

# synMuv vérité—Myb comes into focus

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Over the past 25 years, tremendous progress has been made in the identification of the genetic alterations that cause cancer. The discovery of oncogenes and tumor-suppressor genes relied largely on studies of animal retroviruses, consistent chromosomal aberrations in human cancers, transfection of human tumor DNA into rodent cells, DNA tumor viruses, and rare familial cancer syndromes (Bishop 1995). A major challenge has been to understand the function of the protein products of these genes in both normal and malignant cells. In many cases studies of developmental genetics in model organisms have provided key insights that have illuminated the pathways that go awry in human cancer. In particular, the awesome power of beast genetics in the nematode and fruit fly has benefited from the speed and relatively low cost of forward genetics, their much lower degree of gene (and genome) duplication relative to vertebrates, their relatively small genome size, the accumulated knowledge about the genetics and development of these organisms, and the strong and highly interactive communities of researchers devoted to their study. However, the discovery of genetic pathways alone is not sufficient to understand the intricate workings and assembly of the machinery within the cell. In addition, biochemistry and cell biology are required to reveal the possible functions of the pieces of this machinery. In the happy event that genetic and biochemical approaches converge on the same picture of the world, each gains confidence and insight from the other. This article focuses on recent results that display just such a convergence that bears directly on our understanding of the *RB-E2F* pathway that is mutated in most, if not all, human cancers (Gagrica et al. 2004; Korenjak et al. 2004; Lewis et al. 2004).

## 'S wonderful! 'S Muv-Vul-ous!

In the early 1990s a remarkable series of discoveries revealed the existence and function of a genetic and biochemical pathway that leads from extracellular peptide growth factors to receptor tyrosine kinases through RAS, RAF, and MAP kinase to ETS family transcription fac-

tors. The marriage of oncogene research, cell biology, biochemistry, the genetics of *Drosophila* eye development, and the genetics of *Caenorhabditis elegans* vulval development all contributed in important ways to our current understanding of this key signaling pathway (Rubin et al. 1997; Sternberg and Han 1998; Malumbres and Barbacid 2003). The identification, genetic ordering, and eventual molecular cloning of two classes of nematode mutants were particularly informative—those that caused either a multivulval (Muv) or a vulvaless (Vul) phenotype. In general, mutations that activate the pathway, such as an oncogenic RAS mutation, cause a Muv phenotype, whereas mutations that inactivate the pathway cause a Vul phenotype.

Another result of the same genetic study was the identification of an unusual strain in which two unlinked mutations were required to generate a multivulval phenotype (Horvitz and Sulston 1980). Further analyses showed that neither of these loss-of-function mutations alone was sufficient to cause a Muv phenotype. The mutant alleles of these two genes (*lin-8* and *lin-9*) were then used to identify mutant alleles of additional genes that could cause a similar synthetic Muv phenotype (synMuv), but that by themselves would not display a Muv phenotype (Ferguson and Horvitz 1989). Remarkably, two classes of genes were identified such that any mutant in Class A could synergize with any mutant in Class B, but in no case would two mutants from the same class synergize to cause a Muv phenotype. The interpretation of these results was that two redundant pathways (synMuvA and synMuvB) acted to repress the function of the RAS pathway described above. Molecular cloning revealed that synMuvB genes encoded homologs of the retinoblastoma susceptibility protein (LIN-35/RB), the heterodimeric E2F DNA-binding transcription factor (EFL-1/E2F and DPL-1/DP) that interacts directly with RB family proteins, and a WD40-repeat histone-binding protein (LIN-53/MSI1/RbAp48/CAC3) that also binds to RB (Lu and Horvitz 1998; Ceol and Horvitz 2001). Additional synMuvB genes encode homologs of components of the nucleosome remodeling deacetylase complex (NuRD) including the ATP-dependent nucleosome remodeling subunit (LET-418/Mi-2) and the histone deacetylase subunit (HDA-1/HDAC), along with LIN-53/MSI1/RbAp48/CAC3 (Solari and Ahinger 2000; von Zelewsky et al. 2000).

Careful analysis of the incidence of familial and sporadic retinoblastoma in humans led Knudson to propose

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1274804>.

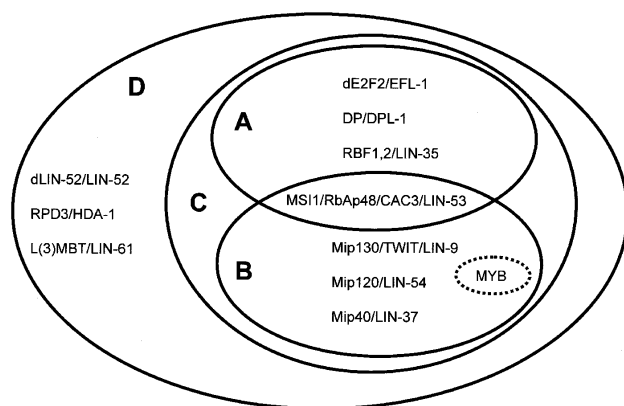
the two-hit hypothesis of tumor suppression (for review, see Knudson 2001). Both hits turned out to be loss-of-function mutations in the retinoblastoma susceptibility gene (*RB*). Molecular cloning of this gene did not immediately reveal its function. However, the direct binding of the nuclear RB protein to the E1A oncoprotein of adenovirus eventually led to experiments showing that E1A displaces RB from the cellular E2F transcription factor in order to promote expression of the adenovirus E2 gene (for review, see Nevins 2001; Classon and Harlow 2002). Initial studies of cellular genes activated by E2F identified essential S-phase components such as ribonucleotide reductase, thymidylate synthetase, and PCNA. Over time it became clear that human E2F was a heterodimeric DNA-binding transcription factor composed of one of six different E2F subunits and one of two different DP subunits. The E2F subunits in vertebrates can be divided into those that predominantly activate transcription (E2F1–E2F3) and those that predominantly repress transcription (E2F4–E2F6). Additional complexity arose when two additional RB-related proteins were identified in vertebrates, p107 and p130. The analysis of this regulatory system is still a work in progress, but a general theme has been that E2F-regulated genes are repressed in G0/G1 by an E2F–DP bound to an RB-related protein. Growth factor signaling via RAS and other pathways increases the expression of cyclin D, thereby sequentially activating the *cycD*–CDK4 and *cycE*–CDK2 protein kinases that phosphorylate and thereby inactivate the RB-related repressors, thus permitting expression of S-phase genes. Acute repression of gene expression by RB-related proteins is generally believed to occur via histone deacetylation, although longer-term repression may utilize other mechanisms. The RB–cyclin D pathway appears to be a key component of the “restriction point” in late G1, after which cells are committed to completing the cell cycle even in the absence of additional growth factors. This pathway is a frequent target of mutations in human cancer cells including loss-of-function mutations or tumor virus inactivation of *RB*, amplification of *cyclin D*, and loss-of-function mutations in *Ink4A*, which encodes the p16 inhibitor of the *cycD*–Cdk4 kinase. Although the *synMuvB* genes in *C. elegans* (including homologs of *RB*, *E2F*, and *DP*) were originally discovered because of their role in regulating the RAS-driven vulval developmental decision, recent studies have shown that the same genes also regulate G1 progression in this organism (Boxem and van den Heuvel 2002).

