been suggested that cell cycle synchronization begins anterior to the MF by regulation of the G_2 to M phase transition before arrest in G_1, since there is an increase in the number of mitoses and high levels of the mitotic inducer Stg mRNA immediately anterior to the MF (Thomas et al., 1994; Heberlein et al., 1995). Within the MF, cells are arrested in G_1 and do not express any cell cycle regulators. To confirm these observations and to examine more closely the cell cycle events leading to G_1 arrest and cell synchronization during eye development, we carried out double labeling with antibodies to G_1/S phase (Cyclin E) and G_2-M phase markers (Cyclin A, Cyclin B and Stg). Cyclin E protein distribution correlates with S phase cells, but is absent in G_1-arrested cells in the eye disc (Richardson et al., 1995), while Cyclin A and Cyclin B protein accumulate to high levels in G_2-M, but are degraded at metaphase and anaphase, respectively (reviewed by Follette and O'Farrell, 1997b). The mitotic inducer Stg is specific for G_2-M cells during embryogenesis (Edgar and Datar, 1996). In addition, bromodeoxyuridine (BrdU) incorporation was used to detect S phases, and mitotic cells were detected by DNA staining with propidium iodide (PI) or anti-MPM-2 staining, which strongly stains M phase cells in *Drosophila* (Secombe et al., 1998).

In the anterior region of the eye disc, cells expressing Cyclins A, B or E, or incorporating BrdU were randomly distributed, reflecting the asynchronous mode of these cell cycles (Fig. 1A-C). Cells containing high levels of G_2-M Cyclins had lower levels of G_1/S markers and vice versa, consistent with the expected cell cycle regulated appearance of these proteins. Cyclins were not detected within the MF, consistent with these cells being arrested in G_1.

Posterior to the MF, expression of Cyclin E appeared in a band of cells (Fig. 1B). A band of cells in a similar position were in S phase as determined by BrdU incorporation (Fig. 1A,C). The G_2-M Cyclins, Cyclin A and Cyclin B, were expressed posterior to the MF in a domain that is complementary and slightly posterior to cells with high levels of Cyclin E or BrdU labeling (Fig. 1A-C), consistent with the observation that mitotic cells are observed in this region (Fig. 1D,E). Within these zones, there appeared to be a greater degree of overlap between Cyclin A- or Cyclin E-expressing cells than between cells expressing Cyclin B or incorporating BrdU (compare Fig. 1B with 1C). This suggests that Cyclin A is expressed earlier in the cell cycle than Cyclin B, and supports evidence that Cyclin A may also play a role in S phase in *Drosophila* (Secombe et al., 1998; reviewed by Follette and O'Farrell, 1997b). We observed a larger proportion of cells expressing Cyclin A or Cyclin B than those expressing Cyclin E or incorporating BrdU (Fig. 1), suggesting that Cyclin A and Cyclin B are present for a greater proportion of the cell cycle in eye discs.

Staining for M phase cells using the anti-MPM-2 antibody or DNA staining with PI, showed that immediately anterior to the MF a slightly higher proportion of cells were in mitosis (Fig. 1D,E). Posterior to the MF more mitotic cells were observed, indicating that mitoses are more synchronized posterior than anterior to the MF. Surprisingly, when eye discs were stained with the Stg antibody, we did not observe high levels of Stg protein within the MF as has been observed for *stg* mRNA (Fig. 1F; Thomas et al., 1994; Heberlein et al., 1995). Rather, high levels of Stg protein were only detected in a subset of mitotic cells anterior and posterior to the MF (Fig. 1F). Indeed, more Stg-staining cells were observed posterior than anterior to the MF (Fig. 1F), reflecting the higher degree of synchronization seen in these posterior cells. Although it is possible that low levels of Stg protein may not be detected by the Stg antibody, these results suggest that Stg protein expression is highly dynamic and under post-transcriptional regulation in the eye disc. Stg protein expression is also post-translationally regulated in the embryo, since it is present during S, G_2 and M phases, but degraded at the metaphase to anaphase transition and is unstable or not translated during G_1 (Edgar and Datar, 1996).

Disruption of Dpp signaling results in ectopic cyclin expression and S phases within the MF

Previous results have shown that Dpp receptor mutant clones within the MF ectopically express Cyclin B, suggesting cells were arrested in G_2 (Penton et al., 1997). To more precisely define the cell cycle stage at which Dpp acts, we monitored

