Epigenetic regulation of gene expression by *Drosophila* Myb and E2F2–RBF via the Myb–MuvB/dREAM complex

Hong Wen,¹ Laura Andrejka,¹ Jonathan Ashton,¹ Roger Karess,² and Joseph S. Lipsick^{1,3}

¹Department of Pathology and Department of Genetics, Stanford University, Stanford, California 94305, USA; ²Centre National de la Recherche Scientifique (CNRS), Centre de Génétique Moléculaire, 91198 Gif sur Yvette, France

The *Drosophila* Myb oncoprotein, the E2F2 transcriptional repressor, and the RBF and Mip130/LIN-9 tumor suppressor proteins reside in a conserved Myb–MuvB (MMB)/dREAM complex. We now show that Myb is required in vivo for the expression of *Polo* kinase and components of the spindle assembly checkpoint (SAC). Surprisingly, the highly conserved DNA-binding domain was not essential for assembly of Myb into MMB/dREAM, for transcriptional regulation in vivo, or for rescue of *Myb*-null mutants to adult viability. E2F2, RBF, and Mip130/LIN-9 acted in opposition to Myb by repressing the expression of *Polo* and SAC genes in vivo. Remarkably, the absence of both Myb and Mip130, or of both Myb and E2F2, caused variegated expression in which high or low levels of *Polo* were stably inherited through successive cell divisions in imaginal wing discs. Restoration of Myb resulted in a uniformly high level of *Polo* expression similar to that seen in wild-type tissue, whereas restoration of Mip130 or E2F2 extinguished *Polo* expression. Our results demonstrate epigenetic regulation of gene expression by Myb, Mip130/LIN-9, and E2F2–RBF in vivo, and also provide an explanation for the ability of *Mip130*-null mutants to rescue the lethality of *Myb*-null mutants in vivo.

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A central question in developmental biology is the mechanism by which genetically identical cells stably maintain different phenotypic states during successive rounds of mitotic division. Conversely, the disruption of such epigenetic regulation is widely believed to play a key role in cancer (Lund and van Lohuizen 2004). A powerful model for understanding the mechanisms of epigenetic control was provided by Muller's eversion of displacements in *Drosophila*, which display variegated expression of eye pigment due to the spread of constitutive heterochromatin into adjacent regions of the genome (Muller 1930; Schulze and Wallrath 2007). Parallel systems of epigenetic regulation by Trithorax and Polycomb group proteins were discovered via genetic studies of the *Drosophila* body plan (Lewis 1978; Schuettengruber et al. 2007).

The retinoblastoma (RB) family of tumor suppressor proteins regulates gene expression by binding to E2F–DP heterodimers, which themselves bind directly to DNA (Classon and Harlow 2002). RB family proteins have been proposed to inhibit gene expression by direct inter-

action with components of the classical position effect variegation (PEV) system and/or the Polycomb system (Nielsen et al. 2001; Vandel et al. 2001; Gonzalo et al. 2005; Kotake et al. 2007). However, evidence for epigenetic regulation of gene expression by RB family proteins in vivo has thus far been lacking.

RB is encoded by the retinoblastoma susceptibility gene (*RB1*), loss of which causes both inherited and sporadic retinoblastoma in humans (Classon and Harlow 2002). Two RB-related proteins, p107 and p130, are encoded by the *RBL1* and *RBL2* genes in vertebrates. These three "pocket" proteins can repress gene expression via the E2F transcription factors. This repression is relieved by phosphorylation of RB-related proteins by the Cyclin D–CDK4 protein kinase, which itself is inhibited by the p16 and p15 CDK inhibitor proteins. The importance of this regulatory circuit is underscored by its mutation in the majority of human cancers.

In vertebrates, the RB-responsive E2F family form heterodimeric DNA-binding proteins composed of one E2F subunit (an E2F1–3 activator or an E2F4–5 repressor) and one DP subunit (DP1 or DP2). *Drosophila* contains only two RB-related proteins (RBF1 and RBF2), two E2F subunits (an E2F1 activator or an E2F2 repressor), and a single DP subunit. Biochemical studies revealed that the

³Corresponding author.

E-MAIL lipsick@stanford.edu; FAX (650) 725-6902.

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Drosophila E2F2, DP, and the RBF proteins are present in a large complex (Myb–MuvB [MMB]/dREAM) that includes the Myb protein, as well as four previously identified Myb-binding proteins (Mip130, Mip120, Mip40, and p55CAF1) (Beall et al. 2002; Korenjak et al. 2004; Lewis et al. 2004). Unexpectedly, loss-of-function mutants of *Mip130*, *Mip120*, or *Mip40* can rescue an otherwise lethal *Myb*-null mutant to adult viability (Beall et al. 2004, 2007). With the exception of Myb itself, all of these proteins are conserved in *Caenorhabditis elegans* and form a similar complex (Lipsick 2004; Harrison et al. 2006). Interestingly, mutation of any one of the corresponding genes (*synMuvB*) has been shown to cause a synthetic multivulval (*synMuv*) phenotype in nematodes in combination with mutation of any single gene of another equivalence group (*synMuvA* or *synMuvC*) (Ferguson and Horvitz 1989; Ceol et al. 2006). These results imply that *synMuv* genes act in a redundant fashion to repress the RTK–RAS pathway, activation of which causes a multivulval phenotype. Related MMB/dREAM-like complexes are also present in human cells (Korenjak et al. 2004; Litovchick et al. 2007; Pilkinton et al. 2007; Schmit et al. 2007).

