Drosophila myb is required for the G₂/M transition and maintenance of diploidy

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The *myb* proto-oncogenes are thought to have a role in the cell division cycle. We have examined this possibility by genetic analysis in *Drosophila melanogaster*, which possesses a single *myb* gene. We have described previously two temperature-sensitive, recessive lethal mutants in *Drosophila myb* (*Dm myb*). The phenotypes of these mutants revealed a requirement for *myb* in diverse cellular lineages throughout the course of *Drosophila* development. We now report a cellular explanation for these findings by showing that *Dm myb* is required for both mitosis and prevention of endoreduplication in wing cells. *Myb* apparently acts at or near the time of the G₂/M transition. The two mutant alleles of *Dm myb* produce the same cellular phenotype, although the responsible mutations are located in different functional domains of the gene product. The mutant phenotype can be partially suppressed by ectopic expression of either *cdc2* or *string*, two genes that are known to promote the transition from G₂ to M. We conclude that *Dm myb* is required for completion of cell division and may serve two independent functions: promotion of mitosis, on the one hand, and prevention of endoreduplication when cells are arrested in G₂, on the other.

[Key Words: Cell cycle; transcription factor; proto-oncogene; G₂/M; endoreduplication; diploidy]

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The proto-oncogene *c-myb* was first encountered as a transduced retroviral oncogene *v-myb*, which causes a myeloid leukemia in chickens and transforms myeloid cells in culture (for review, see Lyon et al. 1994; Thompson and Ramsay 1995). Mutations affecting *c-myb* have since been implicated in tumors of mice and humans (Lyon et al. 1994; Thompson and Ramsay 1995). The product of *c-myb* (*Myb*) is a transcription factor that binds a specific sequence in DNA (Biedenkapp et al. 1988; Weston and Bishop 1989). The protein is divided into at least three discrete functional domains (Fig. 1): one for binding to DNA, a second for activation of transcription from other genes, and a third that governs the biochemical activity of the protein (Gonda et al. 1996; Kanei-Ishii et al. 1996). Vertebrates possess two additional genes that are related to *c-myb*, known as *A-myb* and *B-myb* (Nomura et al. 1988). The proteins encoded by these genes (*MybA* and *MybB*) also function as transcription factors (for review, see Kanei-Ishii et al. 1996).

It is generally believed that *Myb* plays a role in the cell division cycle, specifically at the G₁/S transition (Lyon et al. 1994; Thompson and Ramsay 1995). The most direct evidence has come from experiments with antisense

oligonucleotides directed against *c-myb*, which block entry of cells into the S phase of the cell cycle (Calabretta 1991). The validity of these results has been challenged, however, because the antisense oligonucleotides are said to inhibit cellular proliferation in a nonspecific manner (Burgess et al. 1995). *MybB* has also been implicated in the G₁/S transition (Lyon et al. 1994; DeGregori et al. 1995; Kanei-Ishii et al. 1996; Robinson et al. 1996), whereas *MybA* is more likely to be involved in cellular differentiation (Trauth et al. 1994; Kanei-Ishii et al. 1996). Genes that encode transcription factors with DNA-binding domains related to that of *Myb* have also been identified in yeast and plants (Paz-Ares et al. 1987; Tice-Baldwin et al. 1989; Ohi et al. 1994). One of these (the *cdc5* gene of *Schizosaccharomyces pombe*) has been implicated in the G₂ phase of the cell cycle (Ohi et al. 1994).

In an effort to define further the cellular function of *myb* genes, we have turned to genetic analysis of the single *myb* gene found in *Drosophila melanogaster*, *Dm myb* (Katzen et al. 1985). The protein encoded by *Dm myb* (*DMyb*) is approximately the same size as vertebrate *Myb* (Peters et al. 1987), and the two proteins share four regions of homology, including the DNA-binding domain (Fig. 1). Similar regions are also present in *MybA* and *MybB*. Because *Dm myb* is equally similar to each of the vertebrate *myb* genes and is currently the only *myb*-

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related gene identified in *Drosophila*, it may represent the progenitor of all three genes and encompass their combined functions.

Previously, we have described two temperature-sensitive mutants of *Dm myb* whose effects revealed a requirement for *myb* in diverse cellular lineages throughout the course of development (Katzen and Bishop 1996). It appeared that *myb* might be essential only during periods when cells are mitotically active. We now report a cellular explanation for these findings by demonstrating that *Dm myb* may have two distinctive functions during the cell-division cycle: promotion of mitosis, on the one hand, and prevention of endoreduplication of DNA when cells are arrested in G₂, on the other. The two mutant alleles of *Dm myb* produce the same cellular phenotype, although the responsible mutations are located in different functional domains of DMyb. These results implicate *myb* in the mitotic portion of the cell division cycle for the first time and represent the first direct implication of a transcription factor in the G₂/M transition.

Results

The nucleotide sequences of *Drosophila myb*¹ and *myb*²

Before extending our analysis of the phenotypes caused by the mutant alleles of *Dm myb*, we characterized the nature of the genetic damage affecting the two alleles. Analysis with restriction enzymes revealed no evidence

of chromosomal rearrangements or deletions. We therefore cloned the two mutant alleles and their wild-type counterpart and then determined the nucleotide sequence of the *myb*-coding domains within the clones. The results are summarized in Figure 1. Each mutant allele contained a single nucleotide substitution resulting in the change of an amino acid perfectly conserved between DMyb and its vertebrate counterparts (Fig. 1). The amino acid substitution in *myb*¹ (Gly → Ser) is considered to be neutral, whereas the substitution in *myb*² (Arg → Lys) is highly conservative (McLachlan 1971). These relatively mild changes are consistent with the temperature-sensitive and hypomorphic natures of the mutant phenotypes (see Discussion).

