Genomic profiling and expression studies reveal both positive and negative activities for the Drosophila Myb–MuvB/dREAM complex in proliferating cells

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Myb–MuvB (MMB)/dREAM is a nine-subunit complex first described in Drosophila as a repressor of transcription, dependent on E2F2 and the RBPs. Myb, an integral member of MMB, curiously plays no role in the silencing of the test genes previously analyzed. Moreover, Myb plays an activating role in DNA replication in Drosophila egg chamber follicle cells. The essential functions for Myb are executed as part of MMB. This duality of function lead to the hypothesis that MMB, which contains both known activator and repressor proteins, might function as part of a switching mechanism that is dependent on DNA sites and developmental context. Here, we used proliferating Drosophila Kc tissue culture cells to explore both the network of genes regulated by MMB (employing RNA interference and microarray expression analysis) and the genomic locations of MMB following chromatin immunoprecipitation (ChIP) and tiling array analysis. MMB occupied 3538 chromosomal sites and was promoter-proximal to 32% of Drosophila genes. MMB contains multiple DNA-binding factors, and the data highlighted the combinatorial way by which the complex was targeted and utilized for regulation. Interestingly, only a subset of chromatin-bound complexes repressed genes normally expressed in a wide range of developmental pathways. At many of these sites, E2F2 was critical for repression, whereas at other nonoverlapping sites, Myb was critical for repression. We also found sites where MMB was a positive regulator of transcript levels that included genes required for mitotic functions (G2/M), which may explain some of the chromosome instability phenotypes attributed to loss of Myb function in myb mutants.

Keywords: Drosophila; Myb-MuvB/dREAM; transcription

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Metazoan chromosomes contain cis-acting elements called enhancers and silencers that regulate many nuclear functions of the cell including gene expression and DNA replication. Enhancers and silencers serve as binding sites for proteins that control the state of chromatin proximal to transcription start sites (TSS) or replication start sites by controlling the ability of the so-called core or basal factors to initiate RNA or DNA synthesis (Carey and Smale 2000; Levine and Tjian 2003). These DNA modules thus serve as sites for processing information that define the epigenetic state of the cell [Ptashne 2007].

For purposes of classification, there are many examples of “modular enhancers” where the cis-acting elements are composed of clustered submotifs with flexible spacing between the DNA-binding sites that recruit different DNA-binding factors combinatorially. A model for such enhancer elements is the eve stripe 2 enhancer in Drosophila [Small et al. 1991]. “Enhanceosomes” are another distinct type of enhancer element, where again, different DNA-binding factors are recruited to DNA. However, the exact spacing and geometry of the motifs are critical and when bound by proteins, allow for the creation of a higher-order nucleoprotein structure that is...
critical for enhancer function. The interferon-β response element provides the paradigm for this type of enhancer where spatial differences of a single base pair can affect enhancer function [Thanos and Maniatis 1995a,b]. For each of these enhancer types, the protein assembly may occur cooperatively but the path to building the functional site begins with individual DNA-binding factors. The discovery of complexes containing multiple site-specific DNA-binding proteins such as Drosophila Myb–MuvB [MMB]/dREAM and tMAC [Korenjak et al. 2004; Lewis et al. 2004; Beall et al. 2007] suggest another model for assembly at enhancers and sinkers. MMB contains DNA-binding proteins with both activating and repressing activities. It has been postulated that binding sites within sinkers and enhancers might allow for a switch in function in a developmental pathway when bound by this complex [Beall et al. 2004, 2007; Lewis et al. 2004].

MMB is ~700 kDa, and contains a unique ensemble of nine proteins, of which five are capable of binding to DNA: Myb, E2F2/DP, and Mip120 are site-specific DNA-binding factors, and a fifth protein, Mip130, contains an A–T hook domain capable of binding to AT-rich DNA. Although E2F2 and Myb have been widely studied individually, nothing has yet been explored as to how these DNA-binding proteins behave in an ensemble. Perhaps the more biologically relevant question is: How do the different site-specific DNA-binding proteins choose where to bind among the potentially large number of binding sites within the genome of a cell? It is likely that all or some combination of the DNA-binding activities may participate in MMB targeting to specific DNA sites. The other MMB factors—RBF1 or RBF2, Caf1/p55, Mip40, and Lin-52—are not known as DNA-binding proteins, but may contribute indirectly to DNA targeting through association with histones.

The Drosophila Myb protein can function as a transcriptional activator [Hou et al. 1997; Jackson et al. 2001; Okada et al. 2002; Lee and Orr-Weaver 2003]. In addition, roles for Myb in the G2/M transition and chromosome stability have been inferred from genetic studies of myb mutants [Katzen et al. 1985, 1998; Fung et al. 2002; Manak et al. 2002, 2007]. The extent to which these roles for Myb are directly dependent on altered gene expression in the absence of Myb have not been resolved, in part, because Myb-dependent target genes have not been clearly identified. Myb is also required for DNA-mediated gene amplification in Drosophila follicle cells where MMB functions as an activator through binding to the ACE-3 replication enhancer [Beall et al. 2002]. MMB can also repress replication at other genomic sites, perhaps both directly and transcriptionally through limiting the levels of transcripts encoding for replication factors [Cayirlioglu et al. 2003; Beall et al. 2004, 2007]. As all of the Myb protein is associated with MMB and the steady-state level (but not mRNA level) of Myb is dependent on Mip40, Mip120, and Mip130 [Beall et al. 2004; Korenjak et al. 2004; Lewis et al. 2004], a systematic study of the role for Myb in gene expression in terms of MMB association is needed.

E2F2 is also a stoichiometric component of MMB and E2F2 steady-state protein levels are similarly dependent on Mip120 and Mip130. The role of E2F2 in gene repression has been studied for the most part only in the context of association with the RBFs. Dimova et al. [2003] used RNA interference (RNAi) to deplete E2F2 and identified the set of genes requiring E2F2 for repression in Drosophila S2 cells. With the discovery of MMB/dREAM, a number of genes in this list were chosen to assess the codependence for repression with the additional MMB factors. Initially, we and others showed that E2F2-dependent gene repression requires Mip120 and Mip130 but not Myb, even though Myb is found at these target gene promoters [Korenjak et al. 2004; Lewis et al. 2004]. Importantly, the present literature defines MMB/dREAM solely as a complex required for gene repression. We proposed that Myb is a silent partner for repression at these gene promoters, and might be involved in derepression or activation of these genes at a later point in development [Beall et al. 2004; Lewis et al. 2004].