The *Myb* gene family was discovered due to the oncogenic transduction of *c-Myb* by an acutely transforming chicken retrovirus (Lipsick and Wang 1999). All vertebrates examined have three related *Myb* genes, whereas *Drosophila* has a single *Myb* gene (Katzen et al. 1985; Davidson et al. 2004). The highly conserved Myb DNA-binding proteins can regulate gene expression in a variety of experimental systems. Vertebrate A-*Myb* (*MYBL1*) and *c-Myb* (*MYB*) are tissue-specific with regard to gene expression and loss-of-function phenotypes in the laboratory mouse (Mucenski et al. 1991; Toscani et al. 1997). In contrast, vertebrate B-*Myb* (*MYBL2*) is expressed in all dividing cells, and loss-of-function mutants cause early embryonic lethality (Tanaka et al. 1999).

Drosophila Myb is an essential gene, and loss-of-function mutants cause early prophase arrest, failure of progression of chromosome condensation, aneuploidy, polyploidy, aberrant mitotic spindles, and abnormal centrosome number (Katzen et al. 1998; Fung et al. 2002; Manak et al. 2002, 2007). Similar cell cycle defects occur in B-*Myb* mutant zebrafish (Shepard et al. 2005). Interestingly, vertebrate B-*Myb*, but neither A-*Myb* nor *c-Myb*, can complement the cell cycle defects in *Myb*-null *Drosophila* hemocytes (Davidson et al. 2005). These results imply that B-*Myb* is the vertebrate ortholog of *Drosophila Myb*. Importantly, increased levels of B-*Myb* expression correlate with poor prognosis in a variety of human malignancies including breast cancer (Amatschek et al. 2004; Paik et al. 2004).

The RB–E2F axis has primarily been studied as a regulator of the G1/S transition of the cell cycle (Dimova and Dyson 2005). However, *Drosophila Myb* and vertebrate B-*Myb* mutants disrupt the G2/M transition. Therefore, we sought to understand the mechanism of the mitotic cell cycle defects in *Myb* mutant cells and to determine the role of MMB/dREAM in G2/M control. We found that *Polo*, a regulator of spindle pole assembly and func-

tion, is directly regulated by Myb in vivo, as are key components of the spindle assembly checkpoint (SAC). Unexpectedly, we found that *Polo* displays a variegated pattern of expression in the absence of Myb and Mip130 or of Myb and E2F2. These results demonstrate a role for MMB/dREAM in epigenetic regulation of gene expression.

Results

Loss of Drosophila Myb causes mitotic delay with spindle pole abnormalities

Drosophila Myb-null mutants die as late third instar larvae with aberrant chromosome condensation, ploidy, and mitotic spindle formation in brain and imaginal disc cells (Manak et al. 2002, 2007). To better understand the nature of these mitotic defects, we performed real-time analysis of the cytologically favorable giant larval neuroblasts. We used an RFP-histone H2avD fusion protein to identify those neuroblasts that had undergone chromosome condensation and a GFP-trap microtubule-associated protein fusion protein to visualize the spindle poles and spindle fibers (Morin et al. 2001; Pandey et al. 2005). We found that *Myb*-null neuroblasts that displayed characteristics of chromosome condensation were dramatically delayed in prometaphase/metaphase with persistently abnormal spindles (Fig. 1A,B; Supplemental Fig. S1). The initially bipolar spindle poles migrated improperly, and the spindle itself was often rotated, bowed, and/or misattached. In addition, the spindle poles in *Myb*-null cells displayed an abnormal and persistent accumulation of GFP-TACC with a larger apparent volume and a much more diffuse, multicentric character than spindle poles in wild-type cells (Supplemental Fig. S1; Barros et al. 2005). Similar spindle abnormalities were observed regardless of the overall length of mitotic delay. These results demonstrate that in addition to the prophase delay with failure of chromosome condensation, *Myb*-null cells that do eventually progress into metaphase experience subsequent abnormalities of the mitotic spindle.

Myb is required for expression of Polo and the SAC

The spindle pole abnormalities in *Myb*-null larval neuroblasts pointed us toward Polo kinase, a key regulator of spindle pole assembly and function (Glover 2005). We used compartment-specific rescue, which permits the side-by-side analysis of mutant and rescued cells in the same tissue at the same time without direct competition (Manak et al. 2007). We first examined the expression of a GFP-Polo fusion protein under control of the normal *Polo* transcriptional regulatory elements in larval imaginal wing discs, which increase from 50 to 50,000 cells during the third instar period of development and eventually give rise to adult wings (Fig. 1C, panels a,g; Moutinho-Santos et al. 1999). We found that in the absence of Myb, levels of GFP-Polo were greatly reduced in mitosis and during interphase.

Figure 1. Loss of *Myb* causes mitotic delay, and *Myb* is required for expression of *Polo* and the SAC. (A) Wild-type or *Myb*-null larval neuroblasts expressing GFP microtubule-associated protein (GFP protein trap G147) and RFP-histone H2avD were visualized by time-lapse laser scanning confocal microscopy. Only GFP is shown here. RFP-H2avD is also shown in Supplemental Figure S1. *Myb* mutant cells entering mitosis spent a much longer time in metaphase and displayed abnormal spindle morphology. The time is in hours:minutes:seconds with T_0 set at the start of imaging. Broken circles indicate cells in mitosis. (B) Bar graph showing distribution of the duration of larval neuroblast mitosis for wild-type (wt, $N = 22$) and *Myb*-null mutant ($N = 37$). The time in minutes indicates the duration of mitosis from spindle pole apposition to onset of anaphase. (C,D) Imaginal wing discs from *Myb*-null larvae in which RFP-Myb or unfused Myb protein was expressed in the posterior compartment via the GAL4-UAS binary system. (C, panels a–f) Fluorescence of indicated fusion proteins expressed from native promoters. Shown directly below each panel are fluorescence of RFP-Myb (panels g,h,j–l) or RFP coexpressed with Myb (panel i) in the same imaginal discs. (D, panels a–f) RNA in situ hybridizations for expression of endogenous genes as indicated. (Panels g–l) Shown directly below each panel are RNA in situ hybridizations for *Myb* in the same imaginal discs. In this and other photomicrographs of wing imaginal discs, anterior is to the left.