RbAp48 was discovered as an RB-associated protein in experiments in which cellular extracts were passed over an RB protein affinity column (Qian et al. 1993). Intriguingly, this human protein was found to be related to MSI1 (multicopy suppressor of *ira1*), a protein of budding yeast that negatively regulates RAS signaling (Ruggieri et al. 1989). *IRA1* encodes a RAS-GAP related to the human neurofibromatosis gene product (NF-1) that normally functions to inactivate RAS signaling. Remarkably, human RbAp48 was shown to be capable of suppressing heat sensitivity of budding yeast carrying an

activated *RAS* allele or an *ira-1* loss-of-function allele. In retrospect, these results are of particular interest for two reasons: First, *synMuvB* genes themselves are negative regulators of RAS signaling, albeit in a redundant fashion with *synMuvA* genes. Second, *synMuv* loss-of-function mutants generally display a heat-sensitive decrease in viability.

The MSI1/RbAp48/CAC3 protein, and its close relative RbAp46, have been rediscovered many times in various protein complexes that acetylate newly synthesized histones (Hat1), assemble nucleosomes during DNA replication (CAF-1), assemble nucleosomes independently of DNA replication (HIRA), remodel chromatin during gene transcription (NURF), deacetylate histones during short-term gene repression (Sin3), remodel chromatin and deacetylate histones to repress gene expression (NuRD), and that cause long-term gene silencing by histone methylation (PRC2) (for review, see Kaufman 1996; Ridgway and Almouzni 2000; van Nocker 2003). Although the MSI1/RbAP48 protein is often referred to simply as CAF-1 in the literature, this can be confusing because MSI1/RbAp48/CAC3 is only one of the three subunits of chromatin assembly factor 1 (CAF-1). The other subunits are encoded by separate genes (*CAC1* and *CAC2*) and are not present in other complexes that contain MSI1/RbAp48/CAC3. Furthermore, mutation of one of four MSI1 homologs in *Arabidopsis* causes an epigenetic maintenance of reproductive development phenotype that is not caused by mutations in the other CAF-1 subunits (Hennig et al. 2003). The direct binding of histones by the MSI1/RbAp48/CAC3 protein is consistent with the function of these complexes in chromatin assembly, modification, and remodeling (Verreault et al. 1996). Further efforts to identify additional RB-associated proteins and to purify a larger RB–E2F–DP complex from vertebrate tissues and cultured cells have been problematic in that genetic evidence to support the *in vitro* biochemistry has generally been lacking. However, two research groups working independently have now used biochemical purification of *Drosophila* cell extracts to make substantial and surprising progress in identifying such a complex (Korenjak et al. 2004; Lewis et al. 2004).

*Drosophila* has only two E2F-related proteins (dE2F1, an activator; dE2F2, a repressor not to be confused with vertebrate E2F2, an activator), a single DP protein, and two RB-related proteins (RBF1 and RBF2) that function differentially in transcriptional regulation (Du et al. 1996; Stevaux et al. 2002). Genome-wide analyses of transcriptional regulation by these proteins has revealed that dE2F1 is the primary regulator of many genes required for cell cycle progression, whereas dE2F2 represses a much wider variety of genes, many of which do not appear to be cell cycle regulated (Dimova et al. 2003). Purification of a native dE2F2–DP complex has now resulted in the identification of a large multiprotein complex the authors call dREAM (*Drosophila* RBF, E2F, and Myb-interacting proteins) (Fig. 1; Korenjak et al. 2004). In addition to dE2F2 and DP, RBF1 and RBF2 are both present in such complexes, but not within the same indi-



**Figure 1.** The *Drosophila* Myb–MuvB complex. (A) Previously described E2F–RB protein network. (B) Previously described Myb complex. (C) dREAM complex. (D) Myb–MuvB complex. Note that all proteins in the diagram are encoded by homologs of *C. elegans* synMuvB genes, except for Myb (dotted circle), which appears to have been lost during the evolution of *C. elegans*.

vidual complex. The *Drosophila* homolog of MSI1/RbAp48/CAC3 is also present. Surprisingly, this dE2F2–RB complex also contains all of the proteins that were found in a recently described complex that contains the *Drosophila* homolog of the Myb oncoprotein (see below; Beall et al. 2002). Furthermore, all of these proteins except Myb are encoded by the *Drosophila* homologs of synMuvB genes of *C. elegans*. Immunostaining of polytene chromosomes was used to localize the dREAM complex to transcriptionally silent regions, consistent with previous observations that dE2F2 functions as a dedicated repressor. Consistent with a role for this complex in transcriptional repression, the dREAM complex could bind to immobilized histone H4 tails *in vitro*, and this binding was prevented by acetylation of the H4 peptide. The presence of a Myb protein in an E2F–RB complex was presaged by reports showing direct physical association and functional interactions between the vertebrate B-Myb and RB-related p107 proteins (Sala et al. 1996; Joaquin et al. 2002). To understand the origins of the Myb complex itself, we will now turn our attention to *Drosophila* oogenesis.