The *myb*² mutation occurred within the second of three imperfect tandem repeats that comprise the DNA-binding domain of DMyb (Peters et al. 1987; Kanei-Ishii et al. 1996). In contrast, the *myb*¹ mutation occurred near the carboxyl terminus of DMyb, within the fourth region of conservation shared between the *Drosophila* and vertebrate proteins (Bishop et al. 1991). No specific activity has been ascribed to this region, but its strong evolutionary conservation (47% identity between DMyb and chicken Myb) along with the phenotypic defects associated with the *myb*¹ mutation, indicate that this domain has functional importance.

Wing defects observed in *myb* mutants raised at temperatures permissive for viability

Previously, we reported that *myb*¹ and *myb*² are tem-

perature sensitive for lethality [*myb*¹ viable at 18°C, but not at 25°C; *myb*² viable at 18°C and 25°C, but not at 28°C (Katzen and Bishop 1996)]. In addition, the lethal phenotype is tighter when either mutation is carried over a deficiency chromosome that deletes the *myb* gene [*Df(1)sd*^{72b26}] than when either of the mutations is homozygous, indicating that both alleles are hypomorphic rather than null.

*myb*¹ and *myb*² mutants raised at temperatures permissive for viability (18°C and 25°C, respectively) were not grossly malformed, but closer inspection of adults revealed several defects, the most obvious of which occurred in the wings (Katzen and Bishop 1996). These findings indicate that although temperatures of 18°C and 25°C are permissive for viability of *myb*¹ and *myb*² mutants, respectively, they are not completely permissive for *myb* function. We will therefore refer to these temperatures as semi-permissive. When wings from the *myb*¹ mutant and the parental *white* (*w*) strain raised at 18°C were mounted and examined under low magnification, mutant wings were approximately the same size and shape as parental wings, but appeared to be considerably cruder (Fig. 2A,B). In particular, wing veins were thicker and differed slightly in their relative positions. Inspection of wings at higher magnification revealed that the mutant wings had approximately half the number of hairs as wild-type wings, and that mutant hairs were considerably larger than normal (Fig. 2D,E; Table 1). Hairs were less regularly spaced, less uniform in orientation, and occasionally grouped in small clusters, indicating a disturbance of tissue polarity (for a review on tissue polarity, see Adler 1992). Bristles on mutant wings were not obviously reduced in number, but did appear to be larger and less uniform in orientation. One copy of the wild-type *Dm myb* transgene completely rescued the

wing phenotype (Fig. 2C,F; Table 1), demonstrating that the wing defects were attributable to the lesion in the *Dm myb* gene.

Wings dissected from *myb*² mutants raised at 18°C were much less severely affected, with respect to both quantity and orientation of hairs, than were *myb*¹ wings (Fig. 2G; Table 1). However, when wings were dissected from *myb*²/*Df(1)sd*^{72b26} females, which occasionally survive at 18°C, or from *myb*² mutants raised at 25°C, the defects were more extreme, closely resembling the *myb*¹ phenotype (Fig. 2H,I; Table 1). These results reinforce the evidence from viability studies that *myb*² is a hypomorphic allele (retains subpar activity), as two copies are better than one (*myb*² homozygote vs. *myb*²/*Df(1)sd*^{72b26}) and the severity of wing phenotype is temperature sensitive (18°C vs. 25°C).

The wing phenotype results from a defect in cell division

In wild-type wings, each cell that is not specialized for another purpose is represented by a single hair (Postlethwait 1978). Therefore, the reduced density of hairs on mutant wings indicated that either these wings had fewer cells, each of which was larger, or they had the same number of cells as wild type, but only some of the cells made hairs. Because we were unable to stain adult wings for any cellular structure, the correspondence between cells and hairs could not be determined at this stage. We also failed to obtain any useful information from third instar larval wing disks, both because of their complex folding patterns and because of the difficulty of obtaining enough disks at exactly the same stage of development. In contrast, pupal wings proved to be both

Figure 2. Mutant *myb* wings were cruder than wild-type wings and had fewer, larger hairs that were not uniformly oriented. Wings were dissected from female flies raised at 18°C unless otherwise specified. Bars in A and D, 0.1 mm. Complete wings for *white* (A), *myb*¹ (B), and *myb*¹; *P(w⁺,myb⁺)/+* (C) are shown at the same magnification. D–I are shown at the same magnification, and the number of hairs located within the boxed area of each panel is indicated below in brackets. When compared with the parental *w* strain (84 hairs) (D), the number of hairs in *myb*¹ mutants (39 hairs) (E) was decreased by approximately half. This phenotype could be fully rescued by a wild-type *Dm myb* transgene, shown in F [*myb*¹; *P(w⁺,myb⁺)/+* (86 hairs)]. The reduction in hair number was significantly less in *myb*² flies raised at 18°C (68 hairs) (G) but was stronger when *myb*² was carried over a deficiency chromosome at 18°C *myb*²/*Df(1)sd* (43 hairs) (H), or when *myb*² flies were raised at 25°C (46 hairs) (I).

Table 1. The relative cell number on wings of different genotypes under varying conditions, as measured by hair counts

Genotype	Temperature (°C)			18°C–28°C temperature shift ^b
	18	25	25–heat shock ^a	
<i>white (w)</i>	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.04
<i>myb¹</i>	0.52 ± 0.05			
<i>myb¹; P(w⁺, myb⁺)/+</i>	1.00 ± 0.07	1.00 ± 0.09		
<i>myb²</i>	0.82 ± 0.05	0.60 ± 0.09	0.60 ± 0.05	0.55 ± 0.04
<i>myb²/Df(1)sd^{72b26}</i>	0.53 ± 0.05			
<i>myb²; HS–Dm cdc2/+</i>			0.79 ± 0.08	
<i>myb²; HS–Dm cdc2AF/+</i>			0.81 ± 0.03	
<i>myb²; HS–stg/+</i>			0.78 ± 0.12	

In each experiment, the number of hairs in white wings is assigned the value of 1.0. The relative numbers represent the averages with standard deviations of a minimum of five sampled areas. For each experiment, at least two regions of the wing were examined. (HS) Heat shock.

^aFor conditions, see legend to Fig. 5.