Because alterations in ploidy occur in *Drosophila Myb*-null larvae, we also examined components of the SAC (Karess 2005; Musacchio and Salmon 2007). In the

absence of *Myb*, we observed dramatic reductions of GFP-Mad2, a key component of the highly conserved mitotic checkpoint complex (MCC), and of GFP-Rod, a

key component of the animal-specific RZZ complex (Fig. 1C; Buffin et al. 2005). Similar observations were made for GFP-BubR1, another component of the MCC complex (data not shown). However, several other proteins required for proper chromosome behavior during mitosis were unaffected in the absence of Myb, including Mps1, a SAC kinase; Mei-S332/Shugoshin, a regulator of centromeric sister chromatid cohesion; and CENP-C, a conserved inner kinetochore component (Fig. 1C).

We found that levels of a GFP-Mad2 fusion protein expressed under control of the *Ubiquitin* promoter were not affected by the presence or absence of Myb (data not shown). This result implied that Myb did not affect GFP-Mad2 protein stability, but rather affected *GFP-Mad2* mRNA levels only when expressed under control of the normal *Mad2* transcriptional regulatory elements. To determine if the endogenous *Polo* and SAC genes were regulated by Myb at the mRNA level, we performed in situ hybridization on imaginal wing discs from *Myb*-null animals in which cells in the posterior, but not the anterior compartments, were rescued by expression of a

Myb cDNA (Fig. 1D). *Polo*, *Mad2*, *Rod*, and *BubR1* mRNA levels were highly dependent on the presence of Myb. In contrast, the expression of two other genes encoding SAC components, *Bub1* and *Bub3*, were not affected by the absence of Myb.

Loss of Myb results in a failure of progression of chromosome condensation such that the phosphorylation of Ser 10 on histone H3 occurs normally in heterochromatin but fails to spread to euchromatin (Manak et al. 2007). However, this failure of chromosome condensation occurred only after many cell divisions in the absence of Myb. To determine the kinetics of the loss of *Polo* and SAC gene expression in the absence of Myb, we performed genetic mosaic analysis using the FLP-FRT system (Golic 1991). Cells containing a wild-type *Myb* allele were marked by RFP-HP1, whereas *Myb*-null clones were RFP-negative (Fig. 2). Nuclei were counterstained with TOTO-3, a DNA dye with a preference for heterochromatin. We observed that GFP-*Polo* and GFP-*Mad2* expression were extinguished in the absence of Myb, even in very small mutant clones (Fig. 2). Control

Figure 2. Mosaic analysis of *Polo* and *Mad2* regulation by Myb. Imaginal wing discs from heterozygous larvae (*Myb*⁺, *RFP-HP1*⁺/*Myb*⁻) in which mutant clones (*Myb*⁻/*Myb*⁻) and adjacent twin spots (*Myb*⁺, *RFP-HP1*⁺/*Myb*⁺, *RFP-HP1*⁺) were generated by mitotic recombination induced by hs-FLP. Each column displays the same imaginal disc via fluorescence of indicated GFP fusion proteins expressed from native promoters (panels a-d), of the RFP-HP1 clonal marker (panels e-h), of the merge of GFP and RFP (panels i-l), and of the merge of indicated GFP fusion protein fluorescence with that of the DNA dye TOTO-3 (panels m-p). The first and third columns display larger clones at lower magnification, whereas the second and fourth columns display smaller clones at higher magnification. Arrows indicate representative *Myb* mutant clones.

experiments demonstrated that altered levels of the RFP-HP1 marker itself in a *Myb* wild-type background caused no change in GFP-Polo or GFP-Mad2 (data not shown). Mutant clones (*RFP⁺, Myb⁻/RFP⁺, Myb⁻*) were similar in size to adjacent “twin spots” (*RFP⁺, Myb⁺/RFP⁺, Myb⁺*), demonstrating that greatly decreased levels of Polo and SAC components do not inhibit cell proliferation in the short term. In summary, these results imply that Myb is required for the maintenance of normal levels of Polo and Mad2, and that the GFP-Polo and GFP-Mad2 proteins themselves are degraded during each mitotic cell cycle.

The highly conserved Myb DNA-binding domain is not required for activating gene expression in vivo

The Myb DNA-binding domain has been highly conserved during the evolution of animals, green plants, protists, and cellular slime molds (Davidson et al. 2004). Remarkably, the ~150-amino-acid Myb domain displays a 65% identity between the human and *Dictyostelium* proteins (Stober-Grasser et al. 1992). To determine the role of this domain in vivo, we constructed transgenic *Drosophila* in which the C terminus of Myb completely lacking the DNA-binding domain was fused to GFP. To our surprise, we found that this C-terminal protein fragment rescued a uniformly lethal *Myb*-null mutant to adult viability, albeit at a lower frequency than a wild-type Myb protein (Supplemental Table S1). To determine whether this C-terminal Myb fragment was capable of regulating gene expression in vivo, we expressed either a full-length GFP-Myb fusion protein or the GFP C-terminal fusion protein in the posterior compartments of *Myb*-null imaginal wing discs in the presence of an *RFP-Mad2* transgene. Both full-length Myb and the C-terminal Myb fragment were capable of activating *RFP-Mad2* expression (Fig. 3A, panels a,d). The C-terminal Myb fragment lacking a DNA-binding domain was also capable of suppressing the increased frequency of H3S10phospho (PH3)-positive cells and partial chromosome condensation that occurs in vivo in *Myb*-null cells (Fig. 3A, panels c,f; Manak et al. 2007).

