Development 129, 4497-4507 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV5031

Drosophila myb exerts opposing effects on S phase, promoting proliferation and suppressing endoreduplication

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Accepted 19 June 2002

SUMMARY

Drosophila melanogaster **possesses a single gene, *Dm myb*, that is closely related to the vertebrate family of Myb genes, which encode transcription factors that are involved in regulatory decisions affecting cell proliferation, differentiation and apoptosis. The vertebrate Myb genes have been specifically implicated in regulating the G_1/S transition of the cell cycle. *Dm myb* is expressed in all proliferating tissues, but not at detectable levels in endoreduplicating cells. Analysis of loss-of-function mutations in *Dm myb* revealed a block at the G_2/M transition and mitotic defects, but did not directly implicate *Dm myb* function in the G_1/S transition. We have used the Gal4-UAS binary system of ectopic expression to further investigate the function of *Dm myb*. Our results demonstrate that depending upon the type of cell cycle, ectopic *Dm myb* activity can exert opposing effects on S phase: driving DNA replication and promoting proliferation in diploid cells, even when developmental signals normally dictate cell cycle arrest; but suppressing endoreduplication in endocycling cells, an effect that can be overcome by induction of E2F. We also show that a C-terminally truncated DMyb protein, which is similar to an oncogenic form of vertebrate Myb, has more potent effects than the full-length protein, especially in endoreduplicating tissues. This finding indicates that the C terminus acts as a negative regulatory domain, which can be differentially regulated in a tissue-specific manner. Our studies help to resolve previous discrepancies regarding *myb* gene function in *Drosophila* and vertebrates. We conclude that in proliferating cells, *Dm myb* has the dual function of promoting S phase and M phase, while preserving diploidy by suppressing endoreduplication.**

Key words: *Drosophila*, Myb, Transcription factor, Replication, Mitosis, Endoreplication, Endocycle, Endoreduplication, Genomic stability

INTRODUCTION

The proto-oncogene c-*myb* (*MYB* – Human Gene Nomenclature Database) is the cellular homolog of the transduced retroviral oncogene v-*myb*, which induces myeloid leukemia in chickens and transforms myeloid cells in culture. Mutations affecting c-*myb* have been implicated in the genesis of neoplastic disease in mice and humans (Oh and Reddy, 1999). c-*myb* represents a small gene family in vertebrates which includes two other closely related members, A-*myb* and B-*myb* (*MYBL1* and *MYBL2*, respectively – Human Gene Nomenclature Database) (Nomura et al., 1988). These three genes, which constitute the Myb gene family, encode nuclear, sequence specific DNA-binding proteins that can regulate transcription, and have been implicated in regulatory decisions affecting cell proliferation, differentiation and apoptosis (Oh and Reddy, 1999; Weston, 1998).

Several functional domains of the c-Myb protein have been defined (reviewed by Oh and Reddy, 1999): the sequence-specific DNA binding domain positioned near the N terminus; the transcriptional activation domain located in the middle of the protein; and a negative regulatory domain residing at a more C-terminal position (see Fig. 1). C-terminal and N-terminal sequences of the c-*myb* gene are missing in two independently isolated oncogenic viral Myb genes. In cultured cells, C-terminal truncation of the c-Myb protein enhances both its transforming potential and its ability to activate transcription from a reporter construct in cultured cells. A-*myb* and B-*myb* genes encode proteins that share several regions of homology with c-Myb, and are structurally similar, but not identical to the c-Myb protein (Oh and Reddy, 1999).

Drosophila melanogaster possesses a single gene, *Dm myb*, that is closely related to the vertebrate Myb gene family (Katzen et al., 1985). The protein encoded by *Dm myb* (DMyb) shares four regions of homology with vertebrate Myb proteins and is equally related to A-Myb, B-Myb and c-Myb (Bishop et al., 1991) (see Fig. 1). We have recently demonstrated that DMyb binds to a DNA consensus sequence that is similar to vertebrate Myb proteins, and that it can activate transcription from a reporter construct regulated by vertebrate Myb proteins (Jackson et al., 2001). *Dm myb* is expressed in all proliferating tissues, but not at detectable levels in endoreduplicating cells, with the exception of ovarian nurse and follicle cells (Katzen and Bishop, 1996) (G. R., unpublished). Analysis of loss-of-

function mutations has revealed that *Dm myb* is involved in regulating cell proliferation during *Drosophila* development, although the precise nature of the cellular defects differs between affected tissues. Mutant *myb* wing cells arrest in G_2 of their final cell cycle and a proportion of the arrested cells subsequently enter into endoreduplication (Katzen et al., 1998). Abdominal epidermal cells that are mutant for *Dm myb* proliferate much more slowly than wild-type cells and display a variety of mitotic defects, including abnormal numbers of centrosomes, resulting in aneuploidy and polyploidy (Fung et al., 2002). In a recently published report, newly isolated null alleles of *Dm myb* were shown to display mitotic defects in larval imaginal disc and brain cells that closely resemble the defects we observed in abdominal histoblasts (Manak et al., 2002).

Although the finding that *Dm myb* plays a role in the cell cycle superficially agrees with evidence that vertebrate Myb genes are required in at least some cell types for proliferation, there are important differences. Vertebrate Myb genes are generally thought to be required for the G_1/S transition and progression through S phase, whereas our analyses of mutant *myb* phenotypes in *Drosophila* have implicated *Dm myb* in later phases of the cell cycle. Recent studies showing that mutations in several genes known to be involved in DNA replication can lead to a block in mitosis as well as the expected G_1 arrest (Pflumm and Botchan, 2001; Whittaker et al., 2000), raise the issue of whether the *Dm myb* mutant phenotypes could also result from defects that occur during S phase. Data in two recently published papers have some bearing on this issue: the first shows that DMyb induces expression of the *cyclin B* gene in eye imaginal discs, providing support for *Dm myb* having a direct role in regulating the G_2/M transition (Okada et al., 2002); the second provides some indication of S-phase defects in addition to mitotic defects in null alleles of *Dm myb* (Manak et al., 2002).

We have now turned to the Gal4-UAS binary system of ectopic expression to further investigate the activities of wild-type and C-terminally truncated DMyb proteins. These studies have revealed that, depending upon the type of cell cycle, DMyb can exert two opposing effects on DNA replication. Ectopic expression in developing salivary glands of C-terminally truncated DMyb (ΔDMyb), and to a lesser extent the full-length DMyb protein, can suppress endoreduplication. By contrast, ectopic expression of either DMyb protein in diploid cells can drive cells into S phase and M phase, thereby promoting proliferation.

MATERIALS AND METHODS

Transgenic *Drosophila* stocks

To generate *Dm myb* transgenes that would be regulated by the yeast transcriptional activator GAL4, cDNA fragments representing the *Dm myb* transcription unit were cloned into the pUAST P-element vector (Brand and Perrimon, 1993). The 5′-UTR in *Dm myb* transcripts is lengthy (604 bases) and includes seven AUGs upstream of the DMyb AUG (Accession Number XO5939) (Peters et al., 1987). Removal of the upstream AUGs increased the efficiency of translation in vitro and in cultured cells (Sharkov et al., 2002). Therefore, we deleted the majority of the 5′-UTR in both transgenic constructs. For UAS-DMyb, we used a cDNA fragment that started at a *Bgl*II site (position +497 from the beginning of the transcript and –108 with respect to the starting AUG), which resulted in removal of all upstream AUGs and contained the complete coding sequence. For UAS-ΔDMyb, the cDNA fragment started at the same *Bgl*II site and continued to a *Tth*111 I site (position +1895 with respect to the beginning of the transcript), which encodes a truncated protein of 431 amino acids, deleting 226 amino acids from the C terminus (see Fig. 1). Transgenic lines were generated as previously described (Katzen and Bishop, 1996). Some of the transgenic lines were produced from constructs in which the MYC-epitope tag (EQKLISEEDL), which is specifically recognized by the monoclonal antibody Myc 1-9E10.2 (Evan et al., 1985), had been inserted in frame at the C terminus of the DMyb protein. The MYC-epitope tag did not alter the phenotypic effects of ectopically expressing the DMyb proteins, but we were unable to detect the proteins with the 9E10 monoclonal antibodies.