^bFor conditions, see legend to Fig. 7.

accessible and informative. We concentrated on the wings of *myb¹* mutants raised at 25°C, assuming that the mutant phenotype would be most extreme under these conditions.

Developing wings from *myb¹* mutants and the parental *w* strain were dissected out of prepupae at 6 hr after puparium formation (APF) and treated with the DNA stain, DAPI. No difference was detected between the mutant and wild-type wings (Fig. 3A,B). In contrast, for wings dissected out of pupae between 24 and 36 hr APF, the density of nuclei in any given region of the mutant wing was approximately half of that found for the same region of a wild-type wing, and the mutant nuclei were larger than their wild-type counterparts (Figure 3C,D). No apoptotic nuclei were observed in any of the DAPI-stained samples. The more mature wings (36 hr APF) were also stained with rhodamine-labeled phalloidin (an F-actin-specific stain) to visualize the developing hairs (prehairs) (Wong and Adler 1993). For both mutant and wild-type wings, a one-to-one correspondence was found between the number of nuclei and the number of prehairs (Fig. 3C–F). Taken together, these data show that the mutant wings had fewer cells, each of which was larger and produced a hair.

The preceding results point to a defect in cellular proliferation during the final stages of wing development in the *Dm myb* mutants. We can infer the nature of that defect by reference to previous descriptions of how the wings of *Drosophila* mature (Schubiger and Palka 1987). At 3 hr APF, all wing cells except those at the anterior and posterior margins arrest synchronously in G₂. The arrest persists until 12 hr APF, when mitosis occurs, followed by an additional cell division cycle that is completed by 24 hr APF. Thereafter, the cells remain in G₀. When examined during the early G₂ arrest (at 6 hr APF), the number of wing cells in *myb¹* mutants was normal, but in postmitotic wings (after 24 hr APF), the number of wing cells in *myb¹* mutants was half that of wild type. For technical reasons (see below) it was not possible to examine the wings between 7 and 24 hr APF, the period during which the final cell cycles occur. If both of the

cell cycles that normally ensue were defective, the number of cells should be reduced by a factor of four. Thus, we conclude that it is likely that the final cell division fails to occur in the mutant wings.

Mutant wing cells are blocked before the G₂/M transition

The preceding experiments did not define the stage during the last cell cycle at which the mutant *Dm myb* wing cells were halted. We decided to ask whether these cells entered S phase during the last cell cycle by testing for their ability to incorporate BrdU. Unfortunately, because the wing cells have secreted pupal cuticle that is very securely attached to the cell surface, pupal wings at the developmental stage of interest (12–18 hr APF) are very difficult to handle, but at 24 hr APF, the cuticle detaches from the wing surface and can be dissected away from the wing. Consequently, we used an approach described by others for injecting BrdU into developing pupae, and allowing them to mature to a convenient stage before dissecting out the wings for further processing and analysis (Schubiger and Palka 1987; Hartenstein and Posakony 1989). Using this method, Schubiger and Palka (1987) showed that the BrdU pattern observed in imaginal disks corresponds to a short pulse (1–2 hr) of BrdU labeling after injection. Their studies also showed that only one S phase (spanning ~12 to 20 hr APF) occurs in the wing cells of interest during pupal development. Therefore, any labeling observed should correspond to the final S phase.

We selected pupae that were either 12–13 hr APF formation or 15–16 hr APF for injection with BrdU and allowed them to develop until 36 hr APF. For both time periods, BrdU incorporation was detected in the mutant as well as in the parental wings (Fig. 4A,B), indicating that both wild-type and mutant disk cells were actively replicating DNA. The patterns of BrdU incorporation for the *myb¹* wings indicated that they were less mature than the wild-type wing, a finding that is consistent with our observations that *myb¹* mutants developed more

Figure 3. *myb*¹ mutant wings had half the number of nuclei as wild-type wings, a defect that occurs during the last cell division. Shown are regions of developing wings dissected from animals raised at 25°C. Wings were treated with either DAPI alone or DAPI and rhodamine-labeled phalloidin (a stain that highlights wing prehairsts because they contain high levels of F-actin). The number of nuclei or hairs located within the box in each panel is indicated below in parentheses. Bars, 0.025 mm. DAPI staining of prepupal wings at 6 hr APF showed that at this stage, the nuclear density in A [*w* (parental strain) (152 nuclei)] and B [*myb*¹ (155 nuclei)] was the same. DAPI staining of pupal wings at 36–37 hr APF showed that at this stage, the nuclear density in C [*w* (153 nuclei)] was approximately twice the density in *myb*¹ (83 nuclei) (D). Note that the *myb*¹ nuclei were larger than the *w* nuclei. Phalloidin staining of the same wings as shown in C and D, E (155 hairs) and F (82 hairs), respectively, showed a one-to-one correspondence between numbers of nuclei and hairs for both wild-type and mutant animals.

slowly than the parental flies. We conclude that *myb*¹ mutant wing cells entered into their final S phase but did not undergo a final division, presumably leaving them with a 4C content of DNA (see below).

To assess further the cell cycle state of the mutant *Dm myb* wing cells, we tested whether the mutant phenotype could be suppressed by overexpression of either wild-type or activated alleles of *Dm cdc2* (the cyclin-dependent kinase that regulates the G₂/M transition) or *string* (the *Drosophila* homolog of *cdc25*, the protein tyrosine/threonine phosphatase that regulates *cdc2* activity) (Edgar and O'Farrell 1990; O'Farrell 1992; Stern et al. 1993). Each of these transgenes, under the transcriptional control of the *hsp70* heat shock promoter, was mated into strains carrying the mutant *myb* alleles.

*myb*¹ mutant pupae raised at 18°C were unable to tolerate the heat shock treatment and none survived to adulthood. *myb*² mutant pupae raised at 25°C with or without each of the transgenes, were heat shocked when they were between 18 and 22 hr APF, and then allowed to continue development at 25°C. Overexpression of either *cdc2* or *string* was able to partially suppress the *myb*² wing phenotype, increasing the number of hairs by 30% to 35% (see Table 1) and correcting the orientation of the hairs (Fig. 5). Overexpression of these transgenes via heat shock treatment during the same stage of development had no effect on wings in the parental strain (not shown). These results support the conclusion that the mutant *myb* wing cells were arrested at the G₂/M transition.