We next asked whether this C-terminal Myb protein fragment can assemble into MMB/dREAM complexes. Flag-tagged versions of either full-length Myb or the C-terminal fragment lacking a DNA-binding domain were expressed in *Drosophila* S2 cells, and immunoprecipitations with anti-Flag antibodies were performed on nuclear extracts. Both full-length Myb and the C-terminal fragment lacking a DNA-binding domain specifically coprecipitated Mip130, Mip120, Mip40, p55CAF1, and E2F2 (Fig. 3B). In summary, the highly conserved Myb DNA-binding domain is not required for association with the MMB/dREAM complex, activation of gene expression in vivo, or rescue of chromosome condensation defects that occur in the absence of Myb.

Variation of GFP-Polo expression in the absence of both Myb and Mip130

Although *Myb*-null mutants are lethal and *Mip130* mutants are viable, the lack of both *Myb* and *Mip130* re-

Figure 3. The Myb DNA-binding domain is not required for regulation of gene expression, for progression of chromosome condensation, or for entering the MMB/dREAM complex. (A) Imaginal wing discs from *Myb*-null larvae in which either a GFP full-length Myb fusion protein (panels a–c) or GFP C-terminal Myb fusion protein lacking a DNA-binding domain (panels d–f) were expressed in the posterior compartment via the GAL4-UAS binary system. Each row displays the same imaginal disc using three different markers: RFP-Mad2 visualized by staining with anti-RFP antibody (panels a,d), GFP-Myb fusion (panels b,e), or anti-histone PH3 antibody (panels c,f). (B) Coimmunoprecipitation with anti-Flag M2 antibody $\pm$ ethidium bromide (EtBr) in *Drosophila* S2 cells transfected with full-length Flag-Myb, Flag-C-terminal Myb, or control vector. Stability of these interactions in the presence of ethidium bromide implies that these protein–protein interactions are not dependent on DNA (Lai and Herr 1992). Western blotting was performed with indicated antibodies. Input represents 5% of total.

stores viability (Beall et al. 2004). In the absence of Myb, the remaining members of the Myb complex might act together as an unrestrained repressor of the *Polo* and SAC genes, thereby resulting in mitotic defects. This model predicts that (1) loss of Myb but not Mip130 alone would reduce *Polo* and SAC gene expression; and (2) loss of both Myb and Mip130 would restore expression of *Polo* and SAC genes in vivo. As expected, GFP-Polo expression was uniformly decreased in *Myb* single mutant, but not in *Mip130* single mutant imaginal discs (Fig. 4A).

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GFP-Polo expression was, indeed, observed in *Myb*, *Mip130* double mutant imaginal discs, as predicted. Unexpectedly, the *Myb*, *Mip130* double mutant imaginal discs displayed a variegated pattern of fluorescence in which clusters of adjacent cells alternatively expressed either high or low levels of GFP-Polo (Fig. 4A). Similar variegation was observed with two independent insertions of the *GFP-Polo* transgene. These insertions have been mapped by inverse PCR and DNA sequencing to euchromatic regions of the genome at cytological bands 45D (in *CG13955*) on Chromosome II and 76D1 (between *Fibp* and *Deaf1*) on Chromosome III. Discovery of this variegated expression was possible because GFP-Polo functions as a visible, cell-autonomous marker with single-cell resolution. In contrast, cell-by-cell resolution of gene expression is difficult to achieve by RNA in situ hybridization in imaginal wing discs.

Ectopic Myb suppresses and Mip130 enhances the variegation of GFP-Polo expression

In wild-type imaginal wing discs GFP-Polo is ON, whereas variegated expression occurs in *Myb*, *Mip130*

double mutant tissue. By analogy with PEV, enhancers are genetic modifiers that increase the proportion of cells in the OFF state, whereas suppressors decrease the proportion of cells in the OFF state (Schotta et al. 2003). The expression of a *Myb* cDNA in the posterior compartment of a *Myb*, *Mip130* double mutant imaginal wing disc suppressed the variegated pattern of GFP-Polo, resulting in uniformly high levels consistent with stabilization of this gene in the ON state (Fig. 4B, panels a–c). *Myb* protein has been shown to be destabilized in the absence of *Mip130* (Beall et al. 2004). However, our results imply that at sufficiently high levels, *Myb* can function in a *Mip130*-independent fashion to regulate *Polo* gene expression. No alteration in *GFP-Polo* expression was observed when *Myb* was ectopically expressed in wild-type imaginal wing discs (data not shown). Taken together, these results imply that the MMB/dREAM complex, and that *Myb* in particular, is required for the epigenetic maintenance of *Polo* expression.

The expression of *Mip130* in the posterior compartment of a *Myb*, *Mip130* double mutant imaginal wing disc enhanced the variegated pattern of GFP-Polo expres-

Figure 4. *Myb* suppresses and *Mip130* enhances the variegation of GFP-Polo expression. (A) Expression of GFP-Polo in imaginal wing discs from single or double mutant genotypes as indicated. (B) Imaginal wing discs from double mutant larvae in which RFP-Myb, *Mip130* alone, or RFP and *Mip130* were expressed in the posterior compartment via the GAL4-UAS binary system as indicated. Each row displays the same imaginal disc using two different markers (first and second columns) and a merged image (third column). (Panels a–c) *Myb*⁻, *Mip130*⁻ disc with GFP-Polo and UAS-RFP-Myb. (Panels d–f) *Myb*⁻, *Mip130*⁻ disc with GFP-Polo and UAS-Mip130 visualized by anti-Mip130 antibody. (Panels g–i) *Myb*⁻, *Mip130*⁻ larvae with UAS-RFP and UAS-Mip130 stained with anti-histone PH3 antibody. Note variegation of GFP-Polo expression only in the *Myb*, *Mip130*-disc (A) and unrescued anterior compartments of such discs (B).

sion, in this case resulting in uniformly low levels consistent with stabilization of this gene in the OFF state (Fig. 4B, panels d–f). Ectopic Mip130 had no effect on GFP-Polo expression in a wild-type or a *Mip130* mutant background (data not shown). Although Myb and Mip130 also opposed one another in regulating the expression of *GFP-Mad2* and *GFP-Rod*, no variegation of these genes was observed in *Myb*, *Mip130* double mutants (Supplemental Fig. S2; data not shown).