All other transgenic lines were generously provided by other investigators and have been previously described: UAS-RBF from Wei Du (via Bruce Edgar) (Xin et al., 2002); UAS-GFP (=UAS-GFPnls) from Bruce Edgar (Neufeld et al., 1998); *en*-Gal4 (=Scer\GAL4$^{en-e16E}$) (FlyBase, 1999) from Andrea Brand (Fietz et al., 1995); *sd*-Gal4 (=P{GAL4}sd$^{SG29.1}$) from Shelagh Campbell and isolated by Veronica Rodrigues (Roy et al., 1997); *fkh*-Gal4 from Steven Beckendorf [contains the salivary gland-specific enhancers of *fkh* defined by Zhou et al. (Zhou et al., 2001)]; HS-*E2F*,HS-*DP* from Bob Duronio (Follette et al., 1998); and Actin5c-Gal4 on chromosome 3 from the Bloomington stock center (FlyBase, 1999).

Preparation and fluorescent staining of imaginal discs and salivary glands

Animals were raised at 24°C unless otherwise specified. For heat shock induction of HS-*E2F*; HS-*DP*, animals were incubated at 37°C for 30 minutes once every 12 or 24 hours, as noted. Salivary glands and imaginal discs were dissected from larvae (either wandering third instar or timed in hours after egg deposition, AED, if so noted) and fixed in 4% paraformaldehyde in PBS and 0.1% Triton-X for 30 minutes at room temperature. Immunostaining was performed as previously described (Audibert et al., 1996; Theurkauf, 1994) with the following dilutions for primary antibodies: 1:700 for the polyclonal rabbit antibody against the DMyb DNA-binding domain (Jackson et al., 2001); 1:1000 for PH3 (Upstate Biotech); and 1:5 for Cyclin B (mouse monoclonal supernatant, F2F4, Developmental Studies Hybridoma Bank of the University of Iowa). For BrdU (5-bromo-2-deoxyuridine) labeling, dissected imaginal discs or salivary glands were incubated in Schneider's media (Gibco) containing 1 mg/ml BrdU for 30 minutes (discs) or 1 hour (salivary glands). Afterwards, they were fixed as above, washed three times, denatured in 2N HCl and neutralized in 100 mM sodium tetraborate. Samples were blocked with bovine serum albumin (BSA) and incubated with mouse anti-BrdU monoclonal antibody (Sigma) at 1:20. Secondary antibodies conjugated to either FITC or rhodamine (Boehringer Mannheim) were used at recommended dilutions. After immunostaining, samples were treated with DAPI at 0.5 μg/ml for 10 minutes, rinsed and mounted in Vectashield (Vector).

To identify apoptotic cells, discs were stained with Acridine Orange or the TUNEL assay. For the former, wing discs were dissected in 5 μg/ml Acridine Orange solution, rinsed in PBS, and then mounted on slides for viewing. For the latter, we used the ApopTag kit (Intergen) and followed the protocol described by White et al. (White et al., 1996).

Samples were imaged either by confocal microscopy (Zeiss LSM 550) or by wide-field microscopy (Zeiss Axioplan2) using a Princeton Instruments Micromax cooled CCD camera.

Quantitation of DNA content

We followed the protocol described by A. Weiss and colleagues (Weiss et al., 1998) for quantitation. All salivary glands were dissected from climbing stage third instar larvae that were aged to ~120 hours AED. For each genotype, a total of 35-65 salivary gland nuclei from

four to six salivary glands were measured. Then the ratios for each salivary gland nucleus to the average fat body nucleus for each genotype (derived from 20-30 nuclei) were determined and the mean ratio and standard deviations were calculated.

RESULTS

Ectopic expression of DMyb proteins in *Drosophila melanogaster* during development had potent consequences

To take advantage of the Gal4-UAS binary system of expression (Brand and Perrimon, 1993), DNA fragments from *Dm myb* cDNA clones were inserted into the pUAST vector and transgenic lines were generated. The fragments were designed to either encode the full length protein, DMyb, or a C-terminally truncated protein, ΔDMyb (Fig. 1). The C terminus of the vertebrate c-Myb protein has been shown to contain a negative regulatory domain that downregulates the DNA-binding and transcriptional activation abilities of the protein. By analogy, removal of the C terminus is expected to produce an activated version of DMyb.

In initial experiments, we found that driving ubiquitous ectopic expression of either DMyb protein during development was detrimental. For example, when UAS-DMyb expression was driven by Actin5c-Gal4 at 25°C, less than 1% of the animals survived to adulthood. Viability improved when temperatures were lowered, with about half of the pupae emerging as adults at 21°C and more than 80% emerging at 18°C (the Gal4-UAS system of expression shows temperature sensitivity, driving higher levels of expression at higher temperatures) (Greenspan, 1997; Morimura et al., 1996). By comparison, when UAS-ΔDMyb was driven by Actin5c-Gal4, the result was 100% lethality at all three temperatures, demonstrating that ΔDMyb is a more potent effector than full-length DMyb, as predicted.

Ectopic expression of DMyb induced proliferation in imaginal disc cells

We then focused on the consequences of ectopic DMyb expression in cells of the larval imaginal discs from wandering third instar larvae. Two Gal4 drivers were used for these experiments: *engrailed* (*en*)-Gal4, which drives expression of UAS-reporter constructs in the posterior compartment of each imaginal disc (Fig. 2A), and *scalloped* (*sd*)-Gal4, which drives expression throughout the wing pouch (Fig. 2C). An antibody against the DMyb protein (Jackson et al., 2001) detected increased levels of DMyb protein in the appropriate regions of the discs when the UAS-DMyb constructs were ectopically expressed via the Gal4 drivers (Fig. 2B and not shown). When DMyb expression was driven by either Gal4 driver, wing discs were malformed, appearing to be overgrown (or bulging) in the areas where DMyb was ectopically expressed (Fig. 2E and not shown). Ectopic expression of ΔDMyb caused similar, albeit often stronger, morphological effects on the wing discs (Fig. 2B,F).