myb loss is lethal in Drosophila, but such lethality is suppressed by loss of mip130, mip120, or mip40 [Beall et al. 2004, 2007]. Furthermore, loss of mip40 or mip130 results in genome-wide DNA replication in amplification-stage follicle cell nuclei, establishing a repressive function for MMB in DNA replication [Beall et al. 2004, 2007]. If one of the functions of Myb was to help induce genes already repressed by MBB, loss of Myb might be lethal due to the inability to derepress an essential gene or set of genes. When both the repressing and activating functions of MMB are removed, severe phenotypes can result such as in the case of myb mip130 double mutants, which have shortened life spans [Beall et al. 2004]. This is perhaps due to the inappropriate expression of many genes, but is less severe than loss of myb alone. One of the predictions of this model is that removal of Myb results in a partial MMB complex that is still targeted to a few vital gene(s). Furthermore, Myb would have an essential role in either derepression of MMB and/or transcriptional activation of these vital genes. We showed recently that Myb targeting to polytene chromosomes is dependent on Mip120 and Mip130. Consistent with our model, Mip120 and Mip130 are still found on polytene chromosomes in myb mutant animals where some or all of the bound Mips may assume hypermorphically repressive activity [Beall et al. 2007]. A more detailed view of the network of genes regulated by MMB and the sites within the genome bound by MBB are required to test this model more stringently.

All of the points raised thus far concern the mechanism[s] of targeting MMB to DNA, and highlight the importance of determining the actual sequences bound by MBB at such a resolution to enable further genetic testing of the cis-element requirements for MBB function. Moreover, deciphering the set[s] of genes regulated by MBB may help explain some of the observed phenotypes in animals mutant for members of MMB. To address these points, we targeted each of the MMB proteins using RNAi in Drosophila Kc tissue culture cells to assess the transcripts affected by removal of specific MMB
subunits using microarray analysis. In addition, we performed a genome-wide analysis of the location of Myb, E2F2, Mip130, Mip120, and Lin-52 in the same cell line. We found that MMB was bound to thousands of sites throughout the entire *Drosophila* genome. Interestingly, MMB was bound to far more sites than would have been predicted by the transcriptional profiling studies. Our data also indicated that targeting of MMB to DNA relied either on E2F2 or Myb, but generally not both. Unexpectedly, previous observations, we found that Myb was involved in silencing at many sites where E2F2 was bound but not required for repression. Finally, a percentage of genes affected by the RNAi ablations required the MMB factors for normal levels of expression and had promoter-proximal binding sites for MMB. These data provide evidence that MMB can also participate in transcriptional activation.

**Results**

**Long-term MMB depletion results in decreased growth rates and genome instability**

To analyze the network of genes regulated by MMB, we first measured the kinetics of protein loss after targeting individual proteins of the complex by RNAi treatment. This kinetic analysis allowed us to determine the earliest time point for analyzing direct transcriptional effects of MMB member loss, while avoiding potential downstream secondary effects resulting from extended MMB member depletion. Loss of core factors can influence the accumulation of other MMB members in other cell lines and in vivo (Beall et al. 2004), and such data was needed for Kc cells.

RNAi targeting individual components of the complex (i.e., Mip40, Caf1/p55, Myb, Mip120, Mip130, E2F2, RBF2, and Lin-52) was achieved by adding double-stranded RNA (dsRNA) to *Drosophila* Kc cells. The efficiency of MMB subunit depletion was measured by immunoblot analysis [see Fig. 1A,B, Supplementary Fig. 1 for the quantification of the immunoblot data]. After 4 d of treatment, levels of individually targeted MMB proteins were at least 10-fold reduced, while no significant cell cycle progression defects were observed during this period [Fig. 1B]. As reported for other cell types, Myb was the most affected by the loss of other complex members; its accumulation was significantly reduced in Mip130-, Mip120-, Caf1/p55-, and Mip40-depleted cells [Fig. 1A; Supplementary Fig. 1]. Conversely, loss of Myb had no effect on the steady-state levels of the other factors. We also noticed that the accumulation of the transcriptional repressors RBF2 and E2F2 was dependent on the presence of other MMB members. In contrast, depletion of E2F2 only affected the abundance of RBF2. After RNAi targeting, Lin-52 levels were decreased with similar kinetics as the other MMB members, but it uniquely maintained a wild-type steady-state level in the absence of any other single MMB factor [data not shown]. Targeting of Caf1/p55, Mip120, and Mip130 led to the most significant decrease in the accumulation of other MMB members, and we refer to these three proteins as the “core” essential for the complex integrity. A schematic diagram illustrating the interdependence of each protein’s steady-state levels after RNAi treatment is summarized in Figure 1A. As the Caf1/p55 protein is also a component of other complexes active in these cells and involved in chromatin functions, we did not include this protein in our network analysis.

While cells treated with dsRNA targeting various MMB members could be maintained for serial passages, over time they proliferated more slowly than did the control samples [Fig. 1B]. Analysis of these cells at late time points using FACS analysis revealed that loss of Myb resulted in an increase in the proportion of cells in G2/M while depletion of Mip120 and Mip130 caused an accumulation of the cells in G1 [Supplementary Fig. 2]. The accumulated growth defects observed in the absence of MMB were also associated with chromosomal abnormalities. To document such abnormalities in more detail, cells were treated with demecolcine after 14 d of continuous RNAi treatment and analyzed for aberrant metaphase chromosomes. We found that depletion of Mip120, Mip130, or Myb led to genome instability [Fig. 1C]. Three different chromosome classes were monitored: (1) wild type [tetraploid female karyotype] [Echaller 1997]; [2] spreads where chromosomes exhibited impaired sister chromatid cohesion (ISSC), chromosome fragmentation [F], and/or condensation defects [CD]; and [3] polyploidy [P] [Fig. 1C]. For example, Myb-depleted cells exhibited up to a fourfold increase in the number of ISCC, F, and CD karyotypes, compared with control cells. These results corroborated previous in vivo data demonstrating that Myb is involved in genome stability [Katzen et al. 1998; Fung et al. 2002; Manak et al. 2002]. It is interesting that *myb*-mutant larval brains show massive polyploidy (Manak et al. 2002), yet the Kc cells treated with dsRNA targeting Myb showed no such phenotype [Fig. 1C]. Based on data presented below, we anticipated that cell type-specific chromosomal defects would be observed when MMB members were depleted following RNAi. We came to this view in light of the extensive network of genes regulated by the complex in Kc cells, the rather large number of MMB-binding sites found in the genome, and the recent finding that individual MMB members have different roles in different tissues [Beall et al. 2007]. In conclusion, we chose to probe the gene expression network regulated by MMB after 93 h of RNAi treatment—a point at which Kc cells have significantly reduced target protein levels and very little overt cell cycle or genome stability phenotypes.