A subset of nuclei in myb mutants entered endoreduplication cycles

The results to this point suggested that the wing cells in mutant flies failed to complete the final mitosis in their developmental lineage. We explored this possibility further by using high-resolution, three-dimensional wide-field fluorescence microscopy to examine the amount of DNA contained in nuclei of individual wild-type and mutant cells (Fig. 6). Postmitotic wings from wild-type, *myb*¹, and *myb*² animals raised at 18°C (72 hr APF) or 25°C (36 hr APF) were dissected and fixed, and the DNA in the nuclei was stained with DAPI. Results from the

Figure 4. *myb*¹ mutant wing cells entered S phase during the final cell cycle. Shown are developing wings from pupae that were raised at 25°C, injected with BrdU at 16 hr APF, allowed to continue developing at 25°C until 36 hr APF, and then dissected and processed to visualize BrdU incorporation: (A) *w* (parental strain); (B) *myb*¹. Bar, 0.05 mm.

Figure 5. Overexpression of *Dm cdc2* or *string* during pupal development can suppress the mutant *Dm myb* wing defect. Wings were dissected from flies that were raised at 25°C, heat shock treated for 20 min at 37°C when they were between 18 and 22 hr APF, and then returned to 25°C. Bars in A and D, 0.05 mm. The number of hairs within the box in each panel is indicated below in parentheses. Shown in A–C at the same magnification is a region near the distal tip between longitudinal veins III and IV. When compared with *myb*², overexpression of either *Dm cdc2AF* or *stg* in *myb*² mutants resulted in an increase in the number of hairs in *myb*² mutants: (A) *myb*² (103 hairs); (B) *myb*²; HS-*Dm cdc2AF*/+ (135 hairs); (C) *myb*²; HS-*stg*/+ (134 hairs). The wild-type version of *cdc2* under the heat shock promoter also suppressed the *myb* phenotype. To demonstrate that suppression occurred in multiple regions of the wing, a more proximal section is shown at a lower magnification: (D) *myb*² (105 hairs); (E) *myb*²; HS-*Dm cdc2*/+ (137 hairs).

analysis of 120 nuclei for each genotype and temperature are shown in Figure 6. At 18°C, the average DNA content of mutant *myb*¹ nuclei was double that of wild-type nuclei, whereas the average for *myb*² nuclei was only slightly elevated (Fig. 6A). Surprisingly, the average DNA content of mutant *myb*¹ nuclei was even greater at 25°C than at 18°C, and the average for *myb*² nuclei increased to approximately double that of wild-type (Fig. 6B).

As anticipated, the results indicated that the mutant cells generally contained more DNA than wild-type cells. But there was substantial heterogeneity in the data for mutant cells, first apparent as relatively large standard deviations for the measurements of DNA content (Fig. 6A,B). Analysis of the data for individual cells provided an explanation (Fig. 6C,D). At the semipermissive temperature of 18°C, the amount of DNA in *myb*¹ nuclei varied from 2C to well in excess of 4C. At 25°C, the majority of nuclei contained more than 4C DNA. We conclude that the mutation causes endoreduplication in a fraction of the arrested wing cells, the severity of which varies from one cell to another.

Temperature shifts demonstrate a direct role for myb in the final cell cycle of the wing

It remained possible that the defective cell cycle associated with the *Dm myb* mutants was a secondary consequence rather than a primary effect of the deficiency in *myb* function. In an effort to explore this possibility, we performed temperature-shift experiments with *myb*². Two factors facilitated this strategy: the temperature-dependent expressivity of *myb*² within a viable range of temperatures (see above, Fig. 2G,I); and the well-defined window of time within which the final cell division oc-

curs in the maturing wing (see above and Schubiger and Palka 1987). We could not perform this sort of analysis with *myb*¹ because even at 18°C, the majority of cells do not undergo their final mitosis. Since shifting to restrictive temperature earlier in development (late third instar larval or prepupal stages) invariably results in lethality (Katzen and Bishop 1996), it was also not feasible to effect a block in the initial cell cycle of the emerging wing.

In *myb*² mutants raised at 18°C, the majority of wing cells underwent the final mitosis and contained the normal complement of DNA (Fig. 6C), resulting in adult wings that contained 80% of the hairs found in wild-type wings (Table 1; Fig. 2G). In contrast, most of the wing cells in *myb*² mutants raised at 25°C failed to complete their final cell cycle, resulting in adult wings that contained slightly more than half the number of hairs found in wild-type wings (Table 1; Fig. 2I), and cells that contained on average, twice the normal complement of DNA (Fig. 6B). When *myb*² flies were transiently exposed to the higher temperature during the period of time corresponding to the final cell division, the number of wing cells was reduced to the maximum extent, that is, approximately one-half the normal number (Fig. 7; Table 1). We conclude that the failure of wing cells to complete the final cell division cycle is likely to be an immediate consequence of the deficiency in *myb* function.

Discussion

Conservative changes in single amino acids are responsible for the mutant phenotypes of myb¹ and myb²

Mutations in *myb*¹ and *myb*² arose from changes in

Figure 6. Quantitative microscopic analysis of nuclei from mutant and wild-type wings. Developing wings were dissected from wild-type (WT), *myb*¹, and *myb*² pupae at 72 hr APF for animals raised at 18°C, or 36 hr APF for animals raised at 25°C. Wings were then fixed and stained with DAPI. Four samples from each genotype and temperature were optically sectioned using high-resolution, three-dimensional wide-field fluorescence microscopy (see Materials and Methods). Relative fluorescent intensities and volumes of at least 30 nuclei for each sample (≥ 120 /genotype) were analyzed. Shown are the calculated averages (means) of nuclear fluorescent intensities for each genotype at 18°C (A) and 25°C (B) with standard deviations indicated by vertical bars. To illustrate the heterogeneity within the population of mutant nuclei, the fluorescent intensities of 50 nuclei from each genotype at 18°C (C) and 25°C (D) are plotted individually. (Solid diamond) Wild-type; (shaded square) *myb*¹; (open triangle) *myb*².

single nucleotides that cause amino acid substitutions (Fig. 1). In *myb*¹, serine replaces glycine in one of the four structural domains that are conserved among the *myb* loci of *Drosophila* and vertebrates. The fact that the relatively neutral change of serine to glycine engenders a mutant phenotype suggests a strong requirement for serine at the residue. Because the function of the mutant

protein domain is not known, the apparent requirement for serine cannot presently be explained.