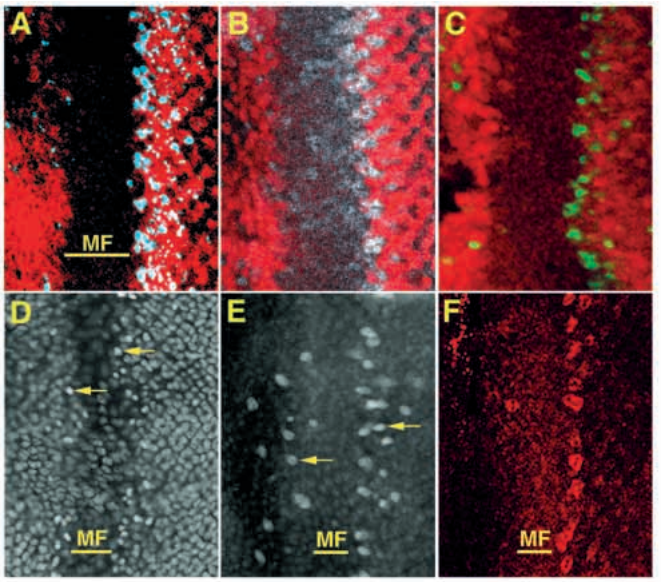

Fig. 1. Expression of cell cycle markers bordering the MF in wild-type eye discs. (A-C) Confocal images of eye discs stained with antibodies to Cyclins A, B or E or BrdU labeled to reveal S phases. (A) Anti-Cyclin A (red) and BrdU labeling (blue). (B) Anti-Cyclin A (red) and anti-Cyclin E (grey). (C) Anti-Cyclin B (red) and BrdU labeling (green). Note that cells containing high levels of Cyclin A or Cyclin B are complementary to cells containing Cyclin E or labeling with BrdU, although there is some overlap. (D-F) Fluorescent microscope images of eye discs. (D) Propidium iodide (PI) staining of DNA. The brighter staining indicates condensed DNA in mitotic cells (examples are indicated by the arrows). (E) Staining with anti-MPM-2, showing strong staining of mitotic cells (examples are indicated by arrows). (F) Anti-Stg staining. Note, staining with PI, MPM-2 or Stg reveal a greater number of mitotic cells posterior to the MF than immediately anterior to it, indicating that mitoses are more synchronized posterior to the MF. Anterior is to the left in this and all other Figures.

G_1/S, S phase and G_2-M phase markers in cells unable to respond to Dpp. Antibody stainings were used to detect Cyclins A, B or E proteins, and BrdU incorporation was used to label S phases. Mitotic recombination was used to generate clones null for the Tkv or Sax type I Dpp receptors and null clones were identified by antibody detection of the Myc epitope, which is lost in the homozygous mutant clones (Xu and Rubin, 1993).

As determined previously (Penton et al., 1997), *tkv* null clones located within the anterior part of the MF contained cells that inappropriately expressed Cyclin B (Fig. 2F,H). In addition, we found that cells within these clones also ectopically expressed Cyclins A or E, or incorporated BrdU (Fig. 2B-D,G,H). Double labeling with Cyclin A and Cyclin E (Fig. 2D) or Cyclin B and BrdU (Fig. 2H) revealed that the ectopic expression of G_1/S and G_2-M markers occurred in distinct subsets of cells in *tkv* clones. The random distribution of cells expressing different cell cycle markers and the generally non-overlapping nature of cell cycle markers in *tkv* clones was similar to that observed in the asynchronously cycling cells anterior to the MF in wild-type eye discs. Similar results were observed with clones defective for *sax* (data not shown). Therefore lack of Dpp signaling does not appear to

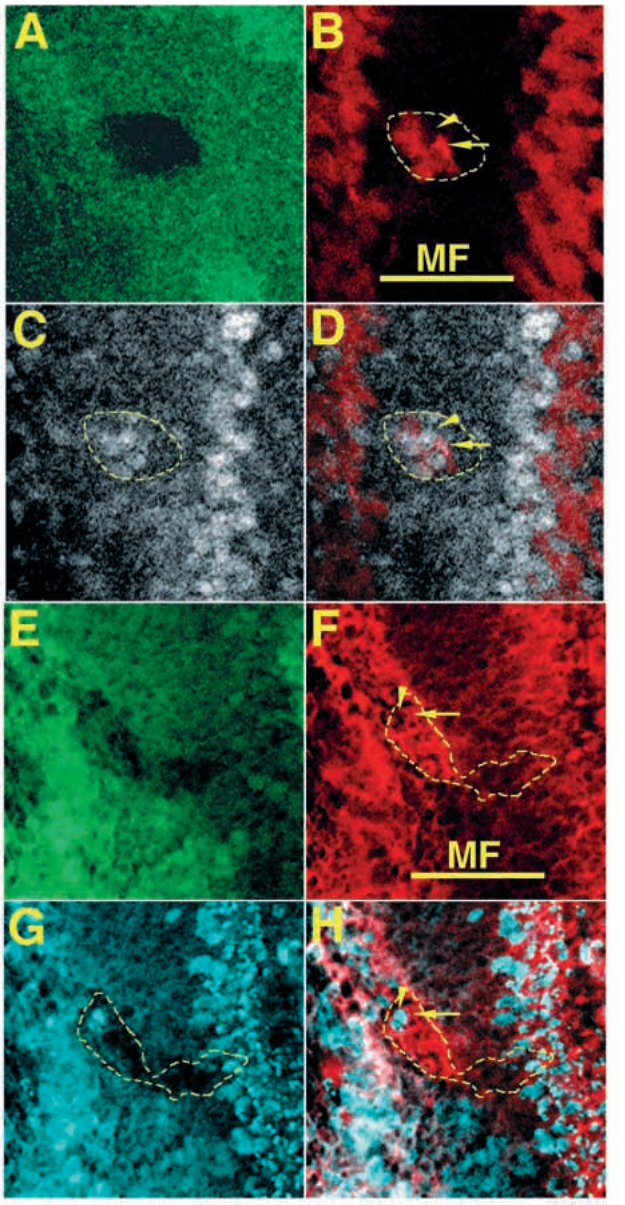

Fig. 2. *tkv* clones within the MF exhibit ectopic G_1/S and G_2-M phase cell cycle markers. (A-D) Ectopic Cyclin E and Cyclin A in a tkv^5 clone. (A) Lack of anti-Myc staining (green) indicates the presence of a *tkv* clone. (B) Anti-Cyclin A staining (red) shows that Cyclin A is ectopically expressed within the *tkv* clone (outlined). (C) Anti-Cyclin E staining (grey) shows that Cyclin E is ectopically expressed in a few cells within the *tkv* clone. (D) Merged image of B and C. Note that cells with high levels of Cyclin A (example indicated by arrow in D,B) and Cyclin E expression (arrowhead in B,D) do not overlap within the *tkv* clone. Cells outside the clone but within the MF do not contain Cyclins A or E (B-D). (E-H) Another *tkv* clone exhibiting ectopic Cyclin B and ectopically incorporating BrdU. (E) The *tkv* clone spans the MF and was identified by lack of anti-Myc staining (green, outlined in F,G and H). (F) Anti-Cyclin B staining (red) showing ectopic expression of Cyclin B in the *tkv* clone. (G) BrdU labeling showing ectopic S phases in the clone (blue). (H) Merged image of F and G. Note BrdU-labeled cells (example indicated by arrowhead in F,H) and Cyclin B-stained cells (an example indicated by arrow in F,H) are distinct from one another. No ectopic cyclin expression or S phases are observed in *tkv* clones in the posterior part of the MF.