### Waltzing on eggshells

The search for direct transcriptional targets of the Rb–E2F pathway initially led to genes encoding essential S-phase proteins. Therefore, one might expect that mutations of this pathway would lead to defective S-phase phenotypes. The basic machinery that regulates eukaryotic DNA replication has been highly conserved from yeast to metazoans (Bell and Dutta 2002). However, the Rb and E2F gene families have thus far been found in animals and plants, but not in fungi. In contrast to studies in yeast, well-defined origins of DNA replication have been difficult to identify in metazoans with the exception of animal viruses that use virally specific ini-

tiation proteins. One interesting exception has been the amplification of the chorion (eggshell) loci in the ovarian follicle cells of *Drosophila* (Calvi and Spradling 1999; Tower 2004). Follicle cells undergo a stereotypic sequence of mitotic cell cycles, followed by endoreplication cycles without mitosis, followed by cessation of genome-wide DNA replication and initiation of site-specific DNA replication that results in amplification of a few specific loci to very high copy number. Because the chromosomes in these cells are polytene and homologs are paired, each amplifying locus can be readily visualized as a single spot of BrdU incorporation (Calvi et al. 1998). In addition, antibodies directed against specific DNA replication proteins can be used to visualize assembly of prereplication complexes and to distinguish initiation and elongation (Claycomb et al. 2002).

Not surprisingly, failure of proper chorion gene amplification results in female sterility with thin eggshells (for review, see Calvi and Spradling 1999; Tower 2004). In this regard, several previously identified female sterile, thin eggshell mutants were found to be hypomorphic alleles of genes that encode essential components of the DNA replication machinery. It was therefore gratifying to learn that mutations of the *Drosophila* homologs of *E2F*, *DP*, and *RB* can cause female sterility (Royzman et al. 1999; Bosco et al. 2001; Cayirlioglu et al. 2001, 2003). *dE2F1* mutants displayed normal genome-wide endoreplication in ovarian follicle cells, but neither localized the origin recognition complex (ORC) proteins to the chorion loci or amplified these loci. Surprisingly, *dE2F1* mutants that lacked a transcriptional activation domain were still able to function in chorion amplification. *dE2F2*, *RBF1*, and *DP* mutant follicle cells somewhat unexpectedly displayed a persistence of genome-wide DNA replication rather than completely shifting to site-specific replication of the chorion loci. In addition, the ORC proteins remained diffusely localized on replicating chromosomes in these mutants, rather than concentrating solely at the chorion loci. Recently, a phenotype very similar to that seen in *RBF1* and *dE2F2* mutants was observed in follicle cell clones mutant for *RPD3*, which encodes a histone deacetylase (HDAC), and in follicle cells treated with HDAC inhibitors (Aggarwal and Calvi 2004). Furthermore, targeted modulation of histone acetylation at the origin of replication directly affected amplification of the chorion locus. Together these results suggest that Rb–E2F is required to target histone deacetylase to flip the switch from genome-wide to site-specific DNA replication.

Meanwhile efforts were under way to identify site-specific DNA-binding protein(s) that might localize the relatively nonspecific ORC DNA-binding complex to the *Drosophila* chorion loci. Nuclease footprinting was used as an assay to purify multiprotein complexes that bound specifically to a minimal enhancer (ACE3) and origin (oriB) of gene amplification at the chorion locus on chromosome III (Beall et al. 2002). The purified complex contained five proteins: Myb, a sequence-specific DNA-binding protein encoded by the *Drosophila* homolog of a vertebrate oncogene; Mip130/Twilight (TWIT), a *Dro*-

*sophila* homolog of the *lin-9* synMuvB gene product; Mip120, a previously unknown sequence-specific DNA-binding protein that turns out to be homologous to the product of the *lin-54* synMuvB gene (C. Coel and H.R. Horvitz, pers. comm.); p55, the *Drosophila* homolog of MSI1/RbAp48/CAC3/LIN-53; and Mip40, a "pioneer" protein (Fig. 1). Recent analyses suggest that Mip40 may in fact be a distant relative of the *lin-37* synMuv B gene product (Korenjak et al. 2004). In support of a role for this Myb complex in chorion gene amplification, deletion of the Myb- and Mip120-binding sites from an amplifiable chorion transgene caused a significant reduction in amplification. In addition, clones of ovarian follicle cells homozygous for a null allele of *Myb* failed to amplify the chorion loci and produced very thin patches of eggshell. However, the molecular phenotype of *Myb*-null ovarian follicle cells differs from that of *RBF1*, *dE2F2*, and *Rpd3/HDAC* mutants in that genome-wide DNA replication ceases. In addition, the ORC, CDT1/DUP, and MCM proteins appear to localize normally to the chorion loci in the absence of *Myb* (Beall et al. 2002; J.R. Manak and J.S. Lipsick, unpubl). These results suggest that *Myb* is required at a step subsequent to RB-E2F action and the assembly of the prereplication complex during the initiation of chorion gene amplification.

Heat-sensitive hypomorphic alleles of *Drosophila Myb* cause partially penetrant lethality at all periods of development (Katzen and Bishop 1996). Null alleles of *Drosophila Myb* cause uniform lethality in late larval and early pupal phases, presumably due to maternally deposited gene products from heterozygous mothers (Manak et al. 2002). In both cases, mitotic aberrations are observed including increased frequencies of aneuploidy, polyploidy, and abnormal spindle morphology (Katzen et al. 1998; Fung et al. 2002; Manak et al. 2002). Overexpression of *Drosophila Myb* can inhibit endoreplication in salivary gland cells (Fitzpatrick et al. 2002). However, *Drosophila Myb* protein is normally present in endoreplicating cells and concentrates on newly replicated DNA in both mitotically cycling and endoreplicating cells (Beall et al. 2002; Manak et al. 2002).