The mutation in *myb*² substitutes lysine for arginine at a position within the DNA-binding domain of the DMyb protein, a conservative change that should not necessarily disrupt function. Arginine has been conserved at the comparable positions in the Myb, MybA, and MybB proteins of vertebrates (Gonda et al. 1985; Gerondakis and Bishop 1986; Majello et al. 1986; Nomura et al. 1988), and in the more distant Myb-related proteins from yeast and plants (Ohi et al. 1994). We conclude that the mutated arginine residue is essential to the function of Myb.

Insight into the role of the conserved arginine comes from examination of the manner in which Myb interacts with its specific target site in DNA. Ogata and colleagues have used nuclear magnetic resonance to analyze this interaction, using a complex between the isolated DNA-binding domain of mouse Myb and the DNA sequence to which it binds (Ogata et al. 1994). The DNA-binding domain of Myb is composed of three imperfect tandem repeats designated R1–R3 (Kanai-Ishii et al. 1996). The second and third of three repeats are required for DNA binding. Each repeat contains three helices, the third of which recognizes the target DNA sequence. Arg-177 in the *Drosophila* protein, the amino acid replaced by Lys in *myb*², corresponds to Arg-133 in the mouse Myb protein. This arginine is in the middle of the recognition (third) helix of R2, and although it does not specifically interact with any of the base pairs of the target sequence, it does form a contact with the phosphate backbone and also interacts by a salt bridge with Asp-100 (Asp-144 in the *Drosophila* protein), interactions that presumably stabilize the protein–DNA complex. In addition, the third helix within R2 was shown to be thermally less stable than any other part of the DNA-binding domain (Ogata et al. 1994). Together, these findings provide an explanation for why the substitution of lysine for arginine results in a temperature-sensitive phenotype.

The wing phenotype is the consequence of a G₂/M block and failure to prevent endoreduplication of DNA

Analysis of pupal wings during different stages of development showed that, for the mutant alleles of *Dm myb* in hand, wing development is apparently normal into early pupation, when cells are normally arrested in G₂. Postmitotic wings in *myb*¹ and *myb*² mutants raised at semipermissive temperatures, however, have approximately half as many cells as wild-type wings. Since wild-type wing cells normally undergo two mitotic cleavages during pupal development, the majority of mutant cells must divide once. It seems likely that mutant *myb* cells progress through the first mitosis and are blocked during their final cell cycle. *myb*¹ wing cells incorporate BrdU during the period corresponding to the final S phase, indicating that the block occurs after entry into DNA syn-

Figure 7. The mutant *myb*² wing phenotype is enhanced by shifting from permissive to restrictive temperature for 1 day during pupal wing development. Bar in A, 0.05 mm. The number of hairs within the box in each panel is indicated below. Shown is a region near the distal tip between longitudinal veins IV and V. Wings were dissected from white flies that were raised at 18°C until 26 hr APF, transferred to 28°C for 24 hr, and then returned to 18°C, 222 hairs (A), *myb*² flies that were raised continuously at 18°C, 178 hairs (B), or *myb*² flies that were raised at 18°C until 26 hr APF, transferred to 28°C for 24 hr and then returned to 18°C, 115 hairs (C).

thesis. This is supported by the fact that in mutant wings, the nuclei, cells, and hairs are all larger than in wild-type wings, an effect known to correlate with increased DNA content.

When overexpressed at the appropriate stage of pupal development, either of two key regulators of the G₂/M transition, *Dm cdc2* or *string/cdc25*, can at least partially suppress the wing defect. These results suggest that S phase has been completed and that the mutant cells are arrested in G₂, as mitosis is generally dependent on the completion of DNA synthesis. In *S. pombe*, however, it has been shown that dependence of mitosis on DNA synthesis is lost in strains that either overproduce the *cdc25* protein or carry mutations in *cdc2* that make it independent of *cdc25* activity (Enoch and Nurse 1990). In those strains, the abnormal divisions lead to disturbances in nuclear morphology and reduced cellular viability. We did not observe any signs of these cellular disturbances in our experiments. It appears that it is more difficult to overcome the dependence of mitosis on completion of DNA synthesis in *Drosophila* than in yeast, as overproduction of *string/cdc25* in *Drosophila* embryos was unable to drive S-phase-arrested cells into mitosis, perhaps because the *string* protein is unstable prior to G₂ (Edgar and Datar 1996; B.A. Edgar, pers. comm.). Therefore, we conclude that the wing cells in *Dm myb* mutants enter and complete DNA synthesis, but are blocked during G₂ or at the G₂/M transition. It is still possible, however, that although S phase is largely completed, a few replication forks are stalled and that at this late stage, overexpression of the G₂/M regulators can overcome the S phase checkpoint.