To determine whether the abnormalities were due to increased proliferation, imaginal discs were incubated with BrdU to label cells in S phase. In discs where *en*-Gal4 was used to drive DMyb expression, increased levels of S phase could be observed in the posterior compartments of wing, haltere and leg discs (Fig. 3). To quantitate this effect, percentages of cells in S phase were calculated by counting the number of DAPI-staining and BrdU-incorporating nuclei in comparable regions of the anterior (A) and posterior (P) compartments from several wing discs of each genotype: control *en*-Gal4/+ (~1300 cells/compartment counted), *en*-Gal4/UAS-DMyb (~5000 cells/compartment) and *en*-Gal4/ΔDMyb (~3500 cells/compartment). In control discs, the P:A ratio of the

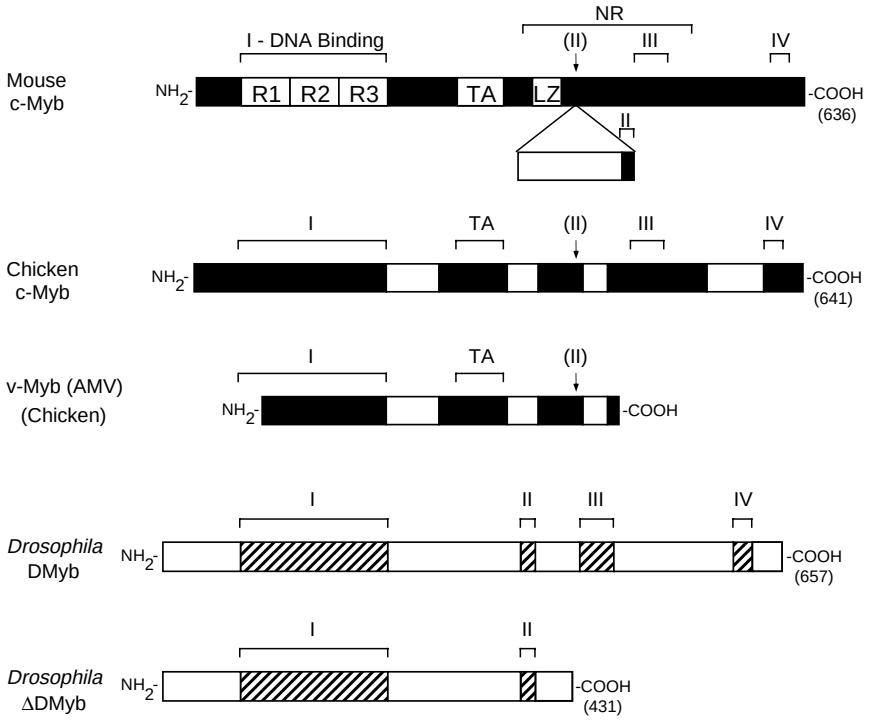

Fig. 1. Topographies of vertebrate and *Drosophila* Myb proteins. Schematic representations of the mouse and chicken c-Myb proteins and v-Myb protein from avian myeloblastosis virus (AMV; derived from the chicken gene) (Oh and Reddy, 1999). Abbreviations: R1, R2 and R3, three imperfect tandem repeats that comprise the DNA binding domain (region I); TA, transcriptional activator domain; LZ, leucine zipper; NR, negative regulatory domain. Also depicted is an additional region encoded by an alternatively spliced exon that contains the majority of conserved region II. The DNA-binding domain is also indicated. Regions of the c-Myb protein that are highly conserved between mouse and chicken are shown in the chicken protein in black. The four regions of conservation shared between vertebrate and *Drosophila* Myb proteins are indicated by Roman numerals (hatched in the DMyb proteins). The v-Myb protein has suffered both N- and C-terminal truncations, but the latter has been shown to be sufficient to activate transcriptional activation and transformation potentials of the Myb protein. Below are schematic representations of the two DMyb proteins produced by the fragments cloned into pUAST: DMyb, the full-length protein; and ΔDMyb, the C-terminally truncated protein which is expected to be hyperactive by analogy to the v-Myb protein.

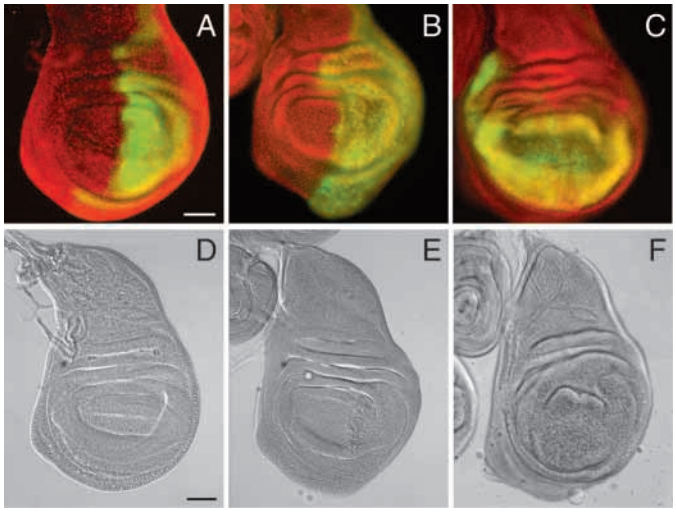

Fig. 2. Ectopic expression of DMyb causes malformation in the larval wing disc. Imaginal wing discs dissected from wandering third instar larvae, posterior to the right in all figures. (A-C) Fluorescent micrographs showing the expression patterns of the *en*-Gal4 and *sd*-Gal4 drivers: (A) *en*-Gal4/UAS-GFP, (B) *en*-Gal4/UAS-ΔDMyb and (C) *sd*-Gal4/UAS-GFP. Red, DAPI-staining to visualize nuclei; green, either (A,C) GFP fluorescence or (B) staining with an antibody raised against the DNA-binding domain of DMyb (Jackson et al., 2001). Note the difference in the shape of the disc when ΔDMyb is expressed in the posterior compartment.
(D-F) Micrographs using differential interference contrast (DIC) optics show a comparison between the appearance of (D) a control *en*-Gal4/+ wing disc and discs in which DMyb has been ectopically expressed; (E) *en*-Gal4/UAS-DMyb; and (F) *sd*-Gal4/UAS-ΔDMyb. Scale bars: in A, 0.05 mm for A-C; in D, 0.05 mm in D-F.

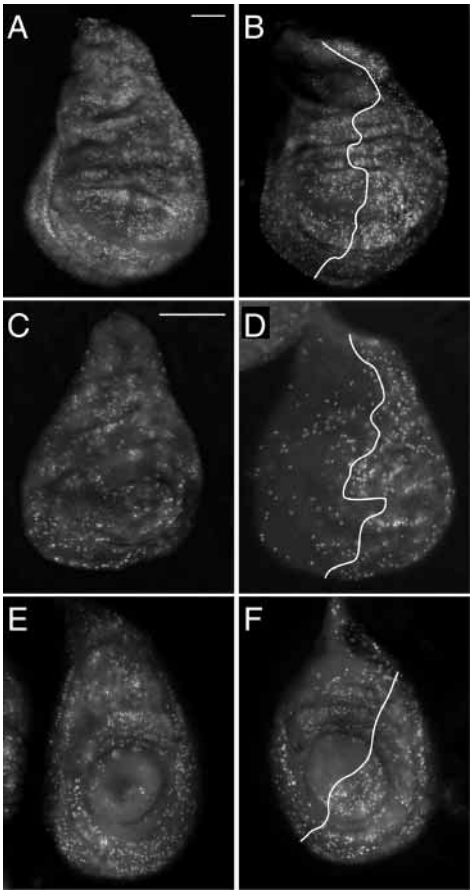

Fig. 3. Ectopic expression of DMyb promotes increased S phase in imaginal discs. Dissected wing (A,B), haltere (C,D) and leg (E,F) imaginal discs from wandering third instar larvae were labeled for DNA synthesis by BrdU incorporation (white dots). S-phase cells were similarly distributed in the anterior and posterior compartments of a control *en*-Gal4/+ disc (A,C,E). By contrast, higher levels of BrdU incorporation were observed in the posterior compartment of each disc when *en*-Gal4 was used to drive expression of either UAS-DMyb (B,F) or UAS-ΔDMyb (D) in posterior compartments. White lines indicate compartment boundaries, posterior towards the right. Scale bars: in A 0.05 mm for A,B; in C, 0.05 mm for C-F shown at same magnifications.

percentage of cells in S phase was 0.9, whereas the P:A ratio was 1.6 for discs in which either DMyb or ΔDMyb was expressed in the posterior compartment. The approximately twofold increase in the ratio of P:A cells in comparison with wild type when DMyb is ectopically expressed in the posterior compartment, is similar to the changes in S phase reported by Neufeld and colleagues (Neufeld et al., 1998) when *cyclin E* or *E2F* were ectopically expressed using the *en*-Gal4 driver. As the cells in the wing disc are not synchronized, it is possible that no greater increase is possible or that inadequate amounts of other factors such as E2F and/or cyclin E limit entry into S phase.