A marked increase in the frequency of PH3-positive cells was observed when a *Mip130* cDNA was expressed in the posterior compartment of a *Myb*, *Mip130* double mutant imaginal wing disc (Fig. 4B, panels g–i). The increased frequency of partial chromosome condensation was similar to that seen in *Myb*-null mutant cells in comparison with adjacent *Myb*-rescued cells (Manak et al. 2007). These results demonstrate that the mitotic defects in *Myb*-null cells are dependent on the presence of Mip130.

E2F2-RBF represses the expression of Polo and the SAC

To test whether E2F2-RBF also regulates the *Polo* and SAC genes, we decided to focus on E2F2 for two reasons. First, E2F2 but not E2F1 is present in the MMB/dREAM complex. Because DP associates with both E2F1 and E2F2, loss of DP is equivalent to loss of both E2F1 and E2F2 (Frolov et al. 2001). Second, although RBF2 has been reported to have some preference for E2F2 over E2F1, both RBF1 and RBF2 can bind to and repress gene expression via E2F2 (Dimova et al. 2003).

In contrast to the results with Myb and Mip130, the ectopic expression of E2F2 in the posterior compartment of an otherwise wild-type wing imaginal disc was sufficient to repress the expression of Polo-GFP (Fig. 5B, panels a–c). In an *E2F2*-null background, the baseline expression of Polo-GFP appeared to be elevated, and the expression of E2F2 in the posterior compartment caused a nearly complete loss of Polo-GFP expression (Fig. 5B, panels d–f). Similar observations were made with *Mad2*-GFP (Supplemental Fig. S3). The expression of RBF2, a corepressor for E2F2, also decreased the expression of GFP-Polo in otherwise wild-type imaginal discs (Fig. 6d–f). Effects of RBF1 were difficult to assess because of increased cell death caused by its ectopic expression (Fig. 6a–c; Neufeld et al. 1998). Ectopic expression of E2F1 in a wild-type background had no effect on GFP-Polo or GFP-Mad2 levels, even in the presence of ectopic DP (Fig. 6g–i). Together these results imply that E2F2 is a limiting factor in the repression of *Polo* and *Mad2* gene expression in vivo. Furthermore, these results suggest that Myb and E2F2 play adversarial roles in the regulation of *Polo* and *Mad2*. Additional evidence for an adversarial role of Myb and E2F2 in vivo was provided by the observation that the slow growth phenotype of *Myb*-null larvae can be suppressed in *Myb*, *E2F2*/+ mutants (Supplemental Fig. S3). These results are reminiscent of the suppression of the slow growth phenotype of larvae

mutant for *E2F1* in *E2F1*, *E2F2* double mutants (Frolov et al. 2001).

Because MMB/dREAM contains three different DNA-binding proteins (Myb, Mip120, and E2F2) and a putative AT-hook protein (Mip130), we hypothesized that *Polo* and *Mad2* might be direct targets of transcriptional regulation by the complex. To test whether these proteins were present at the promoters of *Polo* and *Mad2*, we turned to a simpler experimental system, the *Drosophila* S2 cell line. We first verified that the regulation of the *Polo* and *Mad2* genes by Myb that we had observed in vivo could be modeled by RNAi depletion in S2 cells (Supplemental Fig. S4). We then performed chromatin immunoprecipitation (ChIP) in S2 cells and found that Myb, Mip130, Mip120, Mip40, and E2F2 all specifically occupied the *Polo* promoter (Fig. 5C,D). Similar observations were made with the *Mad2* and *Rod* promoters (Supplemental Fig. S5). In contrast to *Polo* and the SAC genes above, *BubR1* appears to be indirectly regulated by MMB/dREAM because its promoter was not brought down in similar ChIP assays and its expression was not altered by RNAi depletion of Myb. Taken together, these results are consistent with a role for MMB/dREAM complexes in directly regulating transcription of *Polo*, *Mad2*, and *Rod*.

Variegation of GFP-Polo expression in the absence of Myb and E2F2

In *Myb*, *E2F2* double mutant imaginal wing discs, GFP-Polo again displayed a variegated pattern of expression (Fig. 5A). The OFF state of expression in *Myb*, *E2F2* double mutants was even lower than that observed in *Myb*, *Mip130* double mutants, suggesting that perhaps the loss of Mip130 abolishes the action of some but not all E2F2-RBF. The expression of Myb in the posterior compartment of *Myb*, *E2F2* double mutant imaginal wing discs strongly suppressed GFP-Polo variegation, resulting in a nearly uniform population of cells in the ON state (Fig. 5B, panels g–i).

Discussion

Regulation of mitosis by Myb and E2F2-RBF via the MMB/dREAM complex

We characterized the mitotic abnormalities that occur in the absence of *Myb* by real-time live microscopy of mutant larval neuroblasts. Our results confirmed and extended previous studies of fixed tissues and pointed toward specific candidate genes that are dependent on Myb for their expression in vivo. In particular, we found that Myb and E2F2-RBF act in opposition to directly regulate the expression of *Polo* and a subset of SAC genes in vivo. The E2F-RB axis has been intensively studied as a regulator of gene expression that controls the G1-S transition of the cell cycle in vertebrates and in *Drosophila* (Duronio et al. 1995; Dimova and Dyson 2005). Our results demonstrate that in *Drosophila*, Myb acts in opposition