High-resolution, three-dimensional wide-field fluorescence microscopy revealed heterogeneity within the population of mutant cells. In wings from *myb*¹ and *myb*² mutants raised at semipermissive temperatures, most nuclei contain twice the normal complement of DNA, but nuclei with the normal complement are also observed, suggesting that some of the cells, perhaps those that divide earliest, retain enough *myb* activity to complete the final division. We also found that many of the arrested cells continued to replicate their DNA. The extent of endoreduplication varied from one cell to an-

other and was particularly pronounced in the wings of *myb*¹ mutants raised at 25°C (nonpermissive for adult viability), at which temperature the majority of cells had DNA contents in excess of 4C. These results suggest that *Dm myb* normally acts to inhibit endoreduplication, a conclusion that is consistent with our previous findings that *Dm myb* is not expressed at detectable levels in polyploid larval tissues (Katzen and Bishop 1996). The endoreduplication also provides an explanation for why ectopic expression of *cdc2* or *string* did not fully suppress the *myb*² phenotype in the wing. Some of the cells may no longer have been capable of undergoing mitosis, as they had already begun to endocycle. Given recent reports that under certain circumstances, *myb* genes can play a role in regulating *bcl-2* expression (Frampton et al. 1996; Taylor et al. 1996), we note that we did not detect any signs of apoptosis in mutant wings.

In adult wings from *Dm myb* mutants raised at permissive temperature, there was not only a reduction in the number of cells, but also a disturbance of tissue polarity as judged by hair orientation and presence of occasional small clusters of hairs protruding from a single position. This prompts the question of whether the defects in cell cycle and polarity are independent of each other or whether one is an indirect consequence of the other. Because regulators of the G₂/M transition suppress both defects (see above and Fig. 5), it seems likely that the polarity defects are an indirect result of the abnormal cell cycle stage in which the wing cells are trapped. In support of this, when mitotic clones of *cdc2* mutations were induced in wings, cells with multiple hairs and abnormal polarity were observed (P.N. Adler, pers. comm.).

On the basis of our studies, two cell cycle checkpoints appear to be disturbed in the wing cells of *Dm myb* mutants: regulation of the G₂/M transition and prevention of re-entry into S phase before M phase occurs. Is one of these defects a consequence of the other or are they independent of each other? In *string* mutant embryos, cells arrest at the G₂/M boundary and do not enter S phase (Edgar and O'Farrell 1989; Smith and Orr-Weaver 1991), indicating that the mechanism for preventing reinitiation of DNA replication before mitosis can remain intact

when cells are prematurely arrested in G_2 . Therefore, it is unlikely that the endoreduplication in mutant *myb* cells is simply a consequence of the abnormal arrest in G_2 . Because overexpression of either *Dm cdc2* or *string* can partially suppress the *myb* phenotype, at least a proportion of the mutant cells must still be competent for mitosis, suggesting that the block in G_2 is not just a consequence of the mutant cells entering into an endocycle and losing the ability to divide. We conclude that *myb* may play active and independent roles in both checkpoints.

Is Dm myb function required in all cell cycles?

The studies reported here focus on the cellular basis of the wing defect. We have reason to believe, however, that *Dm myb* plays a more general role in cell cycle regulation, as we have observed a variety of other cells that appear to be affected in a fashion similar to the wing cells (fewer cells, larger nuclei). Of particular note is a subset of glial cells, identified by staining with a glial-specific antibody (Campbell et al. 1994). We suspect that the defects in these cells may be responsible for the inviability of *myb* mutants at restrictive temperatures (A.L. Katzen et al., unpubl.). In addition, abdominal cuticular defects are observed in *myb* mutants raised at semipermissive temperatures (A.L. Katzen et al., unpubl.), which are similar to other mutant phenotypes that result from a shortage of adult epidermal cells to replace larval epidermal cells (*escargot* and *cdc2*; Hayashi et al. 1993; Stern et al. 1993). There are, however, cell types that appear to be normal, even at restrictive temperature, and in the wings, the cells are blocked from completing only their final cell cycle.

Do these results necessarily mean that *Dm myb* is not required for all cell types and cell cycles? We suspect that this is not the case. Because *Dm myb* is expressed at relatively high levels in most proliferating cells and both of the mutant alleles are hypomorphic and temperature sensitive (Katzen and Bishop 1996), it seems likely that enough *myb* activity is present in most mutant cells for them to divide. The phenotype observed in the wing supports this possibility, as the developing mutant wings appear normal until the final cell cycle, which occurs during a period (12–24 APF) when the level of *Dm myb* message is decreasing. Of course, it is possible that *Dm myb* function is required for cell division in some tissues and not in others, even though the gene is expressed in all mitotic tissues. In this case, we would have to conclude that *Dm myb* expression in unaffected tissues is either gratuitous or functionally redundant with another gene. Final determination of whether *myb* function is required in all cell cycles will have to await generation of stronger alleles of *myb*.

Regulatory pathways in which myb may participate

The biochemical pathways that regulate the G_2 /M transition and ensure the maintenance of diploidy are likely to be complex. *Dm myb* is not the only gene that has

been implicated in both of these regulatory checkpoints. Two other genes, *Dm cdc2* and *Dm cyclin A*, which play critical roles in regulating the G_2 /M transition, have recently been shown to suppress endoreduplication (Sauer et al. 1995; Hayashi 1996). In *Dm cdc2* mutants, the abdominal histoblasts, which are normally arrested in G_2 during larval development, undergo endoreduplication instead, indicating that endoreduplication can be decoupled from premature mitotic arrest (Hayashi 1996). Hayashi also provided evidence that, unlike the G_2 /M transition, which is initiated by the dephosphorylated, kinase-active form of Cdc2, endoreduplication is inhibited by the phosphorylated (kinase-inactive) form, probably complexed with cyclin A. Consistent with the ability of *Drosophila cdc2*, *cyclin A*, and *myb* to prevent re-entry into S-phase before mitosis occurs, none of these genes are expressed in polyploid tissues (Lehner and O'Farrell 1989, 1990; Stern et al. 1993; Katzen and Bishop 1996).