In the wing disc, the ability of DMyb to induce S phase was not limited to cells in regions where proliferation was ongoing, but also applied to cells in the zone of non-proliferating cells (ZNC). The ZNC represents a band of cells at the dorsal/ventral boundary of the third larval instar wing disc that stop proliferating at about 30 hours prior to pupariation (O'Brochta and Bryant, 1985) (see Fig. 4A). All cells in the posterior compartment of the ZNC are normally arrested in G_1 (Johnston and Edgar, 1998), but ectopic expression of either DMyb or ΔDMyb in this region induced these cells to enter S phase, as judged by BrdU incorporation, demonstrating that overproduction of DMyb can bypass the G_1 arrest (Fig. 4B,C). In the anterior compartment, the normal cell cycle arrest is more complicated than in the posterior, with a central zone of cells arrested in G_1, flanked on either side (dorsally and ventrally) by zones of cells arrested in G_2 (Johnston and Edgar, 1998). When Johnston and Edgar (Johnston and Edgar, 1998) ectopically expressed cyclin E in the ZNC, BrdU incorporation was detected in all posterior cells of the ZNC and in the central zone of anterior cells, but not in the flanking zones. By contrast, ectopic expression of DMyb in the wing pouch induced BrdU incorporation throughout the region and no ZNC was formed, suggesting that overproduction of DMyb can bypass both G_1 and G_2 arrests (Fig. 4D).

Immunostaining with an antibody for a mitotic-specific phospho-epitope on histone H3 (PH3) (Hendzel et al., 1997) showed that ectopic expression of DMyb via *en*-Gal4 also induced increased levels of mitosis in the posterior compartment of imaginal discs (Fig. 5A,B). In climbing stage third instar larvae, an accumulation of the G_2 cyclin, Cyc B, is

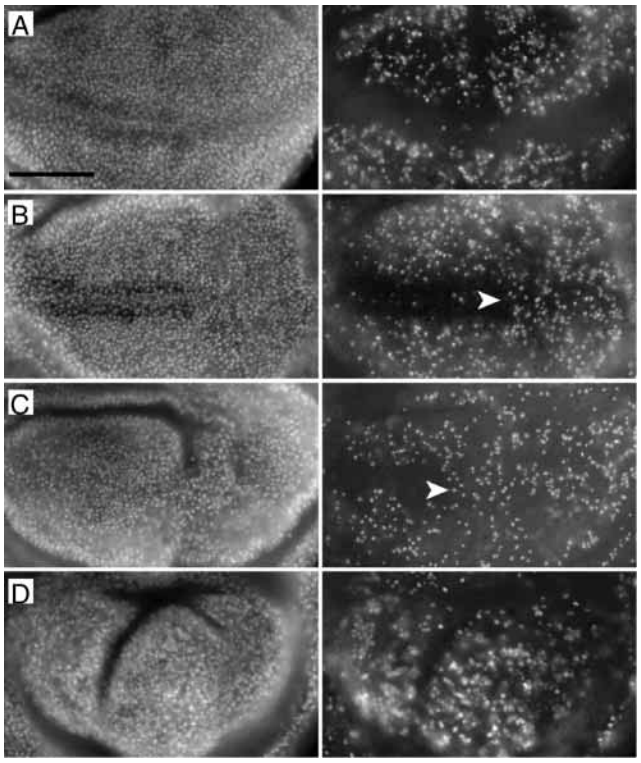

Fig. 4. Ectopic expression of DMyb promotes S phase in the ZNC of larval wing discs. Views of the dorsoventral boundaries of wing discs that were double-stained with DAPI to visualize nuclei (left panels) and for DNA synthesis by BrdU incorporation (right panels). In wild-type control discs, *en*-Gal4/+, BrdU incorporation was not detected in the zone of non-proliferating cells (ZNC), which is composed of cells at the dorsoventral boundary (A). However, when *en*-Gal4 was used to drive expression of either UAS-DMyb (B) or UAS-ΔDMyb (C) in the posterior compartment, BrdU incorporation could be detected in the posterior ZNC (indicated by arrows); and when *sd*-Gal4 was used to drive expression of UAS-DMyb throughout the wing pouch, no ZNC could be detected (D). Scale bar: 0.05 mm.

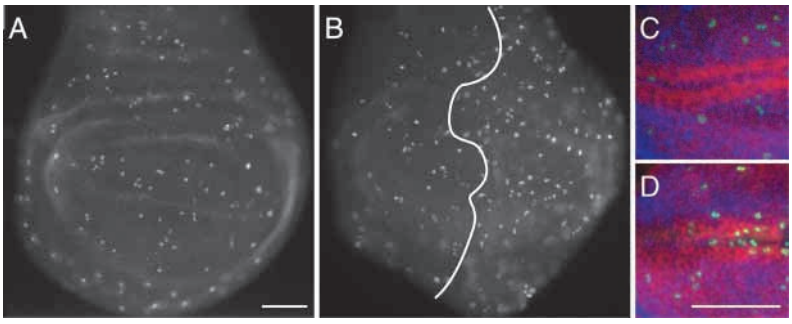

Fig. 5. Ectopic expression of DMyb promotes mitosis in imaginal discs. Mitotic cells identified with the PH3 antibody were similarly distributed in the anterior and posterior compartments of a control *sd*-Gal4/+ disc (A), but were more frequent in the posterior compartment when *en*-Gal4 was used to drive expression of UAS-DMyb (B). White line indicates the compartment boundary, posterior towards the right. (C,D) Higher magnification views of the anterior ZNC in wing discs triply stained with DAPI to visualize nuclei (blue), PH3 antibody to identify mitotic cells (green) and Cyclin B antibody (red). In the control *sd*-Gal4/+ disc, no mitotic cells were detected in the ventral and dorsal domains of the anterior ZNC, where Cyc B accumulates to high levels (C). By contrast, mitotic cells could be detected in these domains when *sd*-Gal4 was used to drive expression of UAS-DMyb in the wing pouch (D). Scale bars: in A, 0.05 mm for A,B; in D, 0.05 mm in C,D.

normally observed in the dorsal and ventral domains of the anterior ZNC (Johnston and Edgar, 1998) (see Fig. 5C). When DMyb was ectopically expressed throughout the wing pouch via *sd*-Gal4, this accumulation of Cyc B was not evident in most discs (not shown). However, in samples where elevated levels of Cyc B could still be observed, PH3 staining cells were detected in the Cyc B expression domains, confirming that overproduction of DMyb can bypass G_2 arrest in the ZNC (Fig. 5D).

Slightly elevated levels of apoptosis were observed when *en*-Gal4 was used to drive expression of either DMyb construct and when *sd*-Gal4 was used to drive expression of full-length DMyb (Fig. 6B,C, and not shown). Considerably higher levels of apoptosis were observed when *sd*-Gal4 was used to drive expression of ΔDMyb (Fig. 6D). We do not have an explanation for this difference at present, but as scalloping of adult wings was frequently observed when the *sd*-Gal4 line was used to drive ectopic expression of full-length DMyb (not shown), we suspect that in these samples, the levels of apoptosis may increase substantially during pupation. The finding that increased apoptosis accompanies the increased cell proliferation induced by ectopic DMyb activity, agrees with results from previous studies, which demonstrated that cell death is often induced when the cell cycle is deregulated in imaginal discs (Asano et al., 1996; Du et al., 1996; Milan et al., 1997; Neufeld et al., 1998).