Recently, null alleles of *Drosophila Mip130/TWIT* have been created (Beall et al. 2004). The animals are viable, but display female sterility similar to that seen in *dE2F2* and *RBF1* mutants—persistent genome-wide endoreplication without chorion amplification. Interestingly, the absence of *Mip130/TWIT* in these animals caused a drastic reduction in *Myb* protein levels, although the converse was not true. Unexpectedly, double mutants of both *Myb* and *Mip130/TWIT* were viable and displayed a phenotype similar to *Mip130/TWIT* null mutants. These results suggest that *Mip130/TWIT*, like *dE2F2* and *RBF1*, acts at an earlier step than *Myb* in the activation of chorion loci for DNA replication. Perhaps the lethality observed in *Myb* null mutants is due to the action of a *Myb*-less *Mip130/TWIT* complex that can be reduced or abolished by removing *Mip130/TWIT*. A second *lin-9/Mip130/TWIT*-related gene called *Always Early (Aly)* is expressed specifically in *Drosophila* testis and mutation of this gene causes a failure of normal ga-

metogenesis (White-Cooper et al. 2000). The role of *RB*, *E2F*, *DP*, and *Myb* in an *Aly* pathway remains unclear, but certainly seems worthy of investigation.

Studies described in this issue of *Genes & Development* (Lewis et al. 2004) have revisited the Myb complex and reached a startling, but gratifying conclusion very similar to that described above (Korenjak et al. 2004). Biochemical purification under less stringent conditions has yielded a larger complex that contains many of the other known synMuvB gene products, including *dE2F2*, *DP*, *RBF1*, and *RBF2*. Three additional synMuvB gene products not present in the dREAM complex were identified in the larger Myb-synMuvB complex: *RPD3/HDAC-1*, *LIN-52*, and *L(3)MBT/LIN-61* (lethal malignant brain tumor) (Fig. 1). The latter gene is of particular interest because it encodes a zinc finger protein that can act as a tumor suppressor in *Drosophila* (Wisnar et al. 1995). Together these results show that the *Drosophila* homologs of many known synMuvB genes encode subunits of a single large multiprotein complex. In addition to synMuvB encoded proteins and *Myb*, the purified fractions of this large complex also contained lesser amounts of NURF, a chromatin remodeling complex (Tsukiyama et al. 1995).

Both the dREAM and Myb-synMuv complexes were shown to localize to promoters of known *dE2F2*-regulated genes. RNAi knockdown studies showed that the *dE2F2* repressor (but not the *dE2F1* activator) and other members of the dREAM and Myb-MuvB complexes are required for repression of endogenous *dE2F2*-regulated genes. The interesting exception is that *Myb* itself can be depleted, but is not required for repression of the particular genes examined in these cell culture assays. One difference between the two studies is that the dREAM complex does not contain HDAC, *L(3)MBT*, *LIN-52*, or NURF components. This may be due to the different purification procedures used by these two research groups. However, another recent paper showed that neither HDAC nor the SWI/SNF chromatin remodeling complex is required for repression of at least some *dE2F2*-regulated genes (Taylor-Harding et al. 2004). Perhaps different arrangements of the various proteins and subcomplexes associate differentially within the cell to regulate different genes.

### A Myb-story wrapped in an enigma

The *Myb* gene family was initially identified because of the transduction of a portion of the *c-Myb* proto-oncogene during the generation of two acutely transforming retroviruses, avian myeloblastosis virus and E26 leukemia virus (for review, see Ganter and Lipsick 1999). All vertebrates studied to date have three closely related *Myb* genes. *c-Myb* and *A-Myb* encode strong transcriptional activators and are expressed in a tissue-specific manner. Disruption of these genes in the laboratory mouse causes a profound and lethal failure of fetal hematopoiesis (*c-Myb*) or a failure of adult spermatogenesis and mammary gland proliferation (*A-Myb*), consistent with a tissue-specific function of these genes (Mucenski

et al. 1991; Toscani et al. 1997). The third family member, B-Myb, appears to be expressed in all dividing cells, and gene disruption in the laboratory mouse causes very early embryonic lethality (Tanaka et al. 1999). In addition, increased expression of B-Myb correlates with poor prognosis in a variety of human malignancies (Raschella et al. 1999; Amatschek et al. 2004). The B-Myb protein lacks the highly conserved central transcriptional activation domain that is present in the c-Myb and A-Myb proteins (Simon et al. 2002). Nevertheless, B-Myb has been reported to activate transcription, particularly when coexpressed with exogenous cyclin A, the limiting subunit of the cyclin A-Cdk2 kinase that is capable of phosphorylating the B-Myb protein (for review, see Joaquin and Watson 2003).

*Drosophila* has a single *Myb* gene that encodes a protein that lacks a transcriptional activation domain related to those of c-Myb and A-Myb (Katzen et al. 1985). However, similar to results with B-Myb, transcriptional activation by *Drosophila* Myb has been detected, particularly when exogenous CBP coactivator is also present, and genetic interactions have been observed between *Myb* and *CBP* (Hou et al. 1997; Fung et al. 2003). In addition, *Drosophila* Myb and related proteins in *Arabidopsis* have been proposed to directly regulate transcription of the *cyclin B* gene (Ito et al. 2001; Okada et al. 2002). Recent experiments have shown that vertebrate B-Myb, but neither c-Myb nor A-Myb, can complement hemocyte and lymph gland abnormalities found in the *Drosophila* Myb-null larvae (Davidson et al. 2004). Indeed, the expression of c-Myb or A-Myb in *Drosophila* results in lethality or tissue-specific developmental abnormalities. Together these results suggest that B-Myb is the vertebrate ortholog of *Drosophila* Myb, and that c-Myb and A-Myb represent neomorphic variants that were retained following two rounds of gene (or genome) duplication and divergence.