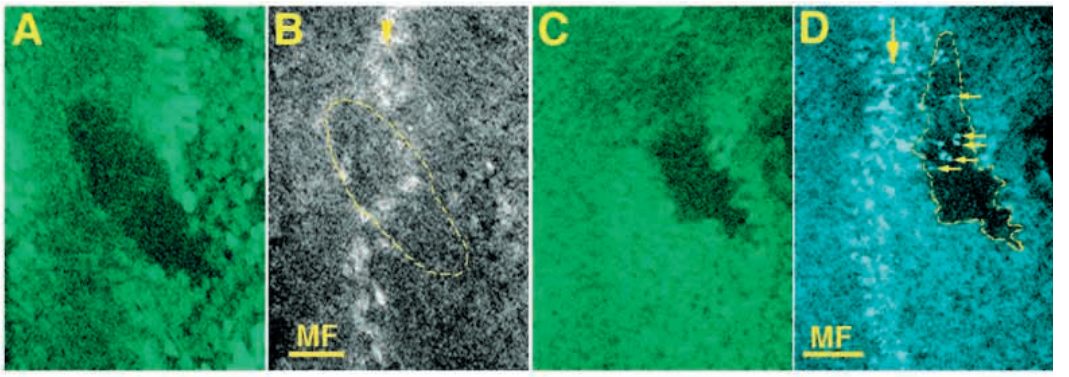

Fig. 3. MF progression is delayed in *tkv* clones. (A-D) Two large *tkv* clones impede progress of the MF through the eye disc. (A) Anti-Myc staining (green) with loss of Myc staining revealing the presence of a large *tkv* clone (outlined in B) in the posterior half of the MF. (B) Anti-Cyclin E staining (grey). Note the marked kink in the band of Cyclin E-containing cells within the clone indicating that re-entry into the cell cycle posterior to the MF has been delayed. (C) Anti-Myc staining (green) indicating the presence of another *tkv* clone (outlined in D) located just posterior to the MF. (D) BrdU labeling (blue) showing ectopic S phase cells within the *tkv* clone that are located in an abnormally posterior position (arrows in D), implying that re-entry into S phase has been delayed within the clone compared with the surrounding normal tissue.

lead to delayed mitosis (Penton et al., 1997), but rather allows the cells to continue cycling asynchronously as if they were anterior to the MF.

Cells that cannot respond to Dpp eventually arrest in G_1 in the posterior part of the MF

Interestingly, ectopic cyclin expression and S phases were not present within *tkv* and *sax* clones in the posterior region of the MF (Fig. 2; Penton et al., 1997). This indicates that other factors must be responsible for the eventual cell cycle arrest in the posterior part of the MF (see Discussion). In addition, large *tkv* clones exhibited a delay in the post-MF expression of Cyclin E (Fig. 3B) and in entry into S phase (Fig. 3D) with respect to the surrounding wild-type tissue. A delay in differentiation also occurs in large *tkv* clones (Penton et al., 1997). Therefore lack of Dpp signaling also appears to delay post-MF S phases and differentiation (see Discussion).

Ubiquitous over-expression of *dpp* inhibits S phase without altering Cyclin E or A protein levels

The inability of cells to appropriately arrest in G_1 in *tkv* and *sax* clones suggests that Dpp is normally required for G_1 arrest within the MF. If this interpretation is correct, it would be expected that ubiquitous over-expression of Dpp should result in a reduction in S phases throughout the eye disc. To test this we used the *GAL4-UAS* system (Brand and Perrimon, 1993) to indirectly over-express *dpp* in the eye disc by heat-shock induction, and monitored entry into S phase using BrdU labeling (see Materials and Methods). Ubiquitous over-expression of *dpp* by heat-shock induction resulted in a dramatic decrease of S phases in the eye disc at 60 minutes after heat shock while control discs were unaffected (Fig. 4F-H compared with 4A,B,E). This effect was transient since S phases had recovered by 90 minutes after heat shock (Fig. 4D,J compared with 4C,I). These results suggest that ectopic expression of Dpp can reversibly inhibit S phases. Ubiquitous Dpp expression also led to a reduction in S phases in antennal discs (Fig. 4F-H), however, S phases in other imaginal discs and in brain lobes were not significantly reduced (Fig. 4K,L; and data not shown). These data suggest that the S phase-inhibitory effect induced by Dpp is tissue specific.

To determine whether ectopic expression of *dpp* leads to G_1 arrest by down-regulating Cyclin E or Cyclin A expression, we stained eye discs with anti-Cyclin E or anti-Cyclin A antisera. In wild-type eye discs (not shown) and non-heat shocked eye discs (Fig. 5A), a distinct band of Cyclin E protein was detected corresponding to cells undergoing a synchronous S phase just posterior to the MF. One hour after heat shock induction of Dpp, expression of Cyclin E protein was unchanged (Fig. 5B). Similarly, the protein distribution of Cyclin A remained unchanged after ubiquitous *dpp* expression (Fig. 5D compared with 5C). These results show that Dpp can inhibit S phases without decreasing Cyclin E or Cyclin A expression or protein stability.