Ectopic expression of DMyb inhibited endoreduplication in salivary glands

Two lines of evidence suggest that *Dm myb* is required for suppression of endoreduplication in diploid cells: *Dm myb* is not normally expressed at detectable levels in larval tissues that undergo endoreduplication (Katzen and Bishop, 1996); and in loss-of-function mutant alleles of *Dm myb*, mutant wings cells that are abnormally arrested in G_2, enter into endoreduplication (Katzen et al., 1998). By contrast, the abdominal histoblast nest cells, which are normally arrested in G_2 throughout larval development (Hayashi et al., 1993), did not show any evidence of endore duplication in *myb* mutants (Fung et al., 2002), indicating that although *Dm myb* function appears to be required to suppress endoreduplication during an aberrant G_2 arrest, it may not be essential for maintaining a normal G_2 phase, even when it is prolonged for an extended period of time.

To determine whether ectopic DMyb activity is capable of suppressing endoreduplication in larval tissues that normally enter into an endocycle, we used two Gal4 lines that drive expression in salivary glands, *fkh*-Gal4 and *sd*-Gal4. *fkh*-Gal4 uses the salivary gland-specific enhancers of *fork head* (*fkh*) (Zhou et al., 2001), a gene required for the formation of embryonic salivary glands (Myat and Andrew, 2000; Weigel et al., 1989). Although the *sd* gene has not been reported to be expressed in salivary glands, we found that like the *fkh*-Gal4 line, the *sd*-Gal4 line induced high levels of green fluorescent protein (GFP) from a UAS-GFP reporter construct in endoreduplicating salivary gland nuclei (Fig. 7A,B). Neither Gal4 line induced expression

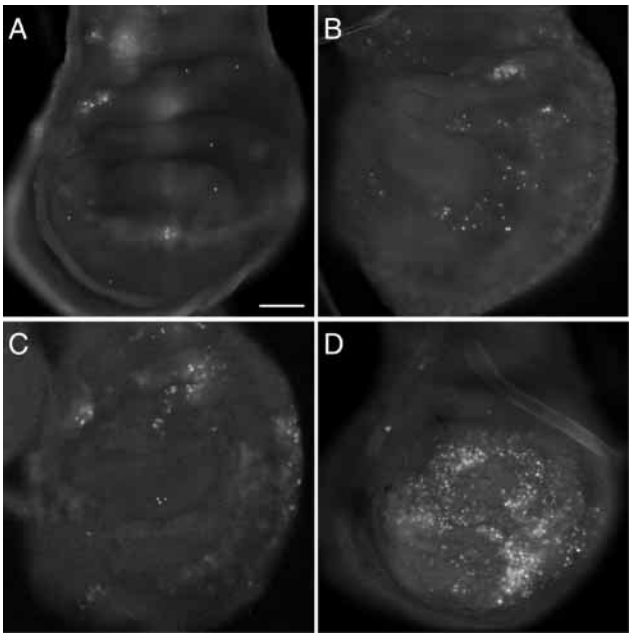

Fig. 6. Ectopic expression of DMyb induces small increases in apoptosis in wing discs. A low level of apoptosis was detected with in a control wing disc (A). Small increases were observed when *en*-Gal4 was used to drive ectopic expression of either DMyb (not shown) or ΔDMyb (B) in the posterior compartment, and when *sd*-Gal4 was used to drive ectopic expression of DMyb (C). Higher levels of apoptosis were observed when *sd*-Gal4 was used to drive ΔDMyb expression (D). Results were similar with Acridine Orange and TUNEL staining. The former is shown in A-C and the latter in D. Scale bar: 0.05 mm.

in imaginal ring cells or in the neighboring fat body, allowing for these cells to serve as internal controls. The results described below were virtually identical with both Gal4 drivers.

As a positive control for this experiment, *fkh*-Gal4 and *sd*-Gal4 were used to drive expression of UAS-RBF, the *Drosophila* homolog of the retinoblastoma protein, a tumor suppressor that inhibits the G_1/S transition in proliferating cells (Dyson, 1998). Ectopic expression of RBF has been previously shown to inhibit growth and DNA endoreduplication in developing salivary glands, and we obtained similar results (Datar et al., 2000) (Fig. 7H). When full-length DMyb protein was ectopically expressed in developing salivary glands, the overall size of the glands from third instar larvae and of the individual nuclei within the glands were smaller than in wild-type controls (Fig. 7D,E), but were still significantly larger than the results obtained with RBF. However, when the C-terminally truncated protein, ΔDMyb, was ectopically expressed using either Gal4 driver, the resulting salivary glands were much smaller than wild type, closely resembling those obtained with RBF (Fig. 7, compare D,F,H). In addition, the salivary gland nuclei were small and exhibited much lower levels of DNA staining. The nuclei of neighboring fat body cells in which ΔDMyb was not expressed, were similar in appearance to wild-type fat body nuclei.

To quantify the differences between the salivary gland nuclei from the various genotypes, the DNA signal ratios of salivary gland nuclei to non-expressing fat body nuclei were determined using the method described by Weiss et al. (Weiss et al., 1998). Our results reinforced the visual impressions that ectopic expression of either DMyb protein inhibited endoreduplication, but that the truncated ΔDMyb was a much more potent inhibitor than the full-length DMyb (see Fig. 7C for graphical representation and Fig. 7D-H for individual examples).

These conclusions were confirmed by in vivo labeling studies of S phase. In larvae raised at 24°C that were between the ages of 72 and 96 hours AED, BrdU incorporation could be detected in imaginal ring cells and the endoreplicating nuclei of salivary glands from wild-type controls and *fkh*-Gal4 or *sd*-Gal4/UAS-DMyb larvae (Fig. 8A,B and not shown). By contrast, no BrdU incorporation was observed in the salivary gland nuclei of *fkh*-Gal4 or *sd*-Gal4/UAS-ΔDMyb larvae, even though it could still be detected in imaginal ring cells (Fig. 8C,D). Similar results were obtained when BrdU incorporation was examined in larvae between the ages of 96 and 120 hours AED (not shown).

These studies focused on salivary glands, but when more broadly expressing Gal4 lines were used to drive DMyb expression, especially ΔDMyb, larval growth was impeded and the nuclei of various endoreplicating tissues were smaller (not shown). These results suggest that the ability of DMyb activity to suppress endoreduplication in salivary glands can be generalized to other larval tissues.

Ectopic expression of a G_1/S regulator can override the DMyb induced inhibition of endoreduplication

To determine whether the inhibition of endoreplication in salivary glands could be overcome, a chromosome carrying the transgenes *E2F* and *DP* under the control of the Hsp70 promoter was mated into flies that also carried either *sd*-Gal4 or *fkh*-Gal4 and UAS-ΔDMyb. *E2F* and *DP* encode the two subunits of the *Drosophila* E2F transcription factor, which promotes DNA replication in cells that are proliferating and in those that are endocycling (Duronio et al., 1995; Dynlacht et al., 1994; Ohtani and Nevins, 1994; Royzman et al., 1997). Daily heat shock treatments (30 minutes at 37°C) resulted in partial rescue of salivary glands, both with respect to the overall size of the glands and the size of individual nuclei (Fig. 7G), and BrdU incorporation could be detected in a number of salivary gland nuclei within a couple of hours after heat shock treatment (Fig. 8E). However, as the heat shock treatment induced expression of E2F in all cells, increased levels of BrdU incorporation were also observed in fat body nuclei.