**MMB factors repress and activate transcription of a wide range of developmentally regulated and cell-autonomous genes**

We combined the use of Affymetrix GeneChip Oligonucleotide arrays to monitor changes in gene expression following removal of the complex in *Drosophila* Kc cells, with Affymetrix GeneChip *Drosophila* Tiling arrays to determine MMB distribution across the entire genome.
We performed RNAi against all individual MMB members except for Caf1/p55 and DP. It has to be noted that RBF1 and RBF2 were targeted as a pair (named RBFs) because of the apparent functional redundancy among the RBF proteins in tissue culture cells [Dimova et al. 2003]. We also targeted the Drosophila lethal (3) malignant brain tumor protein, L(3)MBT, as L(3)MBT is sub-stoichiometrically associated with MMB and required for repression of a number of genes targeted by E2F2 [Lewis et al. 2004]. Association of L(3)MBT with MMB...
was confirmed by coimmunoprecipitation [Supplementary Fig. 3A]). Although histones were not among the proteins copurifying with MMB, we suggested that repression is aided, in part, by the contacts made between L(3)MBT and chromatin-bound histones [Lewis et al. 2004]. In pull-down experiments, purified MBP-MBT repeats derived from L(3)MBT bound to core Drosophila histones, and specifically interacted with intact H3 and H4 tetramers [Supplementary Fig. 3B–D]. Interestingly, Trojer et al. (2007) reported that a human homolog of L(3)MBT, L3MBTL1, is in a complex with core histones, HP1γ and Rb. Most significantly, they showed that L3MBTL1 can compact nucleosomes in a manner that is dependent on mono- or dimethylation of H4 Lys 20. The data shown in Supplementary Figure 3 imply that under our conditions, L(3)MBT showed affinity for essentially all of the H3–H4 tetramers present in 0- to 12-h Drosophila embryos and that specific targeting to defined genetic elements [see below] may be regulated by the site-specific binding of MMB.

We found opposing effects on the mRNA levels of genes following RNAi targeting of MMB members: In the absence of MMB members, many transcripts were up-regulated, extending the network of genes that MMB could repress, while others were down-regulated, showing that MMB played a role in activation. The statistical methods and experimental protocols used for analyzing the microarray data are described in the Materials and Methods. Given the large number of genes affected and the complexity of the data, we created classes wherein all genes in a given class required a common set of MMB factors. This approach is systematic but the choice of class definition was arbitrary. Venn diagrams [Fig. 2] and heat maps [Supplementary Figs. 4–7] were generated to help visualize the observed changes in gene expression when MMB members were removed. A complete list of the genes identified by our analysis as being regulated by MMB is provided in Supplementary Table 1 along with the relevant statistical data for each gene.

For brevity, we will describe the data for four classes termed A–D [Fig. 2; see below]. These classes contain the core MMB factors and known DNA-binding proteins [i.e., Myb, Mip120, Mip130, and E2F2]. Each of these classes [A–D] could be further subdivided according to the requirement for additional MMB members for class member expression [RBFs, Mip40, Lin-52, and L(3)MBT] [Fig. 2, pie charts]. For example, Mip40 and L(3)MBT were required as corepressors for a significant number of genes [61%, 76%, and 51% for Mip40, and 66%, 79%, and 60% for L(3)MBT for Classes A, B, and C, respectively] whereas Lin-52 was important for corepressing many Class A and B genes, but not for many Class C genes [56%, 66%, and 17% in Classes A, B, and C, respectively]. Interestingly, we found that a fraction of the genes in the two classes that did not require E2F2 for repression still required the RBFs for repression [19% of Class C genes and 6% of the Class B genes], supporting the finding that Drosophila RBF2 can function independently of E2F2 [Stevaux et al. 2005]. Based solely on sequence comparison, Mip130 may contain a domain for direct interaction with RBFs [Beall et al. 2007] and may be responsible for RBFs association in MMB, in addition to E2F2.

The results of the gene expression microarray data were confirmed using Northern blot [Fig. 3] and quantitative PCR (qPCR) [data not shown] for ~20 genes representing each of the classes, A–D. We did not find any false positives from these analyses. For the Class A genes, elimination of E2F2, the RBFs, Mip120, or Mip130, but not Myb, led to dramatically increased transcript levels [Fig. 3A]. Class A genes correspond to the previously described set of target genes discovered during the initial investigation of the transcriptional activities of MMB/dREAM, where Myb was not a critical component for repression [Korenjak et al. 2004; Lewis et al. 2004]. For the Class B genes, Myb was needed for repression while removal of E2F2 or the RBFs had no effect on target gene expression [Fig. 3B]. For the Class C genes, Mip120 and Mip130 without Myb or E2F2/RBFs were required for target gene repression [Fig. 3C]. Finally, for the Class D genes, Myb, Mip120, and Mip130 were required for target gene activation because gene expression was decreased in their absence [Fig. 3D]. The Northern blot data and qPCR also confirmed the requirements of Mip40, Lin-52, and L(3)MBT for MMB gene repression and activation [Fig. 3A–D]. From these data, it is clear that MMB can repress or activate a number of target genes in Kc cells, and that each member’s contribution to MMB function is not coordinate.

The proteins encoded by the Class A genes are involved in diverse processes such as oogenesis [micr, SnpE, vasa], courtship behavior [qtc], and development [medsoderm: Myo31DF, how, larval/pupal: yellow-f2] [see Supplementary Table 1 for details]. When comparing the list of the Class A genes to a list of known E2F2/RBF targets in S2 cells [Dimova et al. 2003], we found a 45% overlap between the two sets [see Supplementary Table 1]. As for Class A, Class B genes encode proteins involved in diverse specialized programs such as germ cell development [zpg], cell polarity [crb], and organization of actin filaments [Arp3cB]. The 112 repressed transcripts within Class C are involved in important tissue and cell type-specific processes such as spermatogenesis [achi, vis, and nos], imaginal disc development [ldgf1], wing vein morphogenesis [tok], and RNAi [piwi] [Fig. 2A; Supplementary Table 1].

Similar to the wide range of gene functions requiring MMB for repression, the 49 Class D genes requiring MMB for activation are implicated in many specialized developmental programs [e.g., sta1, sle, squ, and the FGF ligand bnl] [Supplementary Table 1]. However, a significant fraction of these genes (23%) encode proteins involved in the G2/M transition [Table 1]. For instance, Klp61F, CENP-meta, Klp3A, and Pavarotti belong to a Kinesin superfamily that is thought to function as a molecular motor performing distinct roles during mitosis [Goshima and Vale 2003]. Additional Class D genes contain a series of genes involved in chromosome condensation [jil], sister chromatid segregation [jil and pim], and completion of cytokinesis [jil, scrA, and feo] [Table
Interestingly, we found additional genes with known mitosis functions within the classes M, N, P, Q, and R (Table 1). Notable in this group are the genes Bub3, mei-S332, Klp10A, mad2, and polo, all involved in chromosome dynamics during mitosis. Although the expression microarray data indicate that only one or two of the
MBB, we first preprocessed the data using background subtraction (Manak et al. 2006). To identify DNA regions bound by MMB, we used background subtraction to isolate DNA sequences bound by the MMB complex. This approach allowed us to identify DNA sequences bound by the MMB complex.