Dpp-signaling pathway genes interact genetically with *Drosophila cyclin E*

To obtain further evidence for a regulatory role for Dpp in G_1/S during eye development, we examined *dpp* alleles for genetic interactions with the *cyclin E* hypomorphic allele $DmcycE^{JP}$, which exhibits a rough eye phenotype due to insufficient S phases during eye development (Secombe et al., 1998). We have shown that the $DmcycE^{JP}$ rough eye phenotype is sensitive to the dosage of G_1/S regulatory genes (Secombe et al., 1998), and therefore, Dpp-pathway alleles may be expected to show dosage-sensitive interactions with $DmcycE^{JP}$.

$DmcycE^{JP}$ adult eyes are small and disorganized with fused ommatidia (Fig. 6B compared with 6A) and eye discs have reduced S phases anterior and posterior to the MF (Fig. 6F compared with 6E). Initially we tested dpp^{disc} alleles when homozygous or transheterozygous for their ability to suppress the $DmcycE^{JP}$ rough eye phenotype. One *dpp* allele, $dpp^{d\text{-}ho}$, when homozygous, suppressed the $DmcycE^{JP}$ rough eye phenotype and rescued S phases in eye discs (Fig. 6C,G). $Dpp^{d\text{-}ho}$ is a homozygous viable hypomorph that does not show eye roughening. Its suppression of $DmcycE^{JP}$ implies that $dpp^{d\text{-}ho}$ homozygotes may have a reduction of Dpp protein in the eye, although not so severe as to impede MF initiation. Stronger hypomorphic *dpp* alleles, such as dpp^{hr4} and dpp^{d12}, were able to suppress $DmcycE^{JP}$ when transheterozygous with $dpp^{d\text{-}ho}$, but not with other weak *dpp* alleles (not shown). The suppression of the $DmcycE^{JP}$ rough eye phenotype by certain combinations of *dpp* alleles suggests that the proliferation defect caused by $DmcycE^{JP}$ is sensitive to Dpp levels, although if Dpp levels are reduced below a certain threshold then MF

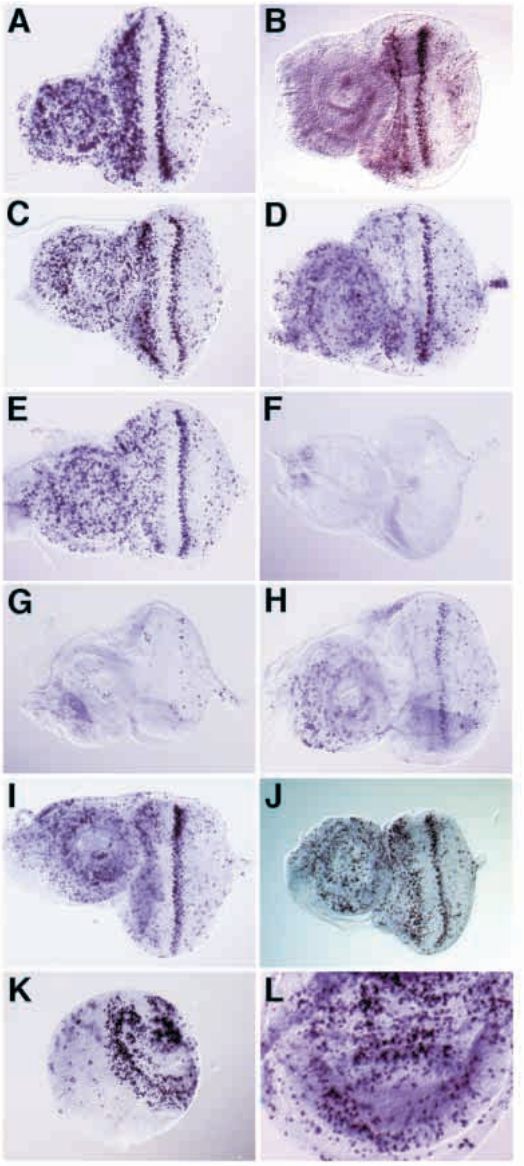

Fig. 4. Ubiquitous expression of Dpp abolishes S phases in the eye disc. Wild-type larvae (w^{1118}) or larvae of the genotype *hsp70-GAL4, UAS-dpp/+* were heat shocked at 38ºC for 1 hour, and allowed to recover at 20ºC for various times before dissection, after which S phases in eye discs were labeled with BrdU. (A) Non-heat shocked wild-type and (B) non-heat shocked *hsp70-GAL4, UAS-dpp/+* eye discs showing a normal pattern of S phases as revealed by BrdU incorporation. (C) Heat-shocked wild type with a 30 minute recovery and (D) heat-shocked *hsp70-GAL4, UAS-dpp/+* with a 30 minute recovery, both showing only a slight decrease in BrdU incorporation due to the heat shock. (E) A heat-shocked wild-type eye disc after a 60 minute recovery showing a normal pattern of S phases. (F-H) Examples of heat-shocked *hsp70-GAL4, UAS-dpp/+* eye disc with a 60 minute recovery, showing that S phases in the eye-antennal discs are considerably reduced compared with the control. (I) Heat-shocked wild type with a 90 minute recovery and (J) heat-shocked *hsp70-GAL4, UAS-dpp/+* with a 90 minute recovery showing normal patterns of S phases. (K) Heat-shocked *hsp70-GAL4, UAS-dpp/+* brain lobe and (L) heat-shocked *hsp70-GAL4, UAS-dpp/+* wing disc (shown at 2× higher magnification than A-K) at 60 minutes recovery showing that S phases appear normal (Richardson et al., 1995; data not shown). Note that the brain lobe and wing disc were taken from complexes where the eye discs showed a dramatic reduction in S phases.