As the ectopically induced DNA synthesis in fat body would presumably lead to excess endoreduplication, the extent of rescue calculated by the ratio of salivary gland nuclei to fat body nuclei is likely to be an underestimate (Fig. 7C). In addition, we suspected that as the GAL4/UAS system is known to be more efficient at higher temperatures (Greenspan, 1997; Morimura et al., 1996), the heat shock treatments might be inducing higher levels of the ΔDMyb protein, which we would expect to further inhibit endoreduplication. To test these possibilities, *sd*-Gal/UAS-ΔDMyb or *sd*-Gal/UAS-ΔDMyb; HS-E2F/DP embryos were collected for 24 hours and then subjected to 30 minute heat-

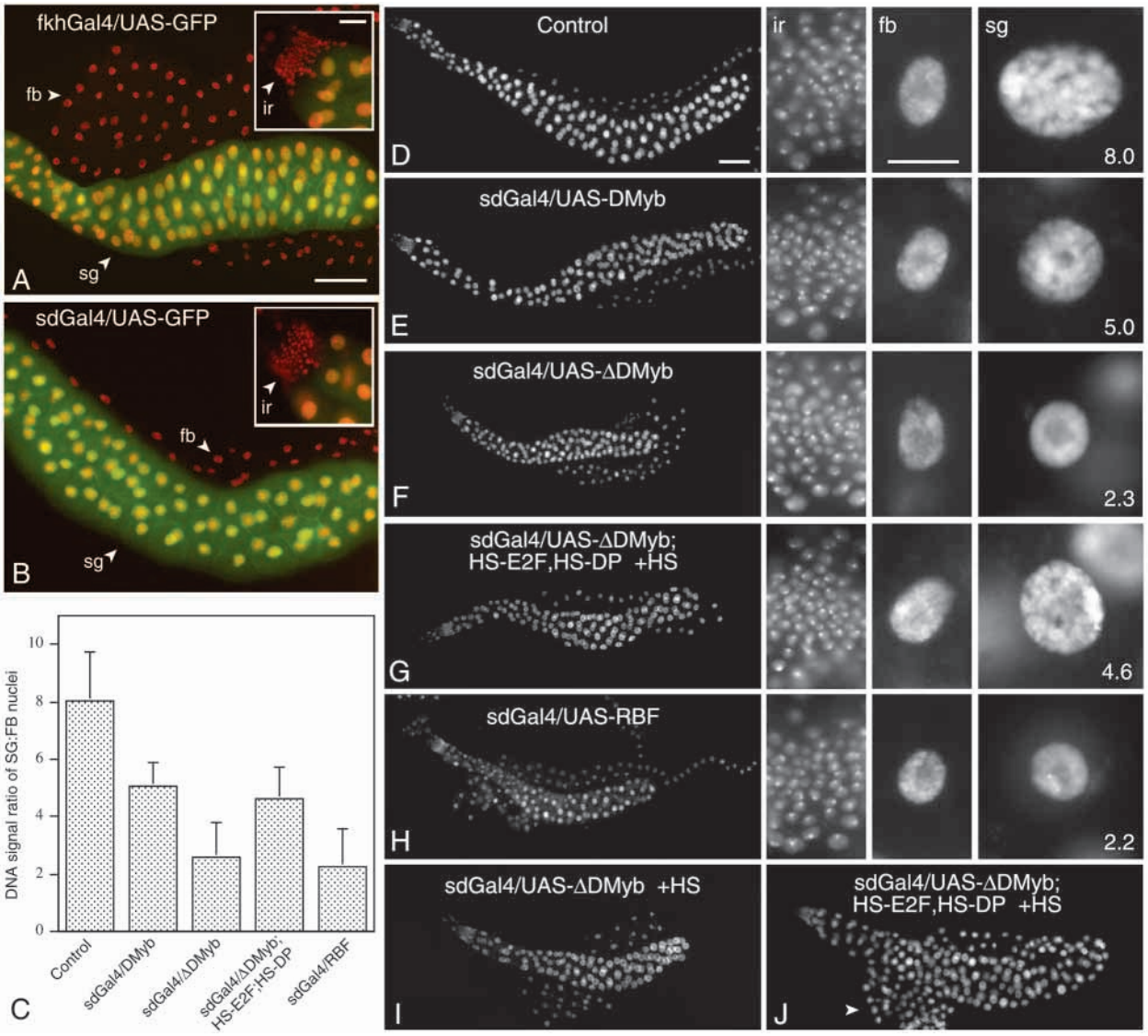

Fig. 7. Ectopic DMyb activity inhibits endoreduplication and growth in salivary glands. (A,B) Fluorescent micrographs of salivary glands dissected from (A) *fkh*-Gal4/UAS-GFP and (B) *sd*-Gal4/UAS-GFP third instar larvae show that both Gal4 lines drive expression in endocycling salivary gland cells (sg), but not in fat body (fb) or imaginal ring cells (ir), which are shown in an inset at higher magnification. (C) Graphical representation for each indicated genotype of the average ratio of the DNA signal from 'expressing' salivary gland nuclei to non-expressing fat body nuclei [using the method of Weiss et al. (Weiss et al., 1998)]. Standard deviations are shown. (D-H) DNA staining of salivary glands and representative nuclei from larvae that were approximately 120 hours AED. Genotypes were: (D) *sd*-Gal4/+ control; (E) *sd*-Gal4/UAS-DMyb; (F) *sd*-Gal4/UAS-ΔDMyb; (G) *sd*-Gal4/UAS-ΔDMyb; HS-*dE2F*, HS-*dDP*/+, which had been subjected to a 30 minute heat-shock treatment every 24 hours after collection; and (H) *sd*-Gal4/UAS-RBF. Panels from left to right show the relative sizes of complete glands and (at a higher magnification), relative sizes of imaginal ring nuclei (ir), fat body nuclei (fb) and salivary gland nuclei (sg) that represent the mean for each genotype. The DNA signal ratio of that nucleus to the average fat body nucleus indicated. (I,J) DNA staining of salivary glands from (I) *sd*-Gal4/UAS-ΔDMyb and (J) *sd*-Gal4/UAS-ΔDMyb; HS-*dE2F*, HS-*dDP*/+ larvae, which had been subjected to 30 minute heat-shock treatments every 12 hours after collection, and which were ~144 hours AED. Arrowhead in J indicates enlarged fat body nuclei resulting from excess endoreduplication in these cells driven by HS-dE2F/DP. Scale bars: in A, 0.1 mm for A,B; in A (inset), 0.025 mm for A,B; in D, 0.1 mm (low magnification) for D-J; in D, 0.025 mm (high magnification) for D-H.

shock treatments every 12 hours. In accordance with our hypothesis that the heat-shock treatments were enhancing the inhibition of endoreduplication, the larvae had to be aged for an additional 24 hours (~144 hours instead of 120 hours) for the *sd*-Gal/UAS-ΔDMyb salivary glands to reach approximately the same size as in samples that had not been heat shocked (Fig. 7, compare I and F). The salivary glands dissected from the *sd*-Gal/UAS-ΔDMyb; HS-E2F/DP larvae were considerably larger than those dissected from the animals that did not carry the HS-E2F/DP transgenes (Fig. 7I,J), and the salivary gland nuclei were also enlarged, but so were the fat body nuclei, confirming the suspicion that the heat-shock treatments produced excess endoreduplication in the fat body.