To identify DNA regions bound by MMB, we used a method called ChIP-seq. This method involves using antibodies against the MMB complex to immunoprecipitate DNA sequences bound by the complex. After immunoprecipitation, the DNA sequences are then sequenced to identify the exact DNA sequences bound by the MMB complex.

To determine the locations of the MMB-bound sites, we performed a ChIP-seq analysis. This analysis showed that the MMB complex is bound to thousands of sites within the Drosophila genome. The ChIP-seq results showed that the entire set of MMB factors analyzed were colocalized to a total of 3538 sites located throughout the entire genome. A view of the distribution of binding sites along the X chromosome was a striking demonstration of this point (Fig. 4A).

We noted that the binding signals within the regions bound by all measured MMB members were not homogeneous. We arbitarily defined a smoothed log2 ratio \( lr \) of the ChIP signal/input of >2 as a “strong” signal and a \( lr \) of <0.5 as “below detection.” A signal between 0.5 and 2.0 was thus defined as “weak.” Based on \( lr \) values for each MMB member analyzed, we created subfamilies (Fig. 4C).

A complete graphical summary and table of the complexity for MMB member colocalization are provided in Figure 5. Consistent with the very large number of binding sites and the many different functions of genes affected by MMB removal, we found no single family of genes that were located nearby the proximal binding sites of MMB. However, we were struck by the finding that many of the genes proximal to the MMB-binding sites showed no change in transcript levels following MMB targeting by RNAi. For example, a role for MMB in the regulation of genes important for S-phase progression in Kc cells was not supported by our microarray data (Class A→D genes).

Nevertheless, we found a series of promoters of S-phase genes such as chif (Dbf4), and the ORC and the MCM complex subunits that were bound by MMB (Supplementary Table 2). Interestingly, in follicle cells, these S-phase genes are actively repressed by E2F2 and RBF1 (Cayirlioğlu et al. 2003). Cross-referencing the previously described transcriptional microarray data following RNAi and the actual MMB-binding sites as determined by ChIP–chip, we found that between ~45% to ~80% of the classes A→R were likely directly regulated by promoter-proximal MMB (Supplementary Tables 2, 3). For example, we found that MMB was bound proximal to the TSS of 72%, 69%, 63%, and 82% of Class A, B, C, and D genes, respectively. Substantiating a direct role for the complex in activating the expression of genes important for the G2/M transition, all five of the MMB proteins were detected at promoters for 28 of the 34 genes listed in Table 1.

Interestingly, we noticed correlations between the ChIP–chip signal strength and the requirement for either

![Figure 3](https://example.com/image-url)
Table 1. *List of MMB-regulated genes involved in cytokinesis and chromosome maintenance*

<table>
<thead>
<tr>
<th>Class</th>
<th>Symbol</th>
<th>Name</th>
<th>Human homolog</th>
<th>Northern/qRT-PCR</th>
<th>Function(s)</th>
<th>Estimate for peak binding location</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>cmet</td>
<td>CENP-meta</td>
<td>CENPE</td>
<td>ND^a</td>
<td>Metaphase chromosome alignment</td>
<td>−358 to −797</td>
</tr>
<tr>
<td>D</td>
<td>CycB</td>
<td>Cyclin B</td>
<td>CCNB1</td>
<td>ClassD</td>
<td>G2/M transition of mitotic cell cycle, cytokinesis</td>
<td>−286 to 175</td>
</tr>
<tr>
<td>D</td>
<td>feo</td>
<td>fascetto</td>
<td>PRC1</td>
<td>ND^a</td>
<td>Cytokinesis, mitotic spindle stabilization</td>
<td>−460 to −748</td>
</tr>
<tr>
<td>D</td>
<td>ial</td>
<td>Ipl-aurora-like kinase</td>
<td>AURKC</td>
<td>ND^a</td>
<td>Cytokinesis, mitotic sister chromatid segregation, mitotic chromosome condensation</td>
<td>−257 to −397</td>
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<tr>
<td>D</td>
<td>Klp3A</td>
<td>Kinesin-like protein at 3A</td>
<td>KIF4</td>
<td>ND^a</td>
<td>Metaphase chromosome alignment</td>
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<td>D</td>
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<td>Kinesin-like protein at 61F</td>
<td>KIF11</td>
<td>ND^a</td>
<td>Proper bipolar spindle assembly</td>
<td>NO^b</td>
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<tr>
<td>D</td>
<td>Klp67A</td>
<td>Kinesin-like protein at 67A</td>
<td>KIP3</td>
<td>ClassD</td>
<td>Spindle assembly, cytokinesis</td>
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<td>pav</td>
<td>pavarotti</td>
<td>KIF23</td>
<td>ClassD</td>
<td>Formation and maintenance of the central spindle, cytokinesis</td>
<td>−128 to −160</td>
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<tr>
<td>D</td>
<td>RacGAP50C</td>
<td>Rac GTPase-activating protein 1</td>
<td>RACGAP1</td>
<td>ClassD</td>
<td>Formation and maintenance of the central spindle, cytokinesis</td>
<td>−57 to 393</td>
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<td>scra</td>
<td>scraps</td>
<td>ANLN</td>
<td>ND^a</td>
<td>Cytokinesis</td>
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<tr>
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<td>ACTL6B</td>
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<td>Cytokinesis</td>
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<tr>
<td>M</td>
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<td>Bub3</td>
<td>BUB3</td>
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<tr>
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<td>karst</td>
<td>SPTBN5</td>
<td>ND^a</td>
<td>Mitotic spindle checkpoint</td>
<td>NO^b</td>
</tr>
<tr>
<td>M</td>
<td>mei-S332</td>
<td>Meiotic from via Salaria 332 (Shugoshin)</td>
<td>Sgo1/2</td>
<td>ND^a</td>
<td>Sister chromatid cohesion</td>
<td>−598 to −749</td>
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<tr>
<td>M</td>
<td>ncd</td>
<td>Non-claret disjunctional</td>
<td>KIFC1</td>
<td>ND^a</td>
<td>Mitotic spindle elongation</td>
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<tr>
<td>M</td>
<td>png</td>
<td>pan gu</td>
<td>KIFC1</td>
<td>ND^a</td>
<td>Regulation of progression through mitotic cell cycle</td>
<td>NO^b</td>
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<td>Rpn9 rhomboid</td>
<td>PSMD13</td>
<td>ND^a</td>
<td>Regulation of exit from mitosis</td>
<td>221 to 572</td>
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<td>M</td>
<td>rho</td>
<td>rhomboid</td>
<td>ECT2</td>
<td>ND^a</td>
<td>Cytokinesis</td>
<td>NO^b</td>
</tr>
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<td>N</td>
<td>barr</td>
<td>barren</td>
<td>BRRN1</td>
<td>ND^a</td>
<td>Mitotic sister chromatid segregation</td>
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<td>Cap-G</td>
<td>Cap-G</td>
<td>HCAP-G</td>
<td>ND^a</td>
<td>Mitotic sister chromatid segregation, cytokinesis</td>
<td>25 to 307</td>
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<td>N</td>
<td>cnn</td>
<td>centrosomin</td>
<td>CDK5RAP2</td>
<td>ND^a</td>
<td>Assembly of mitotic centrosomes</td>
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<td>RASA3</td>
<td>ND^a</td>
<td>Sister chromatid cohesion</td>
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<td>Dgt2</td>
<td>KIF2</td>
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<td>Localization of γ-tubulin to the centrosome</td>
<td>NO^b</td>
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<td>aurora</td>
<td>AURKA</td>
<td>ND^a</td>
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<tr>
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<td>mad2</td>
<td>mad2</td>
<td>MAD2L1</td>
<td>ND^a</td>
<td>Mitotic spindle checkpoint, chromosome segregation</td>
<td>−75 to −729</td>
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*continued on next page*
E2F2 or Myb for target gene regulation. For all of the Class A genes that required E2F2 but not Myb for repression, the Myb ChIP-chip signal was weaker than that of the other MMB members [Fig. 6A, top panel, Supplementary Fig. 9A]. The distribution curves shown in Supplementary Figure 9A show the enrichment for each bound protein proximal to a TSS for each of the MMB factors analyzed via the ChIP tiling study, and we found that the E2F2 enrichment was approximately threefold stronger than the Myb signal. Conversely, the E2F2 signal was approximately threefold lower than the Myb signal in Class B and D genes where Myb was required for either repression or expression [Supplementary Fig. 9B,D, Fig. 6B,D, top panel, respectively]. In Class C, where neither E2F2 nor Myb were required for repression, the enrichment for E2F2 and Myb was similar [Fig. 6C, top panel, Supplementary Fig. 9C]. Such binding biases were not reported previously because of the limited number of target sites analyzed. Prior to this study, we did not know that Myb was involved in gene repression through MMB or that E2F2 was located proximal to active genes. We performed qPCR at a few Class A–D promoters and confirmed the ChIP–chip data and more accurately measured the MMB enrichment at these sites [Fig. 6A–D, bottom panels]. Consistent with the ChIP–chip signals, we found that at Class A promoters, there was a reduced Myb signal and similarly for E2F2 at Class B and D promoters. For both Myb and E2F2, the qPCR signals were fivefold to 10-fold above background demonstrating that Myb and E2F2 were still present at these promoters as part of MMB.