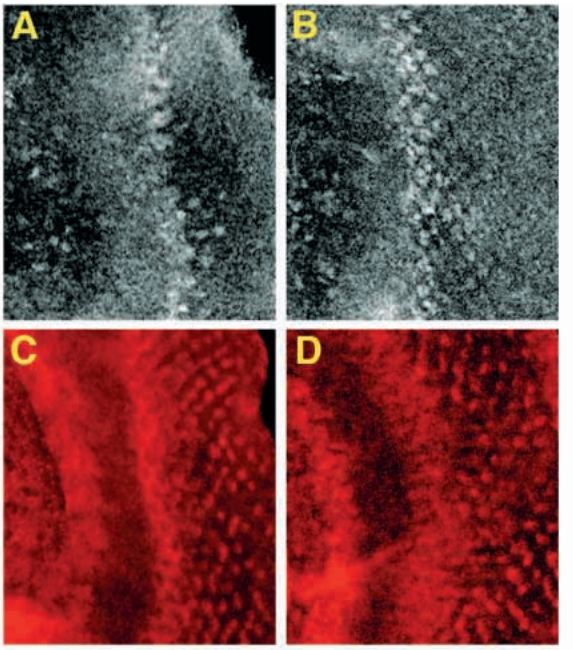

Fig. 5. Ubiquitous expression of Dpp does not affect Cyclin E or Cyclin A protein levels. Eye discs from *hsp70-GAL4, UAS-dpp/+* third instar larvae were either non-heat shocked or heat shocked for 60 minutes followed by 60 minutes recovery before dissection. (A) Anti-Cyclin E staining (grey) of a non-heat shocked sample. (B) Anti-Cyclin E staining of a heat shocked sample. (C) Anti-Cyclin A staining (red) of a non-heat shocked sample. (D) Anti-Cyclin A staining (red) from a heat shocked sample. Note, expression of both Cyclin E and Cyclin A proteins remained unchanged after ubiquitous heat shock expression of *dpp*, at a time when nearly all S phases in the eye disc were abolished.

initiation and/or progression may be impeded, masking the suppression of the $DmcycE^{JP}$ S phase defect.

Downstream effectors in the Dpp-signaling pathway were also tested for interaction with $DmcycE^{JP}$ (not shown). Reducing the dose of the Dpp type I receptor genes *sax* or *tkv* suppressed the $DmcycE^{JP}$ phenotype, although the latter suppressed only in combination with $dpp^{d\text{-}ho}$. In addition, mutant alleles of the type II receptor, *punt*, or the downstream transcription factors *mad*, *med* and *shn*, suppressed the $DmcycE^{JP}$ phenotype when transheterozygous with $dpp^{d\text{-}ho}$.

To determine whether increased expression of *dpp* in the eye disc could enhance the $DmcycE^{JP}$ rough eye phenotype, we generated flies homozygous for $DmcycE^{JP}$ and containing the *hsp70-GAL4* and $UAS\text{-}dpp^{42B.4}$ transgenes. $DmcycE^{JP}$; *hsp70-GAL4*/*UAS-dpp* flies raised at 25ºC, to induce a low level of ubiquitous *dpp* expression, had rougher eyes than $DmcycE^{JP}$ (compare Fig. 6D with 6B) and the number of cells entering S phase posterior to the MF was reduced (compare Fig. 6H with 6F). Flies containing the *hsp70-GAL4* and *UAS-dpp* transgenes alone had no eye defects at 25ºC (not shown). Furthermore, decreasing the dosage of a negative regulator of the Dpp-signaling pathway, *daughters-against-dpp* (*dad*; Tsuneizumi et al. 1997), enhanced the $DmcycE^{JP}$ rough eye phenotype (not shown).

These genetic interactions show that the $DmcycE^{JP}$ rough eye phenotype is sensitive to the level of Dpp signaling.

Together with the observation that over-expression of *dpp* blocks S phases without affecting Cyclin E or A levels, these results are consistent with the idea that Dpp may mediate G_1 arrest in the MF by inhibiting G_1 cyclin-Cdk function.

Dpp-mediated G_1 arrest occurs independently of *dacapo* expression and *roughex*

To address the mechanism by which Dpp mediates G_1 arrest during eye development we examined the expression or function of candidates known to be responsible for cell cycle arrest in the *Drosophila* eye. A *Drosophila* p21/p27 Cdk inhibitor homolog Dacapo (Dap), has been shown to inhibit Cyclin E-Cdk2 activity in vitro and genetically interacts with *DmcycE* (de Nooij et al., 1996; Lane et al., 1996; Secombe et al., 1998). In wild-type eye discs, Dap protein is present at high levels in a broad stripe of cells within the posterior half of the MF, and in differentiating photoreceptor cells immediately posterior to the MF (de Nooij et al., 1996; Fig. 7C). However, it is possible that Dap is present at lower levels in the anterior part of the MF. To determine whether Dpp mediates G_1 arrest by induction of Dap, we examined Dap protein distribution in eye discs where Dpp signaling was disrupted or where Dpp was ectopically expressed (Fig. 7A,B,D). In *sax* null clones Dap protein distribution and expression levels were indistinguishable from the surrounding wild-type cells (Fig. 7B). In addition, Dap protein distribution was unchanged following ectopic Dpp expression when S phases throughout the eye disc were inhibited (Fig. 7D compared with 7C). This indicates that Dpp does not mediate G_1 arrest by inducing Dap expression.