A few puzzles arise from the discovery of the Myb-MuvB supercomplex. The first is that B-Myb itself is perhaps the best studied target of transcriptional regulation by vertebrate E2F proteins (for review, see Joaquin and Watson 2003). Repressive E2Fs and the RB-related p107 or p130 proteins, but not RB itself, are required to prevent B-Myb expression in G0/G1 (Hurford et al. 1997). In vivo DNase footprinting implied that the E2F-binding sites in the B-Myb promoter are unoccupied when the gene is expressed in late G1/S phase (Zwicker et al. 1996). However, genetic studies have shown that the activating E2F3, but not E2F1, is specifically required for B-Myb gene expression (Humbert et al. 2000). In contrast, the *Drosophila* Myb gene does not appear to be regulated by E2F1 or E2F2, but instead may be regulated by DREF, an unrelated transcription factor (Sharkov et al. 2002; Dimova et al. 2003). These results suggest that a feedback loop has evolved in vertebrates in which B-Myb expression is repressed by an E2F-p107 complex that perhaps includes B-Myb itself, but that once expressed, B-Myb in turn binds to p107, thereby reinforcing S-phase progression independently of any transcriptional activation by B-Myb. If a similar functional relationship

exists between *Drosophila* Myb and the RBF proteins, then one might rationalize the failure of anti-Myb RNAi to relieve repression by the *Drosophila* Myb-MuvB complex as follows. *Drosophila* Myb is present in the complex but is not required for repression of dE2F2 target genes. Rather, *Drosophila* Myb might relieve repression by the complex only after it is modified, perhaps by phosphorylation similar to that reported for B-Myb (Robinson et al. 1996).

Second, if the only role for Myb were to relieve repression by the remainder of the complex, then it would be difficult to explain the very different phenotypes of *Myb* mutant follicle cells (cessation of genome-wide replication; proper ORC-Cdt-MCM localization at the chorion loci without gene amplification) versus *RBF1*, *dE2F2*, *DP*, and *Mip130/TWIT* mutant follicle cells (failure of cessation of genome-wide replication; failure to localize ORC solely at the chorion loci). Instead these differing phenotypes imply that Myb acts at a later step in the pathway that leads to activation of an assembled prereplication complex (Beall et al. 2002, 2004). Given that Myb-related domains are contained in a variety of proteins involved in histone acetylation (ADA2), histone deacetylation (N-CoR), nucleosome remodeling (SWI3, I-SWI), telomere maintenance (TAZ1, TRF1, TRF2, RAP1) (for review, see Ganter and Lipsick 1999), it seems likely that the function of Myb during the chorion gene amplification is to permit local alterations in chromatin structure to facilitate the progression of the topologically complex "onion skin" DNA replication. In this regard, recent studies have shown that *Drosophila* Myb is not strictly required for genomic DNA replication in mitotically cycling or in endoreplicating cells (Beall et al. 2002; Manak et al. 2002; J.R. Manak and J.S. Lipsick, unpubl.). Therefore, the same functions of the Myb-synMuv complex may be required in transcriptional regulation and site-specific DNA replication—histone modification and nucleosome remodeling—even though the eventual outputs of the two processes are very different.

The third puzzle is far more interesting, but at present far more perplexing. Although proteins closely related to *Drosophila* Myb have been found in vertebrates, tunicates, urchins, insects, green plants, and cellular slime molds, this gene family appears to have been lost during the evolution of the nematode *C. elegans* (Stober-Grasser et al. 1992; Ganter and Lipsick 1999). This is consistent with the observation that *Drosophila* Myb is not required for transcriptional repression by the Myb-MuvB/dREAM complex. More distantly related Myb-domain proteins are present in *C. elegans*, most notably homologs of the Cdc5 splicing factor and the SNAPc transcription factor (Ohi et al. 1998). Therefore, it remains possible that these proteins might function in place of a canonical Myb in the synMuvB complex. Interestingly, at least some of the LIN-9/Mip130-related proteins of flowering plants carry their own Myb domains, arguing that there may be more than one way to bring a Myb-like function to such complexes (Bhatt et al. 2004). Nevertheless, one wonders what aspect(s) of *C. elegans* biology relieved the selective pressure to retain the Myb gene

family that has been conserved in other animals, plants, and cellular slime molds. Perhaps it is the apparent lack of localized heterochromatin? If so, might one function of Myb in the Myb–MuvB and dREAM complexes be to counteract the repressive effects of localized heterochromatin?

### Histone tales and loose ends

Satisfyingly, direct evidence of a role for vertebrate *LIN-9/Mip130/TWIT* in tumor suppression has now been provided (Gagrica et al. 2004). In particular, the human LIN-9 protein binds directly to RB, can cooperate with RB to suppress the transformed phenotype of RB-deficient osteosarcoma cells, and can prevent morphologic transformation by an activated *RAS* oncogene in an RB-dependent fashion. One surprising wrinkle is that *LIN-9* can cooperate with special alleles of *RB* whose protein products do not appear to bind to E2F proteins. Whether the other members of a dREAM or synMuvB complex are required for this LIN-9 function will be of great interest. Clearly, dREAM-like complexes do exist in human cells (Korenjak et al. 2004).

The relationship of *C. elegans* synMuvA genes to *Drosophila* and vertebrate genes remains unclear. However, another recent publication describes a new class of synMuv genes that acts redundantly with both synMuvA and synMuvB genes (Ceol and Horvitz 2004). These synMuvC genes encode a homolog of the Myc-associated TRRAP protein, a MYST family histone acetyl transferase, and a homolog of the adenovirus E1A-associated p400 SWI/SNF-related ATPase. The broader importance of this work is that a wide variety of chromatin-modifying complexes has now been implicated in the regulation of signaling via the RTK–RAS pathway.