**E2F2 and Myb behave as mutually exclusive targeting factors for MMB**

The data described thus far suggest that E2F2 might be necessary but perhaps not sufficient for targeting MMB to Class A genes and that reciprocally Myb might be necessary but not sufficient for targeting MMB to Class B genes. In addition, E2F2 (for Class A) or Myb (for Class B) may have other essential roles for gene repression such as the recruitment of corepressors. To determine which MMB factors were required for targeting other MMB members to DNA, we used ChIP coupled with qPCR to analyze MMB binding to the promoter-proximal regions of two genes: qtc from Class A and CG31100 from Class B (Fig. 7). For each gene, we chose a series of primers to scan the promoter-proximal DNA to independently map the MMB-binding sites. For both genes, we found a peak for MMB binding that agreed well with the ChIP–chip data. DNase-1 footprinting further identified the specific bases bound by MMB for each gene [data not shown]. Removal of E2F2 but not Myb led to a concomitant decrease in binding of Mip120 and Mip130 [and presumably MMB] to the qtc promoter (Fig. 7B, left panel) whereas removal of Myb but not E2F2 affected targeting of the complex to the CG31100 promoter (Fig. 7B, right panel). We detected a small but reproducible increase of the Myb enrichment upon loss of E2F2 at the qtc promoter and vice versa for E2F2 upon Myb ablation at the CG31100 promoter. Such phenomenon may indicate conformational changes in the complex upon loss of one or another factor, allowing for more efficient cross-linking of the remaining proteins to the DNA [see Discussion]. The loss of E2F2 or Myb following RNAi did not affect the steady-state levels of either Mip120 or Mip130 [Fig. 1; Supplementary Fig. 1]. Thus, the residual binding of Mip120 and Mip130 at the qtc and CG31100 promoters could be due to the independent DNA-binding activities exhibited by Mip120 and Mip130. Furthermore, the incomplete removal of E2F2 or Myb following RNAi treatment could also contribute to the low level of MMB still observed at these promoters. Nevertheless, we concluded that for Class A genes E2F2 clearly contributed to

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**Table 1. (Continued)**

<table>
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<tr>
<th>Class</th>
<th>Symbol</th>
<th>Name</th>
<th>Human homolog</th>
<th>Northern/qRT-PCR</th>
<th>Function[s]</th>
<th>Estimate for peak binding location</th>
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<td>TTK</td>
<td>ND</td>
<td>Mitotic spindle checkpoint</td>
<td>381 to 824</td>
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<td>asp</td>
<td>Abnormal spindle</td>
<td>ASPM</td>
<td>ND</td>
<td>Micrortubule cytoskeleton organization and biogenesis</td>
<td>−291</td>
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<td>R</td>
<td>CG3221</td>
<td>Dgt3</td>
<td>ND</td>
<td></td>
<td>Localization of γ-tubulin to the spindle</td>
<td>NOb</td>
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<tr>
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<td>Cks30A</td>
<td>Cyclin-dependent kinase subunit 30A</td>
<td>CKS1B</td>
<td>ND</td>
<td>Spindle assembly</td>
<td>−309</td>
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<tr>
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<td>SLK</td>
<td>ClassD</td>
<td>Chromosome alignment, cytokinesis</td>
<td>−39</td>
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<tr>
<td>R</td>
<td>Sce</td>
<td>Separase</td>
<td>ND</td>
<td></td>
<td>Mitotic sister chromatid segregation</td>
<td>−253 to 60</td>
</tr>
</tbody>
</table>

More than 20% of Class D genes encode for proteins involved in the G2/M transition. iHOP [http://www.ihop-net.org] and InParanoid [http://inparanoid.sbc.su.se/index.html] databases were used to find human homologs of the described genes. Northern blot and/or qRT–PCR were used to confirm the requirement of Myb, Mip120, and Mip130 in gene expression. The “Estimate for peak binding location” covers the region (minimum and maximum) bound by the entire MMB complex [Myb, Mip120, Mip130, E2F2, and Lin-52].

a(ND) Not determined by Northern blot or qRT–PCR.
b(NO) No binding of the complex within 1 kb of the TSS of the gene of interest.
MMB targeting, and conversely, at Class B genes, Myb contributed to MMB targeting.