Figure 5. E2F2 enhances the variegation of GFP-Polo expression. (A) Expression of GFP-Polo in imaginal wing discs from wild-type (wt), from *E2F2*-null, and from *Myb*, *E2F2* double-null genotypes as indicated. (B) Imaginal wing discs from wild-type (panels a–c), *E2F2*-null (panels d–f), and *Myb*, *E2F2* double-null (panels g–i) mutant larvae in which RFP and either *E2F2* or *Myb* were expressed in the posterior compartment via the GAL4-UAS binary system. Each row displays the same imaginal disc using two different markers (first and second columns) and a merged image (third column). (C,D) ChIP of *Polo* promoter and coding region DNA from *Drosophila* S2 cells by the indicated antibodies or a control (IgG). Occupancy values were determined by real-time PCR. Error bars indicate standard deviation of three independent experiments.

to both *E2F2*–RBF and Mip130 to control key regulators of the G2/M and intra-M-phase transitions, including Polo kinase and the SAC. Our results therefore provide a molecular explanation for the mitotic abnormalities that occur in the absence of *Drosophila Myb* (Fung et al. 2002; Manak et al. 2002).

Our observations in *Drosophila* are supported by studies in vertebrate cell culture showing that E2F4, the ver-

tebrate homolog of *Drosophila E2F2*, occupies promoters of G2/M regulators including *MAD2*, which is constitutively activated in *p107*, *p130* double mutant fibroblasts (Ren et al. 2002). Similar observations were made in *Rb* mutant fibroblasts and in cells expressing the adenovirus E1A protein, an inhibitor of all three Rb-related proteins (Hernando et al. 2004). Recently, several laboratories have reported that E2F4 in vertebrate cells is present in

Figure 6. RBF2 represses *Polo* expression in vivo. Imaginal wing discs from larvae in which the RBF1 (a–c), RBF2 and RFP (d–f), or both the E2F1 and DP (g–i) proteins were expressed in the posterior compartment via the GAL4-UAS binary system. Each row displays the same imaginal disc using two different markers (first and second columns) and a merged image (third column). (a–c) GFP-Polo and RBF1 visualized by anti-RBF1 antibody. Compartment-specific GFP-Polo expression was difficult to assess due to increased apoptosis caused by increased levels of RBF1 in the posterior compartment. (d–f) GFP-Polo and RBF2 coexpressed with RFP. Note the decreased expression of GFP-Polo in the posterior compartment expressing elevated levels of RBF2 and RFP. (g–i) GFP-Polo and E2F1 coexpressed with DP and visualized with anti-E2F1 antibody. No significant alteration of GFP-Polo expression was seen in the posterior compartment expressing elevated levels of E2F1 and DP.

MMB/dREAM-like complexes (Korenjak et al. 2004; Litovchick et al. 2007; Pilkinton et al. 2007; Schmit et al. 2007). Our observations that Myb acts in opposition to E2F2–RBF in *Drosophila* by activating *Polo* and SAC gene expression, together with our previous finding that vertebrate B-Myb can complement cell cycle defects in *Drosophila* Myb-null hemocytes, imply a similar role for B-Myb in vertebrates (Davidson et al. 2005).

B-Myb and cancer

The role of B-Myb in oncogenesis has been paradoxical. Elevated levels of B-Myb/MYBL2 expression correlate with poor prognosis in a variety of human cancers (Amatschek et al. 2004; Paik et al. 2004). In contrast, loss-of-function mutants of *Drosophila* Myb and of zebrafish B-Myb increase the frequency of abnormal mitoses and aneuploidy, hallmarks of cancer cells (Fung et al. 2002; Manak et al. 2002; Shepard et al. 2005). Similarly paradoxical observations have been made for *MAD2*, in that loss-of-function mutants cause genomic instability, whereas increased expression correlates with poor prognosis in human cancer. The expectation was that loss-of-function mutants of SAC genes might be a frequent cause of human cancer (Kops et al. 2005). Although such mutations have been reported, they appear to be the exception rather than the rule. Surprisingly, either increased or decreased levels of *MAD2* expression in mice cause genomic instability and cancer (Michel et al. 2001; Sotillo et al. 2007). *Polo* kinase has yet to be analyzed in such detail. However, increased levels of *Polo* expression

do correlate with poor prognosis in human cancers (Takai et al. 2005). Myb proteins have also been reported to regulate the expression of mitotic cyclins in animals and in plants (Ito et al. 2001; Okada et al. 2002; Zhu et al. 2004). The correlation of increased expression of B-Myb with poor prognosis in human cancer is therefore predicted to result from aberrant expression of G2/M regulators including *Polo* and the SAC genes.

Functional domains of Myb and evolution of the Myb-MuvB/dREAM complex

Surprisingly, the highly conserved DNA-binding domain of *Drosophila* Myb was not required for assembly of Myb into MMB/dREAM, for transcriptional regulation in vivo, or for rescue of Myb-null mutants to viability. Two extreme models that might explain these results are that (1) the Myb DNA-binding domain is not required for positively regulating gene expression or cell cycle progression as implied by our results with *MAD2* (Fig. 3, panels d–f); and (2) the DNA-binding domain-deficient mutant acts as a dominant-negative and permits partial rescue but not robust viability, similar to that seen in double mutants of Myb together with *Mip130*, *Mip120*, or *Mip40* (Beall et al. 2004, 2007). The truth probably lies somewhere in between. The complex and varied nature of gene regulation by MMB/dREAM has been demonstrated in a recent genome-wide analysis of alterations in gene expression following RNAi-mediated knockdown of individual proteins in the complex in a hemocytic cell line (Georlette et al. 2007).