We hypothesize that *Dm myb* participates along with *Dm cdc2* and *Dm cyclin A* in the biochemical circuit that regulates both the G_2 /M transition and maintenance of diploidy. This hypothesis is supported by the ability of *Dm cdc2* to suppress the *myb* wing phenotype, when overexpressed at the appropriate time. Not surprisingly, there are other genes such as *string* and *escargot*, that function to regulate either the G_2 /M transition or suppression of endoreduplication, respectively, but not both (Edgar and O'Farrell 1989; Smith and Orr-Weaver 1991; Hayashi 1996). An illustration of some of the key players that regulate the mitotic and endoreplicative cell cycles is shown in Figure 8. There is little doubt that many other genes participate in regulating one or both of these cell cycles, and it is likely that some of them will be subject to transcriptional regulation by *myb*. Genetic screens designed to isolate suppressors and/or enhancers of the mutant *Dm myb* phenotype should allow us to identify genes that participate in the same biochemical pathways as *myb*. We have preliminary data demonstrating a strong genetic interaction between *Drosophila myb* and *cyclin A*, but the biochemical basis of this interaction is not yet known (A.L. Katzen et al., unpubl.).

Is the role of myb in cell cycle regulation evolutionarily conserved?

Our finding that a mutation in *Dm myb* leads to a cell cycle block superficially agrees with several lines of evidence that vertebrate *c-myb* is required for proliferation in at least a subset of cell types (Mucenski et al. 1991; Badiani et al. 1994; Lyon et al. 1994; Thompson and Ramsay 1995). In contrast to our results in *Drosophila*, vertebrate *myb* genes are generally thought to be involved in regulating the G_1 /S transition (for review, see Lyon et al. 1994), but the most direct evidence for this has recently been challenged (see introductory section).

Could *Dm myb* also be involved in regulating the G_1 /S transition? Since a reduction in *myb* activity leads to inappropriate endoreduplication, it seems unlikely

Figure 8. Schematic of gene products known to play key roles in regulating the mitotic and endoreplicative cell cycles. The data presented in this paper demonstrate that *Dm myb* is a positive regulator of progression from G_2 into M and a negative regulator of endoreduplication, thereby maintaining diploidy. These dual functions are shared with *Dm cdc2* and *Dm cyclin A* (Sauer et al. 1995; Hayashi 1996). In contrast, *string* and *escargot*, function to either activate the G_2 /M transition or suppress endoreduplication, respectively, but not both (Edgar and O'Farrell 1989; Smith and Orr-Weaver 1991; Hayashi 1996). The transcription factor E2F, *Dm cyclin E*, and the *Dm cdc2c* kinase are required for S phase in both mitotic and endoreplicative cell cycle (Duronio et al. 1995; Sauer et al. 1995; Lilly and Spradling 1996). By analogy to the role that vertebrate *myb* genes are thought to play in cell cycle regulation, *Dm myb* may also participate in regulation of the G_1 /S transition, but our studies indicate that it is not required for DNA synthesis in endocycling cells.

that *Dm myb* function is required for DNA synthesis, as such. This conclusion is also supported by previous observations that *Dm myb* transcripts are not detectable in polyploid tissues (Katzen and Bishop 1996) and by our studies of mutants during larval and pupal development, in which no defects in endoreduplication were found (not shown).

Regulation of entry into S phase, however, clearly differs between endoreplicating and mitotically proliferating cells (Smith and Orr-Weaver 1991; Orr-Weaver 1994; Sauer et al. 1995; Lilly and Spradling 1996). Therefore, it is possible that *Dm myb* might play a role in the G_1 /S transition in the mitotic cell cycle, even though it does not appear to be required for replication in endocycling cells. As noted above, *Dm myb* is expressed at relatively high levels in most proliferating cells. If *Dm myb* were required at lower levels for the G_1 /S transition than for G_2 /M, then the hypomorphic *myb* alleles might retain sufficient activity for efficient progression into S, leaving the phenotype to manifest itself at the G_2 /M transition.

Whatever, its role in the G_1 /S transition, there are reasons to suspect that vertebrate *myb* may also act later in the cell cycle. Although expression of both *c-myb* and *B-myb* are specifically induced at the G_1 /S boundary in many cell types, expression levels remain high throughout S phase (Lyon et al. 1994). *B-myb* has also been shown to be a target for transcriptional regulation by the E2F transcription factor (DeGregori et al. 1995; Zwicker et al. 1996). E2F controls a variety of genes encoding proteins required for G_1 /S (e.g., cyclin E), S-phase (e.g., DNA polymerase α), and G_2 - G_2 /M transition (e.g., cyc-

lin A and *cdc2*). Of these genes, the manner in which *B-myb* is negatively regulated by E2F appears to be most similar to that of *cyclin A* and *cdc2* (Bennett et al. 1996; Liu et al. 1996; Hurford et al. 1997). Finally, ectopic expression of *B-myb* or *c-myb* in cell culture induces *cdc2* expression (Sala and Calabretta 1992; Ku et al. 1993). It is therefore tempting to speculate that in *Drosophila*, E2F may regulate expression of *myb*, which in turn regulates one or more of the genes that control the G_2 /M transition and suppression of endoreduplication (e.g., *cdc2*, *string*, *cyclin A*).

Many *myb*-related genes have now been identified in organisms that diverged earlier in evolution than *Drosophila*, such as yeast and plants (Paz-Ares et al. 1987; Tice-Baldwin et al. 1989; Ohi et al. 1994). Only one of these, the *S. pombe cdc5* gene, has been shown to be involved in regulating the cell cycle, having been implicated in the G_2 phase of the cell cycle (Ohi et al. 1994). Our data demonstrate that a *myb* gene can also function to regulate the G_2 /M transition in a multicellular organism. In addition, we have identified a novel activity for *myb* in inhibiting endoreduplication. These results indicate an ancient role for the Myb transcription factor in regulating cell cycle checkpoints other than the G_1 /S transition and raise the possibility that one or more of the vertebrate *myb* genes may have similar functions in cell cycle regulation.