Another candidate for a downstream effector of Dpp is Roughex (Rux), a negative regulator of Cyclin A (Thomas et al., 1997; Sprenger et al., 1997). In *rux* mutant third instar larval eye discs, all cells within the MF ectopically enter S phase, indicating that normal G_1 arrest has failed to occur or is not maintained in these mutants (Thomas et al., 1994; Fig. 7E). To test whether Rux functions downstream of Dpp signaling in the MF, we over-expressed *dpp* by heat-shock induction in a rux^8 background. If Rux acted downstream of the Dpp pathway leading to G_1 arrest, we would expect the *rux* mutant phenotype to be epistatic to the loss of S phases caused by over-expression of *dpp*. However, reduced entry into S phase in response to over-expression of *dpp* was still observed in *rux* mutants (Fig. 7F), indicating that Dpp-mediated G_1 arrest is independent of Rux. Therefore, the mechanism by which Dpp mediates G_1 arrest does not require Roughex.

DISCUSSION

Dpp mediates cell cycle arrest in G_1 phase in the MF

In a previous study it was shown that cells unable to respond to Dpp within the anterior part of the MF ectopically express Cyclin B (Penton et al., 1997). The presence of Cyclin B in these Dpp-unresponsive cells was interpreted to signify a delay in entry into mitosis, leading to the suggestion that Dpp is required to drive cells from G_2 into mitosis anterior to the MF. In this report we show that this interpretation was incorrect and provide evidence that Dpp is important for G_1 arrest within the MF.

First, we show that ectopic expression of Cyclin E or A or BrdU incorporation, as well as Cyclin B, occurs within *tkv* or *sax* clones in the anterior part of the MF. Cells expressing these cell cycle markers were randomly distributed, indicating that cells within Dpp-unresponsive clones are at different stages of the cell cycle. Mitoses were also observed in these clones, predominantly in the posterior region (Penton et al., 1997). From these data we conclude that Dpp-unresponsive cells resemble asynchronously cycling cells anterior to the MF rather then cells that are delayed in G_2. This indicates that Dpp acts to inhibit cells from re-entering the cell cycle once they enter G_1 phase within the MF.

Second, we showed that heat-shock induced ubiquitous over-expression of Dpp transiently inhibited S phases throughout the eye-antennal disc. Entry into S phase resumed by 90 minutes after heat-shock induced *dpp* expression, possibly because Dpp protein is rapidly degraded or its signaling is down-regulated. Our observations are consistent with the results of Pignoni and Zipursky (1997), where a general reduction in BrdU incorporation was observed in third instar larval eye discs after generation of random *dpp*-over-expressing clones.

Since cells in the MF continue to cycle in the absence of Dpp activity and because ectopic *dpp* expression can inhibit S phases we conclude that Dpp triggers G_1 arrest within the MF by inhibiting the re-entry of cells into S phase. It is possible

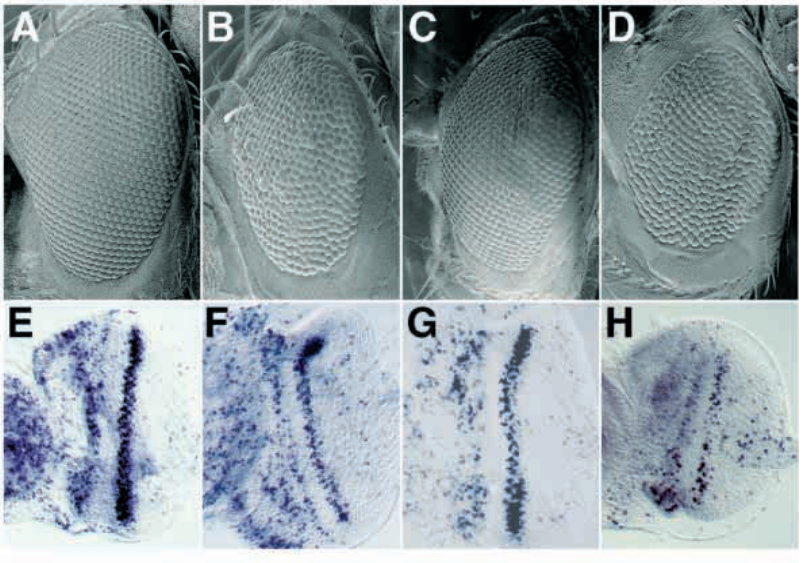

Fig. 6. *dpp* interacts genetically with *DmcycE*. (A-D) Scanning electron micrographs of adult eyes. Anterior is to the left, dorsal is up. (E-H) S phases in eye discs as detected by labeling with BrdU. (A,E) Wild-type (w^{1118}) adult eye and eye disc S phase pattern. (B,F) $DmcycE^{JP}$ homozygote showing disorganization of the adult eye and a reduced number of S phases in the eye disc. (C,G) $DmcycE^{JP}$, $dpp^{d\text{-}ho}$ homozygote, showing that the adult eye is much less disorganized compared with $DmcycE^{JP}$ and there is an increased number of S phases in the eye disc. (D,H) $DmcycE^{JP}$; *hsp70-GAL4/UAS-dpp* flies raised at 25ºC to allow leaky expression of Dpp from the *hsp70-GAL4, UAS-dpp* transgenes, showing enhancement of the $DmcycE^{JP}$ phenotype and a dramatic reduction in the number of S phases in the eye disc.