Our results did prompt us to further probe the evolutionary origins of Myb and the MMB/dREAM complex. The genome of the nematode *C. elegans* encodes all members of the MMB/dREAM complex excepting Myb (Lipsick 2004). However, simpler eukaryotes including cellular slime molds and protists do contain conserved Myb DNA-binding domains. These results led us to speculate that nematodes had lost the *Myb* gene during evolution. Consistent with this hypothesis, analysis of the recently published genome of the sea anemone *Nematostella*, a member of the oldest eumetazoan phylum (Cnidaria), reveals the presence of a *Myb* gene that encodes a protein homologous to vertebrate B-Myb and *Drosophila* Myb (Supplemental Fig. S6). In addition to the Myb DNA-binding domain, the predicted sea anemone Myb protein includes highly conserved regions of the C terminus that are not present in slime mold and protist Myb proteins. The evolutionary conservation of the C terminus is consistent with our finding that the DNA-binding domain of *Drosophila* Myb is not required for some essential functions of the Myb protein in vivo. Remarkably, the *Nematostella* genome also encodes homologs of Mip130, Mip120, Mip40, E2F, and RBF. These results imply that the MMB/dREAM complex existed in the common ancestor of all eumetazoans and that nematodes have indeed lost Myb during their subsequent evolution.

Epigenetic regulation of gene expression by Myb, Mip130, and E2F2-RBF

Only by studying gene expression at the single-cell level in intact tissues were we able to establish a role for the MMB/dREAM complex in the epigenetic regulation of gene expression. In classical position effect variegation (PEV), the *white-mottled* alleles of Muller were caused by X-ray-induced chromosomal inversions and translocations that brought the normally euchromatic *white* and *Notch* genes adjacent to constitutive heterochromatin (Muller 1930). These and other variegating alleles have been instrumental in understanding the establishment and maintenance of constitutive heterochromatin (Eissenberg and Wallrath 2003). In particular, the isolation of enhancers and suppressors of PEV led to the identification of key epigenetic regulators including an H3K9 methylase [*Su(var)3-9*], the major H3K9 methyl-binding protein HP1 [*Su(var)2-5*], and an H3K4 demethylase [*Su(var)3-3*] that acts upstream of H3K9 methylation (Schotta et al. 2003; Rudolph et al. 2007). The normal function of these genes and the proteins they encode is clearly not the modification of PEV. Nevertheless, their function in the context of these unusual *white* alleles has been extremely informative about their function in normal gene regulation.

By analogy, we believe that our studies of *Polo* variegation using single and double mutants of the MMB/dREAM complex are similarly informative about the functions of these proteins in normal gene regulation. Although Myb scores as an “activator” in that *Polo* expression is extinguished in its absence, Myb is actually

not required for *Polo* expression in the absence of both Myb and Mip130, or of both Myb and E2F2. Remarkably, *Polo* expression in such double mutants displays a variegation that fulfills the current definition of epigenetic phenomena in that (1) genetically identical cells display heritable phenotypic differences; (2) these phenotypes exhibit a switch-like behavior; and (3) these phenotypes are stably maintained through successive cell divisions in the absence of an extrinsic signal (Gottschling 2004). From our results, we infer that Myb is required for the stable maintenance of the *Polo* ON state. Conversely, Mip130 and/or E2F2 are required for the stable maintenance of the *Polo* OFF state (Fig. 7).

We note that the normal state of *Polo* expression in late third instar imaginal wing discs is ON. This state is stably maintained in *Mip130* and in *E2F2* single mutants, consistent with the viability of adult animals of these genotypes (Cayirlioglu et al. 2001; Frolov et al. 2001; Beall et al. 2004). In contrast, *Myb*-null mutants fail to maintain expression of *Polo*, consistent with their larval lethal phenotype (Manak et al. 2002). In this regard, other members of the complex were properly targeted to the *Polo* promoter in the absence of Myb in RNAi-treated S2 cells (data not shown). Although *Myb*, *Mip130* double mutants can survive to adulthood, their viability is severely compromised (Beall et al. 2004). The epigenetic instability of *Polo* expression in double mutant imaginal wing discs is consistent with this impaired viability. Presumably, sufficient cell proliferation occurs during development via those epigenetic clones with *Polo* in the ON state in order to permit survival to adulthood of some *Myb*, *Mip130* double mutant animals. However, the loss of those epigenetic clones with *Polo* in the OFF state, either during development or during renewal of essential adult tissues such as the gut and immune system, results in poor overall viability.

We expect that other genes in addition to *Polo* will display a similar epigenetic regulation by MMB/dREAM. However, the magnitude and direction of such regulation may differ from gene to gene, and from cell type to cell type. In this regard, RNAi knockdown studies in two *Drosophila* hemocyte cell lines have shown that proteins of the MMB/dREAM complex regulate an unexpectedly large number of genes with widely divergent biological functions (Dimova et al. 2003; Georlette et al. 2007). In addition, the MMB/dREAM complex was found to be present proximal to the promoters of approximately one-third of all genes in a single cell line (Georlette et al. 2007).

The variegation of *GFP-Polo* expression observed in *Myb*, *Mip130* and in *Myb*, *E2F2* double mutants does not appear to be classical PEV for two reasons. First, we found that the loss of *Myb* itself neither enhances nor suppresses classical PEV (Supplemental Fig. 7S). Secondly, two independent insertions of the same *GFP-Polo* transgene gave similar patterns of expression in *Myb*, *Mip130* double mutant larvae. These euchromatic insertion sites are not consistent with classical PEV, which occurs when normally euchromatic genes are placed adjacent to constitutive heterochromatin. Interestingly, Rb

Figure 7. Epigenetic regulation of GFP-Polo by Myb and E2F2-RBF. In wild-type imaginal wing discs, the entire MMB/dREAM complex is present at the *Polo* promoter, the gene is ON, and all cells are GFP-positive. In the *E2F2*-null mutant, DP and RBF proteins are also absent from the *Polo* promoter, but the gene remains ON, and all cells are GFP-positive. In the *Myb*-null mutant, the remainder of the complex is present at the *Polo* promoter, the gene is OFF, and almost all cells are GFP-negative. In the *Myb*, *E2F2* double mutant, the gene switches between epigenetically stable ON and OFF states. The diagram implies that additional corepressors are dependent on RBF for their recruitment, possibly the HDAC1 or L(3)MBT subunits of the larger MMB complex (Lewis et al. 2004). A similar model can be drawn to explain the data for *Myb* and *Mip130* single and double mutants (not shown).

family proteins have been proposed to function in epigenetic regulation of gene expression (Nielsen et al. 2001; Vandel et al. 2001; Gonzalo et al. 2005; Kotake et al. 2007). However, until now direct evidence for such regulation *in vivo* had been lacking.