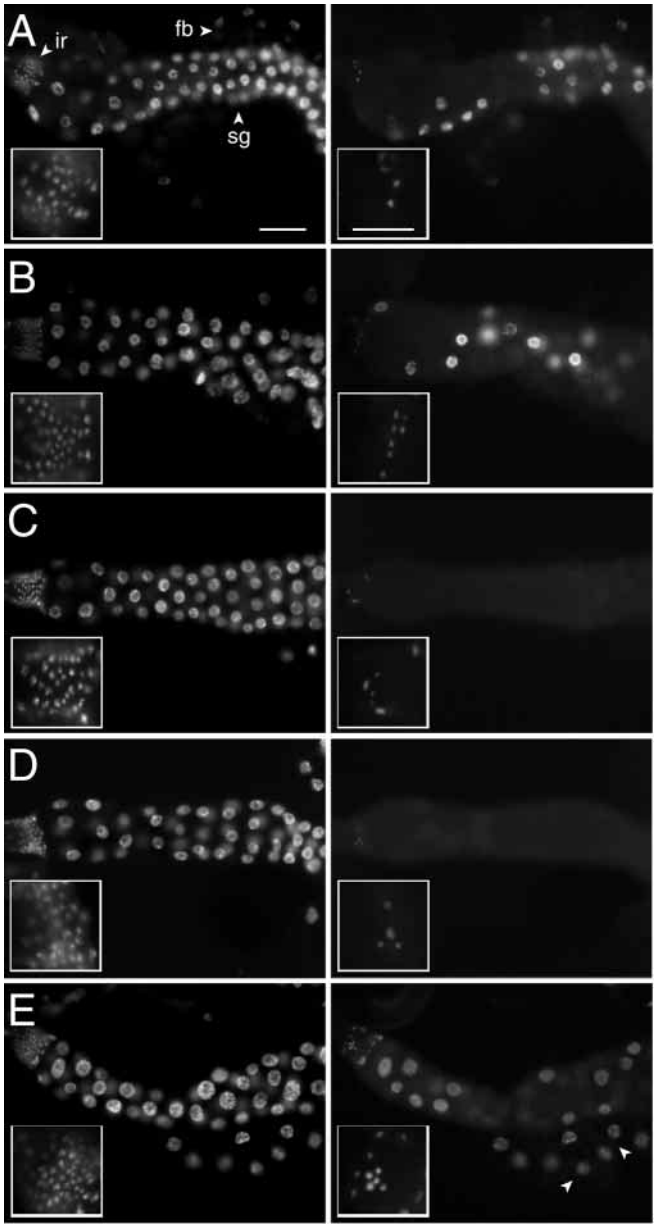

Fig. 8. BrdU incorporation is strongly inhibited in salivary glands when ΔDMyb, but not DMyb, is ectopically expressed. Salivary glands dissected from larvae between 72 and 96 hours AED were double-stained with DAPI to visualize nuclei (left panels) and for DNA synthesis by BrdU incorporation (right panels). Imaginal ring cells (ir) are shown at higher magnification in inset panels. BrdU incorporation was detected in both imaginal ring and salivary gland (sg) nuclei in (A) control *sd*-Gal4/+ glands and in (B) *sd*-Gal4/UAS-DMyb glands. However, BrdU incorporation was only detected in imaginal ring nuclei and not in salivary gland nuclei in (C) *sd*-Gal4/UAS-ΔDMyb and (D) *fkh*-Gal4/UAS-ΔDMyb glands. (E) Heat-shock induction of E2F/DP expression in *sd*-Gal4/UAS-ΔDMyb; HS-*E2F*, HS-*DP*/+ glands was able to override the inhibition of DNA synthesis in salivary gland nuclei, but also induced increased levels of BrdU incorporation in fat body (fb) nuclei, leading to excess endoreduplication in these cells. Examples of these fat body nuclei are indicated with arrowheads. Scale bars: in A (left), 0.05 mm for A-E; in A (inset), 0.025 mm for A-E.

DISCUSSION

The results reported here reveal apparently contradictory roles for *Dm myb*: continuous expression of the DMyb protein promotes S phase in diploid cells, while inhibiting DNA synthesis in endoreplicating cells. Similar results have been obtained with continuous ectopic expression of the Cyclin E protein, a paradox that has been at least partially explained (Follette et al., 1998; Neufeld et al., 1998; Weiss et al., 1998). To maintain genomic integrity in proliferating diploid cells, it is necessary to prevent cells from undergoing more than a single round of DNA replication during each cell cycle. To ensure this, initiation of replication requires the assembly of prereplication complexes, an event that occurs in early G_1 and is dependent on the low level of Cyclin dependent kinase (Cdk) after the mitotic destruction of cyclins (Su et al., 1995). Although cells that undergo endoreduplication do not undergo mitosis (or at least do not complete mitosis), all endocycles exhibit distinct gap phases between each round of DNA replication (Edgar and Orr-Weaver, 2001). Experimental evidence indicates that endocycling nuclei, like proliferating cells, can only regain the competence to re-enter each S phase after a low point in Cdk activity, which appears to be dependent on decreases in Cyclin E levels (Follette et al., 1998; Lilly and Spradling, 1996; Weiss et al., 1998). However, it remains unclear why DNA replication in proliferating cells is not inhibited by the continuously high levels of Cyclin E/Cdk2 activity that occur normally during the early cell cycles of *Drosophila* embryogenesis or that can be driven ectopically in imaginal disc cells (Neufeld et al., 1998; Sauer et al., 1995).

The similarities between the responses of both proliferating and endocycling cells to ectopic expression of DMyb and Cyclin E suggest the possibility that the effects of ectopic DMyb activity might be due to induction (either directly or indirectly) of high levels of Cyclin E, and preliminary results indicate that Cyclin E levels are increased in salivary glands expressing ΔDMyb (C. A. F., unpublished). However, we also found that periodic expression of E2F/DP could overcome DMyb-induced inhibition of endoreduplication, whereas Follette and colleagues (Follette et al., 1998) showed that E2F/DP expression could not override the replication block induced by Cyclin E. The finding that E2F induction can override the inhibition of DNA endoreduplication caused by ectopic DMyb activity, suggests that DMyb induced inhibition may be upstream of E2F or that E2F can circumvent the DMyb-induced block. In addition, it is unlikely that endogenous DMyb plays a role in regulating the levels of Cyclin E in endocycling larval cells, as *Dm myb* transcripts have not been detected in these cells and no deleterious effects on larval tissues have been observed in loss-of-function mutant alleles of *Dm myb* (Katzen and Bishop, 1996; Katzen et al., 1998). For these reasons, and because we do not detect any defects in salivary glands when we ectopically express another DMyb construct, which contains the DMyb DNA-binding domain fused to an engrailed repressor domain (C. A. F., unpublished), we do not believe that DMyb or ΔDMyb are acting to repress, rather than activate expression of target genes in salivary glands, a phenomena that has been observed with other transcriptional activators when they are overexpressed (e.g. Suppressor of Hairless) (Klein et al., 2000). Therefore,

the results from ectopic expression of DMyb reinforce our conclusions from studies of loss-of-function alleles, that one of the functions of *Dm myb* is to suppress endoreduplication and maintain genomic stability in proliferating diploid cells (Fung et al., 2002; Katzen et al., 1998).