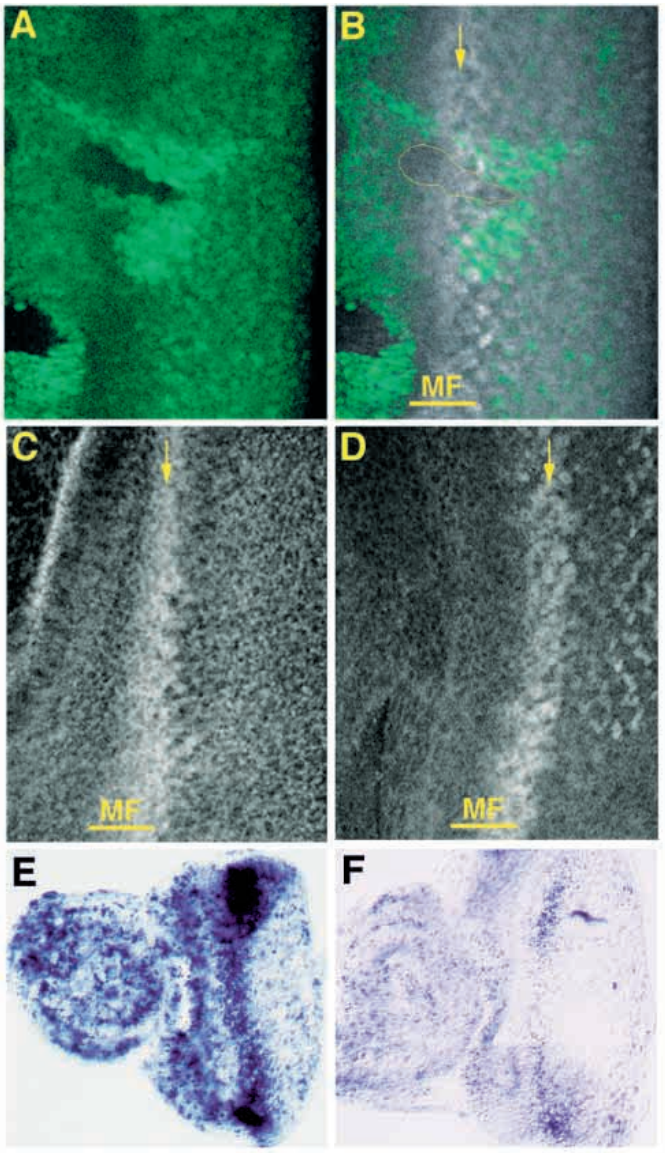

Fig. 7. S phase inhibition by Dpp is not mediated by Dacapo or Roughex. (A-D) Confocal images of eye discs. (A,B) Analysis of Dap in *sax*^{4} clones. (A) The absence of anti-Myc staining (green) reveals a *sax* clone spanning the MF. (B) Merged image of anti-Myc (green) and anti-Dap (grey) staining, showing that the clone (outlined) falls within the normal domain of Dap expression (arrow). Dap protein levels are not reduced within the clone, indicating that Dap expression is not decreased by lack of Dpp signaling. (C,D) Analysis of Dap after ubiquitous over-expression of *dpp*. Eye discs from (C) non-heat shocked or (D) heat shocked *hsp70-GAL4, UAS-dpp/+* 3rd instar larvae were stained with an anti-Dap antibody (grey, arrow). Dap protein abundance and distribution in heat shocked *hsp70-GAL4, UAS-dpp/+* larvae is similar to that in non-heat shocked larvae, indicating that Dap protein is not induced by ubiquitous over-expression of Dpp. (E,F) Light microscopy images of BrdU-labeled eye discs from heat shocked (E) *y*^{2} *cho*^{2} *rux*^{8}*/Y; +/+*, or (F) *y*^{2} *cho*^{2} *rux*^{8}*/Y; hsp70-GAL4, UAS-dpp/+* larvae. (E) Heat shocked *rux*^{8} eye discs, showing that in the absence of Rux, most cells in the MF and some cells anterior to the MF undergo ectopic S phases (Thomas et al., 1994) and that these ectopic S phases are not affected by heat shock. (F) Heat-shock induction of *dpp* in *rux* eye discs showing that S phases are dramatically reduced, indicating that Dpp does not act through Rux to induce G_1 arrest.

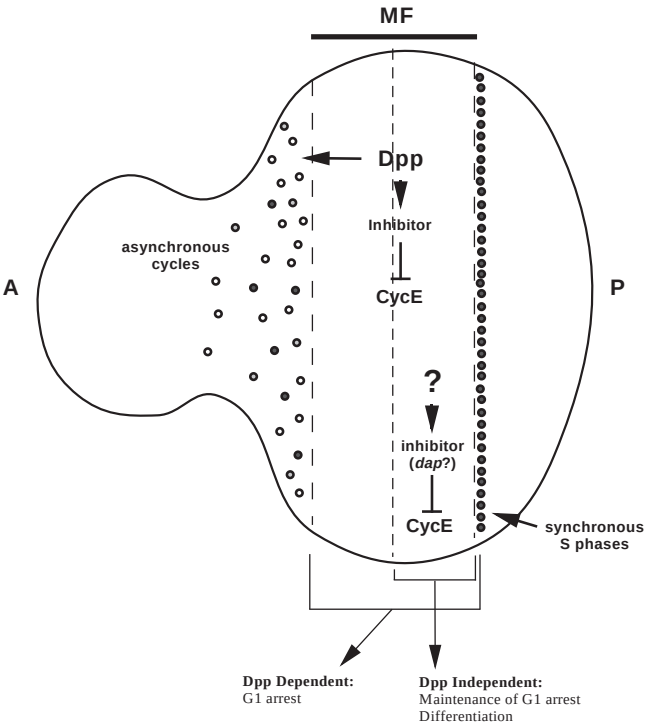

Fig. 8. Model for the role of Dpp within the MF. *dpp* is expressed within the MF (Masucci et al., 1990) and we propose that Dpp diffuses anteriorly to trigger G_1 arrest in cells within the asynchronously dividing region as they approach the MF. Dpp possibly arrests cells in G_1 by leading to the inhibition of Cyclin E-Cdk2 function through an unknown inhibitor. In the posterior half of the MF, G_1 arrest is maintained by a Dpp-independent mechanism, perhaps involving Dacapo.

that Dpp may also drive G_2 cells into M phase anterior to the MF, however we have not observed an increase in mitotic cells after ectopic expression of Dpp (Horsfield and Richardson, unpublished data) and M phase cells are still observed in Dpp-unresponsive clones (Penton et al., 1997).