The focus of much research on transcriptional regulators encoded by oncogenes and tumor-suppressor genes has been to identify critical target genes that might explain the malignant phenotype. However, recent genomic approaches to identify genes regulated by the Myc oncoprotein and the RB–E2F tumor suppressor protein complex have found that the promoters of many, many genes are occupied by and regulated by these proteins (Dimova et al. 2003; Fernandez et al. 2003; Orian et al. 2003). For example, it has been estimated that 10%–15% of all human and *Drosophila* genes may be regulated by the Myc–Mad–Max network. In addition, these proteins have recently been shown to regulate pol III transcription in addition to the more heavily studied regulation of mRNA transcription by pol II (Felton-Edkins et al. 2003). Recent studies in *Drosophila* have also shown that ectopic Myc expression can drive nucleolar enlargement, possibly by stimulation of pol I transcription of rDNA (Pierce et al. 2004).

The genetics of the synMuv phenotype together with recent biochemical and genomic studies cited above suggest that perhaps we have been thinking too narrowly. In addition to searching for a small number of critical target genes, perhaps we should also be thinking about our old friends Myc, Myb, and RB as global regulators of chro-

matin structure that act to homeostatically buffer signaling via various pathways including RTK–RAS. In this regard, the realization that epigenetic states are fluid and that epigenetic alterations can collaborate with genetic mutations in oncogenesis seems quite timely (Ahmad and Henikoff 2002; Lund and van Lohuizen 2004). In the same way that oncogenic mutations in DNA repair enzymes and DNA damage checkpoint proteins offered support for the mutator hypothesis, perhaps oncogenic mutations in global transcriptional regulators like Myc, Myb, and RB are pointing the way to a parallel hypothesis in which an increased frequency of unbuffered, metastable epigenetic states favors oncogenic transformation.

### Acknowledgments

I offer thanks to US taxpayers and the Congress for their generous support of the NIH, which has funded research in my laboratory; to Stanford University for providing salary and research space; to many colleagues and collaborators for lively discussions; and to the members of my family for their understanding.

### References

- Aggarwal, B.D. and Calvi, B.R. 2004. Chromatin regulates origin activity in *Drosophila* follicle cells. *Nature* **430**: 372–376.
- Ahmad, K. and Henikoff, S. 2002. Epigenetic consequences of nucleosome dynamics. *Cell* **111**: 281–284.
- Amatschek, S., Koenig, U., Auer, H., Steinlein, P., Pacher, M., Gruenfelder, A., Dekan, G., Vogl, S., Kubista, E., Heider, K.H., et al. 2004. Tissue-wide expression profiling using cDNA subtraction and microarrays to identify tumor-specific genes. *Cancer Res.* **64**: 844–856.
- Beall, E.L., Manak, J.R., Zhou, S., Bell, M., Lipsick, J.S., and Botchan, M.R. 2002. Role for a *Drosophila* Myb-containing protein complex in site-specific DNA replication. *Nature* **420**: 833–837.
- Beall, E.L., Bell, M., Georgette, D., and Botchan, M.R. 2004. Dm-*myb* mutant lethality in *Drosophila* is dependent upon *mip130*: Positive and negative regulation of DNA replication. *Genes & Dev.* **18**: 1667–1680.
- Bell, S.P. and Dutta, A. 2002. DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* **71**: 333–374.
- Bhatt, A.M., Zhang, Q., Harris, S.A., White-Cooper, H., and Dickinson, H. 2004. Gene structure and molecular analysis of *Arabidopsis thaliana* ALWAYS EARLY homologs. *Gene* **336**: 219–229.
- Bishop, J.M. 1995. Cancer: The rise of the genetic paradigm. *Genes & Dev.* **9**: 1309–1315.
- Bosco, G., Du, W., and Orr-Weaver, T.L. 2001. DNA replication control through interaction of E2F–RB and the origin recognition complex. *Nat. Cell Biol.* **3**: 289–295.
- Boxem, M. and van den Heuvel, S. 2002. *C. elegans* class B synthetic multivulva genes act in G1 regulation. *Curr. Biol.* **12**: 906–911.
- Calvi, B.R. and Spradling, A.C. 1999. Chorion gene amplification in *Drosophila*: A model for metazoan origins of DNA replication and S-phase control. *Methods* **18**: 407–417.
- Calvi, B.R., Lilly, M.A., and Spradling, A.C. 1998. Cell cycle control of chorion gene amplification. *Genes & Dev.* **12**: 734–744.