The transgenic experiments reported here demonstrate that ΔDMyb is a much more potent inhibitor of endoreduplication than DMyb. The C-terminal region of the vertebrate A-Myb and c-Myb proteins has been shown to contain negative regulatory domains that downregulate the DNA-binding and transcriptional activation abilities of the proteins; the equivalent portion of the B-Myb protein contains both negative and positive regulatory sequences (reviewed by Gonda et al., 1996; Oh and Reddy, 1999; Saville and Watson, 1998). Our findings indicate that the C-terminal sequences that were deleted in the DMyb protein (by analogy to c-Myb) act to strongly downregulate DMyb activity in salivary glands. By contrast, ΔDMyb appeared to be only slightly more active than DMyb at promoting proliferation in imaginal disc cells, even those in the ZNC, which should be specifically arrested in either G_1 or G_2. This finding is in agreement with a growing body of evidence from studies with the vertebrate Myb proteins, that their ability to activate transcription is strongly dependent on the presence and/or abundance of other cellular factors (Ness, 1999). Therefore, one rationale for the difference between the behavior of the DMyb proteins in imaginal discs and salivary glands is that imaginal disc cells may contain an 'activating factor' that is absent in salivary glands, that interacts with full-length DMyb to relieve the repression of its transcriptional activating potential that is mediated via the C-terminal domain. Another possibility is that salivary gland cells contain a factor that specifically interacts with full-length DMyb to repress its activity, but this seems less likely as endogenous *Dm myb* expression has not been detected in these cells.

E2F/DP and DREF (DNA replication-related element binding factor) are transcription factors that have been shown to be crucial for cell cycle regulation in *Drosophila*. These factors promote, and are required for DNA replication in both mitotic and endocycling cells (Duronio et al., 1995; Hirose et al., 1999; Royzman et al., 1997). We have now demonstrated that like these factors, DMyb promotes DNA replication in mitotic cells. However, the situation differs in endocycling cells. Previously reported results have shown that DMyb is not required for DNA replication in endocycling cells (see above) (Katzen and Bishop, 1996; Katzen et al., 1998), and the data presented here demonstrate that DMyb can actively inhibit endoreduplication. The ability of DMyb to have directly opposing effects on DNA replication, depending upon cell cycle context, makes DMyb unique among the transcription factors in *Drosophila* that have been implicated in cell cycle regulation (see Fig. 9). Further investigation should elucidate how these transcription factors interact to coordinate cell cycle progression.

Our finding that DMyb activity can induce proliferation throughout the ZNC of the wing disc indicates that it can either override or circumnavigate the G_1 and G_2 blocks established by the Notch and Wingless signaling pathways at the dorsoventral compartment boundary (Johnston and Edgar, 1998). We have preliminary evidence that the levels of protein encoded by some of the genes involved in, or targeted by, these

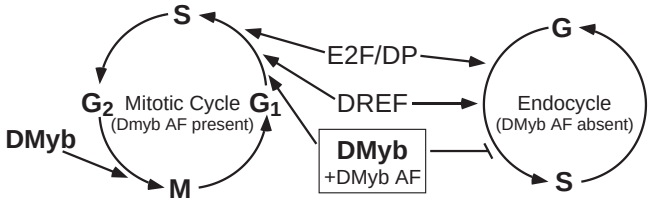

Fig. 9. The roles played by three transcription factors involved in cell cycle regulation in *Drosophila melanogaster*. The E2F/DP and DREF transcription factors have been shown to promote DNA replication (progression from G_1 into S) in mitotic and endocycling cells. Data from previous (Fung et al., 2002; Katzen et al., 1998) and current investigations of the function of *Dm myb* have demonstrated that although in mitotic cells, DMyb shares the function of being a positive regulator of progression from G_1 into S with E2F/DP and DREF, DMyb also acts as a negative regulator of endoreduplication and is able to promote progression from G_2 into M. We propose that the ability of DMyb to inhibit endoreduplication is an important aspect of maintaining genomic integrity in proliferating cells. We have also shown that while the abilities of full-length and truncated (activated) DMyb proteins to promote cell cycle progression in proliferating cells were similar, the truncated protein was considerably more potent at inhibiting S phase in endoreduplicating cells. As indicated in the figure, we hypothesize that one or more factors, to which we refer as DMyb activating factors (DMyb AF), must interact with full-length DMyb to activate its potential as a transcriptional regulator, and that these factors are present in proliferating cells, but absent in endocycling cells.

signaling pathways are decreased (C. A. F., unpublished), but the mechanisms by which this is accomplished are presently obscure. However, disturbances in these pathways may account for the observed increases in apoptosis. Elucidation of these mechanisms should help to further our understanding of how cell proliferation and patterning are coordinately regulated in developing organs.

There is a substantial amount of data indicating that vertebrate Myb genes function to promote the G_1/S transition. By contrast, loss-of-function mutations in *Drosophila*, cause either a block at the G_2/M transition followed by endoreduplication or mitotic defects, which have implicated *Dm myb* in several aspects of cell cycle regulation, but not directly in the initiation of S phase. These discrepancies have prompted the question of whether the functions of the insect and vertebrate Myb genes are really equivalent? However, mitotic defects (chromosome breakage and cells arrested in metaphase) have recently been observed with mutations in several other genes that are known to be required for DNA replication, including *MCM4* (*dpa* – FlyBase) *PCNA* (*mus209* – FlyBase) and three genes encoding proteins crucial for assembly of the pre-initiation complex: *Orc2*, *Orc5* and *dup* (also known as cdt1) (Pflumm and Botchan, 2001; Whittaker et al., 2000). These findings indicate that the mitotic defects observed in *Dm myb* mutants could be secondary consequences of replication defects, a viewpoint supported by Manak and colleagues in a recent paper (Manak et al., 2002). By contrast, the findings that DMyb is an activator of *cyclin B* expression in the imaginal eye disc (Okada et al., 2002) and that DMyb activity can induce mitosis in cells within the ZNC that are normally blocked in G_2 (see Fig. 5), provide support for our earlier conclusions that DMyb has a direct involvement in

promoting mitosis. Additionally, three experimental observations provide strong circumstantial evidence that *Dm myb* function is not an absolute requirement for DNA replication per se: *Dm myb* expression is not detected in larval endoreplicating tissues; endoreduplication in larval tissues appears to occur normally in loss-of-function mutant alleles of *Dm myb*; and de novo endoreduplication is observed in mutant wing cells during pupal development (Katzen and Bishop, 1996; Katzen et al., 1998).

We have presented evidence that, in addition to inducing increased levels of mitosis, *Dm myb*, like its vertebrate counterparts, can promote the G_1/S transition. Our studies also demonstrate that the C termini of the vertebrate and *Drosophila* Myb proteins share the function of downregulating their activities. Finally, the finding that ectopic DMyb can actively inhibit endoreduplication reinforces our conclusions from previous analyses of loss-of-function alleles, that *Dm myb* normally acts in proliferating cells to maintain diploidy by suppressing reinitiation of S phase prior to mitosis. Our demonstration that at least one aspect of *myb* function is conserved between the *Drosophila* and vertebrate Myb proteins, raises the issue of whether one or more of the vertebrate Myb proteins may also act to inhibit endoreduplication and/or to promote mitosis. In conclusion, our studies demonstrate that DMyb functions in multiple aspects of the cell division cycle to promote proliferation and maintain the integrity of the genome.

We thank Steve Beckendorf, Bruce Edgar, Shigeo Hayashi, Bob Duronio, Shelagh Campbell, Wei Du, and Andrea Brand for supplying transgenic fly stocks; and Siau-Min Fung, George Scaria and Karen Artiles for helpful discussion and advice. Work reported here was supported by a National Institutes of Health grant to A. L. K. (CA74221).

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