Materials and methods

Sequence analysis of mutant alleles

Because *myb¹* and *myb²* are temperature-sensitive alleles, both mutants could be maintained as homozygotes. Genomic DNA was prepared from each mutant and the parental *white* strain as described previously (Katzen et al. 1985). Six pairs of oligonucleotides were used to PCR amplify three overlapping segments that span the entirety of the *Dm myb* gene. The oligonucleotides were designed to introduce restriction enzyme sites at the ends of the amplified fragments so that they could be cloned easily into the vector pGEM-7Zf (Promega). The same oligonucleotides (without the added sequences used to create restriction enzymes sites) and additional oligonucleotides corresponding to sequences internal to the fragments, were used for sequencing. The US Biochemical kit (dideoxy [35 S]dATP) was used to sequence 4–5 μ g of denatured, cesium chloride-banded DNA, which was run on gels using Long Ranger gel mix from AT Biochem. Discrepancies in sequences obtained from clones representing the mutant alleles were checked and confirmed by sequencing both strands. For *myb¹* and *myb²*, two or three independent PCR-amplified clones were sequenced, respectively. In the case of *myb¹*, an *EcoRI* fragment spanning the mutation was cloned directly from genomic DNA into the pGEM-7Zf vector. Sequencing of this clone also confirmed the presence of the mutation.

Drosophila stocks

Generation of mutant *Dm myb* alleles on a *white* chromosome, construction of lines carrying a wild-type *Dm myb* rescue transgene, *P(w⁺,myb⁺)* and the *Df(1)sd^{72b26}* chromosome, which deletes a region that includes *Dm myb* have been described pre-

viously (Katzen and Bishop 1996). Transgenic lines carrying either the heat shock (HS)-*string*, HS-*Dm cdc2*, or the HS-*Dm cdc2AF* construct were generously provided by Patrick O'Farrell (University of California at San Francisco; Edgar and O'Farrell 1990; Stern et al. 1993). The *Dm cdc2AF* is an allele of *cdc2* that has an alanine substituted for threonine-14 and a phenylalanine substituted for tyrosine-15, which prevents these two positions from being phosphorylated and makes the kinase activity of the protein *string* independent (Krek and Nigg 1991).

Preparation and fluorescent staining of wings

Adult wings were dissected from flies in isopropanol and mounted with a water-soluble mounting media (Immu-mount, Shandon). Photomicroscopy was performed with a Zeiss Axiophot microscope.

Pupae were staged by transfer of white prepupae (0 hr APF) from healthy bottles maintained at the appropriate temperature to fresh vials and further development at the same temperature. Wing disks and the central nervous system (brain/ventral ganglia) from third instar larvae and prepupae (6 hr APF, 25°C or 13 hr APF, 18°C) were dissected in phosphate-buffered saline (130 mM sodium chloride, 10 mM phosphate at pH 6.8) plus 0.1% Tween (PBST) and fixed for 30–60 min in PBST containing 4% paraformaldehyde, which had been freshly prepared. After being washed at least three times in PBST, wings were incubated for 20 min in the dark in a PBST solution containing 1 µg/ml DAPI. Wings were washed several times in PBST. A drop of glycerol was added to the final rinse solution, and the wings were stored overnight at 4°C in the dark before being mounted in Immu-mount (Shandon). For later stages, a tungsten needle was used to puncture the head of each pupa, which was then put into a 1.5-ml Eppendorf tube containing the same fixation solution as above. After several hours on a rocker, pupae were rinsed and dissected in PBST. The pupal cuticle was gently teased off of each wing. Wings were incubated for 20 min in the dark in a PBST solution containing 5 U/ml rhodamine-labeled phalloidin (Molecular Probes), washed at least three times in PBST, and then stained with DAPI and prepared for mounting as described above. Fluorescence photomicroscopy was performed with a Zeiss Photomicroscope III.

Application of BrdU

Pupae raised at 25°C were selected and aged as described above. They were then injected with BrdU (from Amersham's cell proliferation kit, code RPN 20) at either 12–13 or 15–16 hr APF according to the method described by Hartenstein and Posa-kony (1989) and incubated in a humid chamber at 25°C until 36 hr APF. At this time point, the wings were dissected, fixed in modified Carnoy's fixative [100% ethanol/glacial acetic acid (3:1)], and rinsed several times in PBST. Further processing was according to the instructions of the kit, with the only modification being that 0.1% Tween was included in all solutions. Photomicroscopy was performed with a Zeiss Axiophot microscope.

High-resolution three-dimensional fluorescence microscopy of developing wings

Pupal wings were prepared from wild-type (parental strain) and *myb*¹ and *myb*² mutants at either 36 hr APF for flies raised at 25°C or 72 hr APF for flies raised at 18°C by the protocol described above, except for the following modifications: Wings were fixed in 4% paraformaldehyde prepared in buffer A (15 mM Pipes, 80 mM KCl, 20 mM NaCl, 2 mM Na₂EDTA, 0.5 mM

EGTA, 0.5 mM spermidine, 0.2 mM spermine) plus 0.1% Tween. After DAPI staining and three rinses in the dark, wings were immediately mounted with Vectashield (Vector Laboratories, Inc.), a mounting media that inhibits fading of the fluorescent signal, and cover slips were sealed with clear nail polish. For each genotype and temperature, four samples (two samples each from two different wings mounted under the same cover slip) were examined. Three-dimensional data sets were collected from all wings on the same day (stained wings were kept in the dark when not being analyzed), by use of an Olympus 60× 1.4 N.A. oil immersion lens on a computer controlled wide-field microscopy system and cooled CCD camera as described elsewhere (Hiraoka et al. 1991). Cells were imaged in three dimensions by movement of the sample through the focal plane of the objective lens at 0.5-µm increments and recording of an image with the CCD camera at each position. Out-of-focus light was removed by a constrained iterative deconvolution algorithm using an empirical point-spread function (Agard et al. 1989; Hiraoka et al. 1990). Processed data presented here were examined and manipulated by use of the IVE software package developed for three-dimensional images, which allowed individual nuclei to be selected and modeled interactively with a graphical user interface (Chen et al. 1996). For each data set, a minimum of 30 nuclei were selected for analysis (for a total of ≥120/genotype). After three-dimensional modeling, the integrated fluorescent intensity and volume for each nucleus was calculated.

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