The differential requirement for E2F2 and Myb to target MMB to these promoters led us to ask if consensus DNA-binding sites for E2F2 (TTSSSSS) or Myb (YAACKG) were enriched at Class A and B gene promoters, respectively. Promoter regions spanning 1 kb upstream of each open reading frame that were bound by the entire MMB complex and strongly bound (i.e., lrpeak > 2) by E2F2 (Class A) and Myb (Class B) were analyzed using the motif search program MEME (Bailey and Elkan 1994). The conserved motif TTTGCGCGCAT TTT, which contains an E2F DNA-binding site, was found in 94% (45 out of 48) of the designated Class A promoters [Fig. 8; Supplementary Table 2], supporting the idea that E2F2 was important for targeting MMB at Class A gene promoters. For the Class B promoters, 87% (26 out of 30) of the analyzed sequences were enriched for the CTGCAACTGG motif, which contains a Myb-binding site (Fig. 8; Supplementary Table 4).

We also analyzed the occurrence of all E2F (TTSSSSS) and Myb (YAACKG) consensus binding sites within 1 kb upstream of the 14,395 annotated genes in Flybase, and asked whether the derived consensus sequences were significantly enriched in the MMB-regulated promoters. As shown in Figure 8, the E2F-binding sequence was significantly enriched in the upstream region of the Class A genes than what was expected by chance (P-value 0.007). Furthermore, there was no evidence that the promoters of the Class B, C, and D genes were enriched for the E2F consensus sequence (P-values 0.5, 0.1, and 0.9, respectively). In contrast, the Myb consensus sequence was...
significantly enriched at the promoters of Class B genes (P-value 0.02), while there was no evidence of such enrichment at the promoters of the Class A and C genes (P-value 0.7 and 0.8, respectively) (Fig. 8). In addition, a Myb consensus sequence was overrepresented in the upstream regions of the Class D genes (P-value 0.08) (Fig. 8). These data suggested that Myb was important for targeting MMB to both repressed (Class B) and activated (Class D) genes.

Figure 5. MMB is primarily targeted to DNA as a complex. The main population of the regions called “bound” based on our statistics criteria are enriched for the entire MMB complex; i.e., Myb, Mip120, Mip130, E2F2, and Lin-52 (3538 sites, Category A). This class was subdivided into four subclasses based on the value of the \( l_{r \text{peak}} \); i.e., the smoothed \([\log_2 \text{ratio (ChIP sample/input)}]\): 997 sites where \( l_{r \text{peak}} \) of all members >2 (A1), 301 sites where \( l_{r \text{peak}} \) of all members >2 except Myb (2 > \( l_{r \text{peak}} \) > 0.5) (A2), 429 sites where \( l_{r \text{peak}} \) of all members >2 except E2F2 (2 > \( l_{r \text{peak}} \) > 0.5) (A3), and 1611 remaining sites (other) where 2 > \( l_{r \text{peak}} \) Myb and E2F2 > 0.5 (A4). The number of sites bound by MMB subcomplexes was much smaller compared with that of sites bound by the entire complex (Categories B–E). This behavior was independent of the \( q \)-value used in the statistical analysis (see Supplementary Table 2 for details).
Based on these findings, one would expect a correlation between the MMB-binding pattern and the DNA regions targeted by the complex. We indeed found that the MMB sites where all MMB members were strongly bound except for Myb were significantly enriched for the E2F consensus binding site \((P\text{-value}\ 0.001)\), while the MMB sites where the E2F2-binding signal was weak were significantly enriched for the Myb consensus site \((1E^{-13})\) (Fig. 8).

**Discussion**

**Combinatorial requirements of individual MMB factors for site-specific gene regulation**

The major findings reported here revealed the extraordinary diversity of use and combinatorial requirements for the factors of the MMB/dREAM complex in regulating gene expression. The Venn diagrams and subclasses for each set of genes regulated by MMB members shown in
E2F2 and Myb are indeed responsible for MMB targeting at Class A and Class B gene promoters, respectively. The standard deviations for three independent experiments. MMB association (as measured by Mip120 and Mip130 binding) was decreased after loss of a factor after ablation, may remain bound to a site (Beall et al. 2007). Such limitations might lead to misclassification of a certain gene(s). Focusing on Myb as an example, removal of Myb may critically limit regulation of a class of genes with different kinetics than would be observed following depletion of Mip120 or Mip130. Class Q is one such class for which the depletion of only Myb [and not any other MMB member] resulted in lowered transcript levels for Class Q members. One hypothesis is that RNAi treatment to remove either Mip120 or Mip130 resulted in incomplete loss of Mip120 and Mip130 such that just enough of the MMB complex remained targeted to such sites where MMB levels were sufficient to produce normal transcript levels for that gene. Our inference is that for such sites in the Q class, Myb loss would be most limiting.

Previous genetic and biochemical studies that linked the MMB members as one entity have been substantiated and extended by the data presented here. We found that the five MMB members tested [Myb, Mip120, Mip130, E2F2, and Lin-52] had strikingly coincident genomic binding site profiles [Figs. 4, 5]. However, among the 3538 MMB-binding sites where the five proteins colocalized, there were significant differences in the enrichment signals [Figs. 5, 6]. The observed variations in signal strengths at different sites for MMB members by ChIP-chip provided some intriguing correlations. For Class A repressed genes, the Myb ChIP-chip data [NS] was normalized to a value of 1. Shown are the results and standard deviations for three independent experiments. MMB association [as measured by Mip120 and Mip130 binding] was decreased at the qtc promoter when E2F2 was removed [left] and at the CG31100 promoter when Myb was removed [right], demonstrating that E2F2 and Myb are indeed responsible for MMB targeting at Class A and Class B gene promoters, respectively.