- Cayirlioglu, P., Bonnette, P.C., Dickson, M.R., and Duronio, R.J. 2001. *Drosophila* E2f2 promotes the conversion from genomic DNA replication to gene amplification in ovarian follicle cells. *Development* **128**: 5085–5098.
- Cayirlioglu, P., Ward, W.O., Silver Key, S.C., and Duronio, R.J. 2003. Transcriptional repressor functions of *Drosophila* E2F1 and E2F2 cooperate to inhibit genomic DNA synthesis in ovarian follicle cells. *Mol. Cell. Biol.* **23**: 2123–2134.
- Ceol, C.J. and Horvitz, H.R. 2001. dpl-1 DP and efl-1 E2F act with lin-35 Rb to antagonize Ras signaling in *C. elegans* vulval development. *Mol. Cell* **7**: 461–473.
- . 2004. A new class of *C. elegans* synMuv genes implicates a Tip60/NuA4-like HAT complex as a negative regulator of Ras signaling. *Dev. Cell* **6**: 563–576.
- Classon, M. and Harlow, E. 2002. The retinoblastoma tumour suppressor in development and cancer. *Nat. Rev. Cancer* **2**: 910–917.
- Claycomb, J.M., MacAlpine, D.M., Evans, J.G., Bell, S.P., and Orr-Weaver, T.L. 2002. Visualization of replication initiation and elongation in *Drosophila*. *J. Cell Biol.* **159**: 225–236.
- Davidson, C., Tirouvanziam, R., Herzenberg, L., and Lipsick, J. 2004. Functional evolution of the vertebrate Myb gene family: B-Myb, but neither A-Myb nor c-Myb, complements *Drosophila* Myb in hemocytes. *Genetics* (in press).
- Dimova, D.K., Stevaux, O., Frolov, M.V., and Dyson, N.J. 2003. Cell cycle-dependent and cell cycle-independent control of transcription by the *Drosophila* E2F/RB pathway. *Genes & Dev.* **17**: 2308–2320.
- Du, W., Vidal, M., Xie, J.E., and Dyson, N. 1996. RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in *Drosophila*. *Genes & Dev.* **10**: 1206–1218.
- Felton-Edkins, Z.A., Kenneth, N.S., Brown, T.R., Daly, N.L., Gomez-Roman, N., Grandori, C., Eisenman, R.N., and White, R.J. 2003. Direct regulation of RNA polymerase III transcription by RB, p53 and c-Myc. *Cell Cycle* **2**: 181–184.
- Ferguson, E.L. and Horvitz, H.R. 1989. The multivulva phenotype of certain *Caenorhabditis elegans* mutants results from defects in two functionally redundant pathways. *Genetics* **123**: 109–121.
- Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A., and Amati, B. 2003. Genomic targets of the human c-Myc protein. *Genes & Dev.* **17**: 1115–1129.
- Fitzpatrick, C.A., Sharkov, N.V., Ramsay, G., and Katzen, A.L. 2002. *Drosophila* myb exerts opposing effects on S phase, promoting proliferation and suppressing endoreduplication. *Development* **129**: 4497–4507.
- Fung, S.M., Ramsay, G., and Katzen, A.L. 2002. Mutations in *Drosophila* myb lead to centrosome amplification and genomic instability. *Development* **129**: 347–359.
- . 2003. Myb and CBP: Physiological relevance of a biochemical interaction. *Mech. Dev.* **120**: 711–720.
- Gagrica, S., Hauser, S., Kolfschoten, I., Osterloh, L., Agami, R., and Gaubatz, S. 2004. Inhibition of oncogenic transformation by mammalian Lin-9, a pRB-associated protein. *EMBO J.* (in press).
- Ganter, B. and Lipsick, J.S. 1999. Myb and oncogenesis. *Adv. Cancer Res.* **76**: 21–60.
- Hennig, L., Taranto, P., Walser, M., Schonrock, N., and Gruißsem, W. 2003. *Arabidopsis* MSI1 is required for epigenetic maintenance of reproductive development. *Development* **130**: 2555–2565.
- Horvitz, H.R. and Sulston, J.E. 1980. Isolation and genetic characterization of cell-lineage mutants of the nematode *Caenorhabditis elegans*. *Genetics* **96**: 435–454.
- Hou, D.X., Akimaru, H., and Ishii, S. 1997. Trans-activation by the *Drosophila* myb gene product requires a *Drosophila* homologue of CBP. *FEBS Lett.* **413**: 60–64.
- Humbert, P.O., Verona, R., Trimarchi, J.M., Rogers, C., Dandapani, S., and Lees, J.A. 2000. E2f3 is critical for normal cellular proliferation. *Genes & Dev.* **14**: 690–703.
- Hurford Jr., R.K., Cobrinik, D., Lee, M.H., and Dyson, N. 1997. pRb and p107/p130 are required for the regulated expression of different sets of E2f responsive genes. *Genes & Dev.* **11**: 1447–1463.
- Ito, M., Araki, S., Matsunaga, S., Itoh, T., Nishihama, R., Machida, Y., Doonan, J.H., and Watanabe, A. 2001. G2/M-phase-specific transcription during the plant cell cycle is mediated by c-Myb-like transcription factors. *Plant Cell* **13**: 1891–1905.
- Joaquin, M. and Watson, R.J. 2003. Cell cycle regulation by the B-Myb transcription factor. *Cell. Mol. Life Sci.* **60**: 2389–2401.
- Joaquin, M., Bessa, M., Saville, M.K., and Watson, R.J. 2002. B-Myb overcomes a p107-mediated cell proliferation block by interacting with an N-terminal domain of p107. *Oncogene* **21**: 7923–7932.
- Katzen, A.L. and Bishop, J.M. 1996. myb provides an essential function during *Drosophila* development. *Proc. Natl. Acad. Sci.* **93**: 13955–13960.
- Katzen, A.L., Kornberg, T.B., and Bishop, J.M. 1985. Isolation of the proto-oncogene c-myc from *D. melanogaster*. *Cell* **41**: 449–456.
- Katzen, A.L., Jackson, J., Harmon, B.P., Fung, S.M., Ramsay, G., and Bishop, J.M. 1998. *Drosophila* myb is required for the G2/M transition and maintenance of diploidy. *Genes & Dev.* **12**: 831–843.
- Kaufman, P.D. 1996. Nucleosome assembly: The CAF and the HAT. *Curr. Opin. Cell Biol.* **8**: 369–373.
- Knudson, A.G. 2001. Two genetic hits (more or less) to cancer. *Nat. Rev. Cancer* **1**: 157–162.
- Korenjak, M., Taylor-Harding, B., Binne, U.K., Satterlee, J.S., Stevaux, O., Aasland, R., White-Cooper, H., Dyson, N., and Brehm, A. 2004. Native E2F/RBF complexes contain Myb-interacting proteins and repress transcription of developmentally controlled E2F target genes. *Cell* **119**: 181–193.
- Lewis, P.W., Beall, E.L., Fleischer, T.C., Georlette, D., Link, A.J., and Botchan, M.R. 2004. Identification of a *Drosophila* Myb-E2F2/RBF transcriptional repressor complex. *Genes & Dev.* (this issue).
- Lu, X. and Horvitz, H.R. 1998. lin-35 and lin-53, two genes that antagonize a *C. elegans* Ras pathway, encode proteins similar to Rb and its binding protein RbAp48. *Cell* **95**: 981–991.
- Lund, A.H. and van Lohuizen, M. 2004. Epigenetics and cancer. *Genes & Dev.* **18**: 2315–2335.
- Malumbres, M. and Barbacid, M. 2003. RAS oncogenes: The first 30 years. *Nat. Rev. Cancer* **3**: 459–465.
- Manak, J.R., Mitiku, N., and Lipsick, J.S. 2002. Mutation of the *Drosophila* homologue of the Myb protooncogene causes genomic instability. *Proc. Natl. Acad. Sci.* **99**: 7438–7443.
- Mucenski, M.L., McLain, K., Kier, A.B., Swerdlow, S.H., Schreiner, C.M., Miller, T.A., Pietryga, D.W., Scott Jr., W.J., and Potter, S.S. 1991. A functional c-myc gene is required for normal murine fetal hepatic hematopoiesis. *Cell* **65**: 677–689.
- Nevins, J.R. 2001. The Rb/E2F pathway and cancer. *Hum. Mol. Genet.* **10**: 699–703.
- Ohi, R., Feoktistova, A., McCann, S., Valentine, V., Look, A.T., Lipsick, J.S., and Gould, K.L. 1998. Myb-related *Schizosaccharomyces pombe* cdc5p is structurally and functionally conserved in eukaryotes. *Mol. Cell. Biol.* **18**: 4097–4108.
- Okada, M., Akimaru, H., Hou, D.X., Takahashi, T., and Ishii, S.