Our own results together with those cited above suggest a role for the MMB/dREAM complex in genome-wide epigenetic control, perhaps via the regulation of the facultative heterochromatin that is widely dispersed among bulk euchromatin. We speculate that the specific loss of *Myb* during the evolution of nematodes may be directly related to the absence of heterochromatin in this organism. In addition, our results support the hypothesis that the mutation of some oncogenes and tumor suppressor genes, including *Myb*, *Myc*, *E2Fs*, and *Rb* family members, may cause cancer by a global disruption of epigenetic homeostasis rather than by activating or repressing a small number of critical target genes (Lipsick 2004; Gonzalo et al. 2005; Knoepfler et al. 2006).

Materials and methods

Fly strains and genetics

Flies were raised on cornmeal-molasses-agar medium at 25°C (Greenspan 1997). Meiotic recombination, UAS-GAL4 expression, and FLP-FRT mosaic analysis were performed using standard methods (Greenspan 1997). The genotypes used in this work are described in detail in the Supplemental Material. Transgenic flies were generated by standard methods using the plasmids described in the Supplemental Material.

Time-lapse analysis of neuroblast cell division in larval brain

Chromosomes were visualized using mRFP-histone H2avD under control of its native promoter (Pandey et al. 2005). Mitotic spindles and centrosomes were visualized using either the G147 gene trap expressing a GFP-tagged microtubule-associated protein (Morin et al. 2001), or GFP-TACC under control of *Ubiquitin* promoter (Barros et al. 2005). Details of live microscopy are provided in the Supplemental Material.

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Immunocytochemistry

Experiments were performed as described, except that in some cases TOTO-3 (Molecular Probes) instead of ethidium bromide was used to visualize DNA (Manak et al. 2007). Images were taken using a PCM2000 laser scanning confocal microscope (Nikon) and processed using Adobe Photoshop with no γ -correction. Primary antibodies and dilutions used for immunostaining were as follows: rabbit and mouse anti-PH3 antibodies at 1:500 (Upstate Biotechnology), rabbit anti-Mip130 at 1:500 (from L. Beall), rabbit anti-RFP at 1:500 (from S. Heidmann), mouse anti-RBF1 at 1:500, and rabbit anti-E2F1 at 1:500 (from N. Dyson).

RNA fluorescence in situ hybridization (FISH)

cDNA fragments (~800 base pairs [bp]) of each target gene were amplified by PCR and subcloned into the pCR4-TOPO vector (Invitrogen). Linearized plasmids were used as templates for in vitro transcription. RNA probes were prepared by T7 or T3 RNA polymerase per the manufacturer's instructions (Roche). The *Myb* probe was labeled with biotin, and probes for various other genes were labeled with digoxigenin. Two-color RNA FISH was performed simultaneously with *Myb* and other gene probes as described (Wulbeck and Helfrich-Forster 2007). After hybridization, FISH signals were developed by sequential tyramide detection according to the manufacturer's protocols (TSA-Plus Cyanine 3/Fluorescein System; PerkinElmer LAS, Inc.).

Cell culture and transfection

Drosophila S2 cells were grown at 25°C in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum. Coding sequences for full-length *Myb* and *Myb-Cterm* were amplified by PCR with upstream in-frame insertion of three tandem Flag epitope tags, then subcloned into the Ract-HAdh plasmid (*Drosophila* Genomic Resource Center, Bloomington, IN) to allow the expression under control of the *actin 5C* promoter. S2 cells were diluted to a final concentration of 1×10^6 cells per milliliter, and 30 μ g of Ract-HAdh-Flag-Myb, Ract-HAdh-Flag-Myb-Cterm, or empty vector DNA were transfected using FuGene6 reagent (Roche). Cells were harvested 48 h after transfection for immunoprecipitation experiments.

Immunoprecipitation and Western blots

Nuclear extracts were prepared from the transfected S2 cells 48 h after transfection. Immunoprecipitation, SDS-PAGE, and Western blotting were performed as described in the Supplemental Material.

ChIP

ChIP was performed as described (O'Geen et al. 2006). For each ChIP experiment, 3×10^7 S2 cells were used. The following antibodies were used for ChIP assays: affinity-purified anti-Myb, anti-Mip130, anti-Mip120, and anti-Mip40; unpurified anti-dE2F2 rabbit serum; and rabbit anti-histone H3 (Abcam). Pre-immune rabbit serum was used as a negative control. Gene-specific PCR primers were designed to amplify the promoter and ORF sequences of indicated target genes; these primer sequences are available upon request. Real-time PCR was performed in triplicate on a PRISM 7700 Sequence Detection System using Power SYBR Green PCR Master Mix (Applied Biosystems).

RNAi and quantitative RT-PCR

Gene-specific PCR products (~700 bp) flanked by T7 polymerase-binding sites were used as templates for in vitro transcrip-

tion reactions. dsRNA was transfected into *Drosophila* S2 cells as described in the Supplemental Material.

For quantitative real-time RT-PCR, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen), and cDNA was synthesized using the SuperScript First-Strand Synthesis kit (Invitrogen). RT-PCR was then performed in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems). Gene-specific primers for real-time RT-PCR were designed with Primer Express software (Applied Biosystem); these primer sequences are available upon request.

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Hong Wen, Laura Andrejka, Jonathan Ashton, et al.

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