Figure 7. E2F2 and Myb behave as mutually exclusive targeting factors for MMB. [A] A single MMB complex regulates gene expression in Drosophila Kc cells. Graphical representations of the genomic region surrounding the Class A qtc [left] and Class B CG31100 [right] loci are on the top. The location of the qtc and CG3100 qPCR products, labeled a–g, are shown as boxes on the top. Black arrows indicate the start and direction of transcription. Histograms displaying the qPCR results following ChIP for the proteins indicated in each inset box are summarized at the bottom. The relative fold enrichment for each ChIP was calculated by determining the ratio of intensities of the experimental regions to the actin promoter following qPCR. The mock ChIP data [NS] was normalized to a value of 1. Shown are the results and standard deviations for three independent experiments. For both genes, the qPCR data were in agreement with the MMB location determined by ChIP-chip. [B] E2F2 and Myb are mutually exclusive for targeting MMB. ChIP was performed on chromatin isolated from E2F2- and Myb-depleted Kc cells following RNAi treatment using the antibodies against MMB members indicated on the bottom of each panel. qPCR was performed for the Class A qtc [left] and Class B CG31100 [right] promoters for the PCR products labeled “d” in each case. The relative fold enrichment for each ChIP was calculated by determining the ratio of intensities of the experimental regions to the actin promoter following qPCR. The mock ChIP data [NS] was normalized to a value of 1. Shown are the results and standard deviations for three independent experiments. MMB association [as measured by Mip120 and Mip130 binding] was decreased at the qtc promoter when E2F2 was removed [left] and at the CG31100 promoter when Myb was removed [right], demonstrating that E2F2 and Myb are indeed responsible for MMB targeting at Class A and Class B gene promoters, respectively.
tionship was reversed for the Class B repressed genes (Supplementary Fig. 9). There are many potential reasons for such differences in signal strengths including: different co-factor associations with MMB bound to a particular class of gene promoters, different subunit conformations/accessibilities (resulting in altered pull-down antibody affinities), or perhaps because the formaldehyde cross-linking efficiency was highest for a protein that had a high affinity for a proximal DNA site. Moreover, MMB subunit composition in vivo may be dynamic, and off-rates for a DNA-binding factor may be lowest for those MMB factors that were tightly bound to a DNA site. Hence, for Class A genes that required E2F2 for repression, the cross-linking efficiency and potential targeting of E2F2 might dominate, whereas for Class B genes that required Myb for repression, the reverse would be found.

Consistent with this view, we found a statistically significant enrichment of Myb consensus binding sites nearby Class B genes and E2F2 consensus binding sites nearby Class A genes. We directly examined MMB targeting to one example for both Class A and Class B genes (Fig. 7), and for the Class A gene, E2F2 was critical for DNA binding whereas for the Class B gene, Myb was found to be a key targeting factor. Furthermore, for Class C repressed genes, where neither E2F2 nor Myb were required for repression, equivalent ChIP-chip enrichment signals were found (Supplementary Fig. 9).

The genes repressed by MMB for both Class A and Class B were not cell cycle regulated and there was no biochemical evidence in Drosophila for the existence of two separate Myb- or E2F2-containing MMB complexes. Anti-Myb and anti-E2F2 antibodies co-immunoprecipitated the entire set of MMB factors and hence each other. Furthermore, both E2F2 and Myb were stoichiometric in the defined complex—even after many different biochemical purification protocols of MMB (Lewis et al. 2004; Beall et al. 2007). We also tested this point in DNA binding experiments with purified MMB complex and biotinylated DNA fragments from either a Class A or B gene with a strong E2F2 site or strong Myb site, respectively. Regardless of the DNA fragment used in pull-down experiments, E2F2 and Myb stayed together (data not shown). We thus conclude that the complexity of both the targeting of MMB to DNA and the transcriptional function(s) of MMB are determined by DNA context and other proteins that may have associated with MMB. In this sense, MMB behaves like the multisubunit TFIIID complex, where different TAF subunits determine DNA targeting at a specific promoter (e.g., TAF$_{12}$ at an Inr site, TAF$_{6}$ at a DPE site, and TBP at a TATA box) and the different TAF subunits interact with different coactivators [Hochheimer and Tjian 2003].

**MMB plays a role in regulating genes important for M phase**

Many genes required for the G2/M transition were regulated by MMB. Reduced levels for many of the genes listed in Table 1 could readily account for the chromosome phenotypes that were characterized after RNAi depletion of Myb, Mip120, or Mip130 including: impaired sister chromatid cohesion, chromosome fragmentation, and condensation defects. Furthermore, transcript levels for regulatory checkpoint genes involved in spindle assembly that might indirectly lead to chromosome instability were also affected by loss of MMB. In a recent study, Goshima et al. (2007) conducted a “genome-wide” RNAi screen to identify factors contributing to spindle assembly in Drosophila. Among the unexpected genes revealed by this screen were Myb, Mip130, Lin-52, Mip40, and Caf1/p55. We suggest that...
the MMB member genes were identified in their study, at least in part, because MMB regulates the levels of other “expected” spindle assembly genes such as Klp61F [a kinesin] or lal [Aurora-B kinase] or the “unexpected” spindle assembly genes such as RacGap50C—all of which are MMB-regulated Class D genes. It will be interesting to learn if the human homologs of MMB (Ga-grica et al. 2004; Korenjak et al. 2004; Litovchick et al. 2007; Osterloh et al. 2007; Pilkinton et al. 2007) also regulate genes required for G2/M in humans, because the oncogenic role of Myb in certain cell types may involve misregulation of spindle assembly genes that ensure normal karyotypes. We note that Ren et al. [2002] have shown that the human repressor E2F4 binds to genes involved in chromosomal stability, and one might suspect that this activity is functioning in the context of the paralogous human MMB/dREAM complex.

Many of the genes regulated by MMB and listed in Table I are essential, and in particular, the Class D genes are prominent in this regard. It is possible that the lethality observed for myb-null mutants is the result of misexpression of one or a set of these genes. However, even with a reduction of MMB factors >95% after sustained RNAi treatment, we continued to see proliferation of Kc cells in culture. We point out that the transcript levels for genes requiring MMB for activation [Class D genes] were only modestly reduced [two- to fourfold] in the absence of MMB. Thus, the regulation of these genes by Myb may not be profound or responsible for the lethality of myb mutations in flies. Nevertheless, there are reasons to suspect that the regulation by MMB of these target genes may be relevant at least in part to the essential requirements for Myb in vivo. Recall that myb-mutant lethality is suppressed by loss of mip40, mip120, or mip130 [Beall et al. 2004, 2007]. Following along the lines of our model derived from these genetic studies one might suggest that the critical regulatory step dependent on Myb involves derepression from a quiescent state where cells need to switch on such essential genes for mitotic functions. Such a switching mechanism may be nonessential in cell culture. Hence, repression in a quiescent cell in the developing fly, perhaps mediated by Mip120 and Mip130, may require Myb for induction at a later time or in a specific tissue. In vivo, but not in cell culture, loss of Myb alone [as in myb-null mutants] could result in a “permanently repressed” essential gene whereas loss of the entire MMB complex [as in myb; mip120 mutants] may allow for suboptimal expression levels of an essential gene[s]; for instance, at a two- to fourfold reduced level. Thus, a presently scored Class D gene may behave as a Class C repressed gene in another cell type, where loss of Myb would leave a repressive MMB complex that is unable to be induced.