Furthermore, our results provide evidence for additional controls that occur during eye development. First, in Dpp-unresponsive clones, ectopic cyclin expression or BrdU incorporation does not occur in the posterior half of the MF. This suggests that there is a Dpp-independent mechanism for G_1 arrest in the posterior region of the MF (see below). Second, in Dpp-unresponsive clones extending posterior to the MF there is a delay in re-entry into S phase and in differentiation (this study; Penton et al., 1997). This delay may reflect the need for cells to be in G_1 for a certain time in order to respond to differentiation signals. Alternatively, since Dpp signaling has been shown to induce *hh* expression (Borod and Heberlein, 1998), this delay may indicate that Dpp is required for *hh* expression. Since Hh is required for expression of the proneural gene *atonal* in differentiating cells in the posterior region of the MF (Heberlein et al., 1995), a decrease in *hh* expression may delay the induction of *atonal* expression and therefore differentiation. Likewise, the delayed S phase re-entry in Dpp-unresponsive clones that extend posterior to the MF may also be due to decreased *hh* expression.

How does Dpp act differently in different contexts?

Dpp has a proliferative role in first and second instar eye discs (Masucci et al., 1990, Penton and Hoffmann, unpublished data), and in wing and leg discs (e.g. Singer et al., 1997). How can the proliferative function of Dpp be reconciled with its role in G_1 arrest in the third instar eye disc? One possibility is that Dpp acts through cell cycle regulators that are expressed or activated only when the eye disc begins to differentiate. This would permit Dpp to act as a negative regulator of the cell cycle in a tissue- or temporal-specific manner. This tissue-specific cell cycle regulation may also be present in antennal discs, since ubiquitous over-expression of Dpp also inhibited S phases in antennal discs, but not in other imaginal discs or brain lobes. Tissue-specificity is also exhibited by mammalian TGF-β, which causes G_1 arrest in epithelial cells but induces proliferation in other tissues (reviewed by Wall and Hogan, 1994; Massague and Polyak, 1995).

A model for G_1 arrest and differentiation in the MF

Taken together our results suggest that Dpp triggers G_1 arrest in the MF, but it is not clear how this arrest takes place. Dpp-mediated G_1 arrest occurs downstream of Cyclin E or Cyclin A protein accumulation. Given its similarity to TGF-β (reviewed by Massague and Polyak,1995; Alevizopoulos and Mermod, 1997), it is possible that Dpp induces G_1 arrest by leading to the inhibition of Cyclin E-Cdk2 activity by the induction of a p27 Cdk inhibitor. However, it is unlikely that Dpp-mediated G_1 arrest involves the *Drosophila* p21/p27 homolog, Dacapo, since Dacapo expression is only detected in the posterior region of the MF and in differentiating cells (de Nooij et al., 1996; Lane et al., 1996) and we have shown that ectopic Dpp can inhibit S phases in cells where Dacapo expression is undectable. Furthermore, the Cyclin A inhibitor Roughex (Sprenger et al., 1997; Thomas et al., 1997) is not involved in Dpp-mediated G_1 arrest.

From results of this study we propose a model for G_1 arrest and differentiation during eye development (Fig. 8). Although other models may be possible, this model best explains our results and is consistent with the known action of mammalian TGF-β. Since we have shown that Dpp is unlikely to act through known G_1-S inhibitors, we propose that Dpp acts by inducing a novel inhibitor that abrogates Cyclin E-Cdk2 function, leading to G_1 arrest within the MF. Although *dpp* is expressed within the MF (Masucci et al., 1990), Dpp could diffuse more anteriorly where it may act upon G_1 cells approaching the MF in the asynchronously dividing region. This mechanism alone would result in a higher proportion of cells at later stages of the cell cycle, perhaps accounting for the higher number of mitoses observed immediately anterior to the MF (Thomas et al., 1994).

Since Dpp-unresponsive cells eventually arrest in G_1 in the posterior part of the MF, it appears that a Dpp-independent mechanism operates in this region to maintain cells in G_1. This mechanism may involve the Cyclin E-Cdk2 inhibitor, Dacapo, which is expressed in differentiating cells in the posterior part of the MF (de Nooij et al., 1996) or another unidentified G_1-S inhibitor. The factor that induces expression of this proposed G_1-S inhibitor is also unknown. The Cyclin A inhibitor Rux, also acts throughout the MF to prevent the inappropriate activation of Cyclin A-Cdk1 (Sprenger et al., 1997; Thomas et al., 1997). It is significant that there appears to be at least three G_1-arrest mechanisms operating within the MF. Synchronization of cells in G_1 may be a prerequisite for cells to correctly receive and respond to neural differentiation signals in a coordinated manner. The importance of this G_1 arrest is stressed by the aberrant retinal patterning that occurs when G_1 arrest is disrupted (Richardson et al., 1995; Thomas et al., 1994).

In conclusion, we have provided evidence that *dpp* plays an important role in mediating G_1 arrest in the anterior part of the MF. In addition, it appears that a Dpp-independent mechanism acts in the posterior part of the MF to maintain cells in G_1. Understanding how these controls are integrated to direct eye development may prove to be the key to uncovering the link between tissue patterning and cell cycle regulation.

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