One important issue that needs to be explored is how MMB is targeted to such essential genes. From the genetic suppression data, we infer that MMB is still targeted to essential genes even in the absence of Myb, and therefore does not require Myb for targeting to these gene promoters. If the vital function of Myb is to some-

how induce a MMB-repressed vital gene[s] where loss of Myb was not critical for repression, then the essential activity of Myb may not require the Myb DNA-binding domain at all. In fact, recent data showed that a transgene containing a complete deletion of the DNA-binding domain of Myb is sufficient for myb-mutant viability [J. Lipsick, pers. comm.].

Why are there so many MMB-binding sites in the Drosophila genome?

The number of genomic binding sites for MMB far exceeded what was expected from the MMB gene regulatory network defined by the RNAi analysis. While >80% of the 3538 MMB-binding sites were proximal to promoters, only 25% of proximal genes showed any change in expression when MMB members were depleted following RNAi treatment. Similar observations have been made for other proteins [Martone et al. 2003; Orian et al. 2003]; for example, the number of genes regulated by the Myc, Max, and Mad/Mnt transcriptional network is far lower than the measured number of binding sites for these factors [Orian et al. 2003]. This type of phenomenon may be a simple consequence of biological noise [Struhl 2007]. It is possible that many of the complexes that are bound to sites not regulating transcription of a nearby gene are simply “junk” or vestigial in nature. A majority of such sites would have little selective advantage, or they may simply serve as a nonspecific binding pool to keep the levels of non-DNA-bound MMB low. Alternatively, these sites may play some role in other chromosome function[s] apart from gene expression. For instance, some of these “silent” MMB-binding sites may be directly modulating the selection of replication initiation sites, a point suggested by the role played by the complex in follicle cell gene amplification.

Interestingly, many of the genes that contain “silent” promoter-proximal MMB-binding sites are expressed in Kc cells and transcript levels are unaffected following removal of MMB. Perhaps, at some of these occupied sites, MMB is simply poised to respond to signals that are absent in the culture media. Prominent amongst this list are the genes encoding DNA replication proteins such as Chiffon, the ORCs, and MCMs. An evolutionarily conserved multisubunit complex in human cells that contains homologs of many of the MMB/dREAM subunits, represses cell cycle-dependent genes during quiescence [Litovchick et al. 2007]. It is, of course, possible that similar control in resting cells will be found for the fly complex. This prospect would then add to the established functions of MMB in repressing differentiation-specific genes, and promoting transcription of M-phase genes. Extending this general point, the Kc cells are fixed in one state through their isolation in culture and well-defined passage conditions. It is possible that some of the “silent” MMB-binding sites may function in a developmental pathway that is dependent on the action of a new factor or signal not normally seen in Kc cells in culture.
Materials and methods

Cell culture and FACS

Drosophila Kc cells were grown in suspension at 25°C in Schneider's medium [Invitrogen] supplemented with 10% heat-inactivated fetal bovine serum [HyClone] and Antibiotic–Antimycotic solution [Invitrogen]. FACS analysis was performed as described previously [Bandura et al. 2005], and data were analyzed with MultiCycle for Windows software [Phoenix Flow Systems].

RNAi, immunoblot, and Northern blot

RNAi was performed as described [Beall et al. 2004]. Primer sequences used for dsRNA production are available on request. For immunoblot analysis, total protein, corresponding to 0.3 x 10⁶ cells, was separated using 8%-12% SDS-PAGE and transferred to nitrocellulose; proteins were detected using affinity-purified polyclonal rabbit antibodies against individual complex members as described [Beall et al. 2002; Lewis et al. 2004]. Mouse monoclonal anti-RBF1 and anti-RBF2 antibodies were a kind gift of N. Dyson, and were described in Frolov et al. (2001) and Stevaux et al. (2002). For determination of protein levels, blots were scanned with a Typhoon Fluorimager and signal intensities of the bands were processed using the ImageQuant program [Amersham Biosciences]. Total RNA isolation and Northern blot analysis were performed according to established procedures and are described in Beall et al. (2002) and Lewis et al. (2004).

Metaphase spreads and microscopy

Metaphase spreads were prepared as described in the Supplemental Material.

Affymetrix Drosophila GeneChip

RNAi to deplete Lin-52, Mip40, Myb, Mip120, Mip130, E2F2, both RBFs [RBF1 and RBF2], and L3[MBT] were performed in triplicate. RNAi with a nonspecific RNA derived from a pBSK+ [kind gift of N. Dyson], and were described in Frolov et al. (2001) and Stevaux et al. (2002). For determination of protein levels, blots were scanned with a Typhoon Fluorimager and signal intensities of the bands were processed using the ImageQuant program [Amersham Biosciences]. Total RNA isolation and Northern blot were performed according to established procedures and are described in Beall et al. (2002) and Lewis et al. (2004).

ChIP and qPCR

ChIP was performed as reported [Andrulis et al. 2000] with some modifications detailed in the Supplemental Material. ChIP was assayed by qPCR as described in the Supplemental Material.

ChIP on Drosophila tiled genomic microarrays

In order to identify regions bound by MMB, 10–20 ng of ChIP and control DNA samples were amplified using a randomly primed PCR method, according to Affymetrix recommendations [Affymetrix Chromatin Immunoprecipitation Assay Protocol]. Purified DNAs were then fragmented, TdT labeled, and hybridized to the Affymetrix Drosophila genome Tiling Array 1.0 (reverse part no. 520,054), as described previously [Manak et al. 2006]. ChIP–chip data have been deposited in the GEO database under accession code GSE9087.

Analysis of ChIP tiling array data

Statistical methods used to define and analyze MMB-enriched regions are described in detail in the Supplemental Material.

Acknowledgments

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Genomic profiling and expression studies reveal both positive and negative activities for the *Drosophila* Myb–MuvB/dREAM complex in proliferating cells

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**Supplemental Material**
http://genesdev.cshlp.org/content/suppl/2007/10/31/gad.1600107.DC1

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