2002. Myb controls G2/M progression by inducing cyclin B expression in the *Drosophila* eye imaginal disc. *EMBO J.* **21**: 675–684.
- Orian, A., van Steensel, B., Delrow, J., Bussemaker, H.J., Li, L., Sawado, T., Williams, E., Loo, L.W., Cowley, S.M., Yost, C., et al. 2003. Genomic binding by the *Drosophila* Myc, Max, Mad/Mnt transcription factor network. *Genes & Dev.* **17**: 1101–1114.
- Pierce, S.B., Yost, C., Britton, J.S., Loo, L.W., Flynn, E.M., Edgar, B.A., and Eisenman, R.N. 2004. dMyc is required for larval growth and endoreplication in *Drosophila*. *Development* **131**: 2317–2327.
- Qian, Y.W., Wang, Y.C., Hollingsworth Jr., R.E., Jones, D., Ling, N., and Lee, E.Y. 1993. A retinoblastoma-binding protein related to a negative regulator of Ras in yeast. *Nature* **364**: 648–652.
- Raschella, G., Cesi, V., Amendola, R., Negroni, A., Tanno, B., Altavista, P., Tonini, G.P., De Bernardi, B., and Calabretta, B. 1999. Expression of B-myb in neuroblastoma tumors is a poor prognostic factor independent from MYCN amplification. *Cancer Res.* **59**: 3365–3368.
- Ridgway, P. and Almouzni, G. 2000. CAF-1 and the inheritance of chromatin states: At the crossroads of DNA replication and repair. *J. Cell Sci.* **113** (Pt 15): 2647–2658.
- Robinson, C., Light, Y., Groves, R., Mann, D., Marias, R., and Watson, R. 1996. Cell-cycle regulation of B-Myb protein expression: Specific phosphorylation during the S phase of the cell cycle. *Oncogene* **12**: 1855–1864.
- Royzman, I., Austin, R.J., Bosco, G., Bell, S.P., and Orr-Weaver, T.L. 1999. ORC localization in *Drosophila* follicle cells and the effects of mutations in dE2F and dDP. *Genes & Dev.* **13**: 827–840.
- Rubin, G.M., Chang, H.C., Karim, F., Laverty, T., Michaud, N.R., Morrison, D.K., Rebay, I., Tang, A., Therrien, M., and Wassarman, D.A. 1997. Signal transduction downstream from Ras in *Drosophila*. *Cold Spring Harb. Symp. Quant. Biol.* **62**: 347–352.
- Ruggieri, R., Tanaka, K., Nakafuku, M., Kaziro, Y., Toh-e, A., and Matsumoto, K. 1989. MSII, a negative regulator of the RAS-cAMP pathway in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **86**: 8778–8782.
- Sala, A., DeLuca, A., Giordano, A., and Peschle, C. 1996. The retinoblastoma family member p107 binds to B-MYB and suppresses its autoregulatory activity. *J. Biol. Chem.* **271**: 28738–28740.
- Sharkov, N.V., Ramsay, G., and Katzen, A.L. 2002. The DNA replication-related element-binding factor (DREF) is a transcriptional regulator of the *Drosophila* myb gene. *Gene* **297**: 209–219.
- Simon, A.L., Stone, E.A., and Sidow, A. 2002. Inference of functional regions in proteins by quantification of evolutionary constraints. *Proc. Natl. Acad. Sci.* **99**: 2912–2917.
- Solari, F. and Ahringer, J. 2000. NURD-complex genes antagonise Ras-induced vulval development in *Caenorhabditis elegans*. *Curr. Biol.* **10**: 223–226.
- Sternberg, P.W. and Han, M. 1998. Genetics of RAS signaling in *C. elegans*. *Trends Genet.* **14**: 466–472.
- Stevaux, O., Dimova, D., Frollov, M.V., Taylor-Harding, B., Morris, E., and Dyson, N. 2002. Distinct mechanisms of E2F regulation by *Drosophila* RBF1 and RBF2. *EMBO J.* **21**: 4927–4937.
- Stober-Grasser, U., Brydolf, B., Bin, X., Grasser, F., Firtel, R.A., and Lipsick, J.S. 1992. The Myb DNA-binding domain is highly conserved in *Dictyostelium discoideum*. *Oncogene* **7**: 589–596.
- Tanaka, Y., Patestos, N.P., Maekawa, T., and Ishii, S. 1999. B-myb is required for inner cell mass formation at an early stage of development. *J. Biol. Chem.* **274**: 28067–28070.
- Taylor-Harding, B., Binne, U.K., Korenjak, M., Brehm, A., and Dyson, N.J. 2004. p55, the *Drosophila* ortholog of RbAp46/RbAp48, is required for the repression of dE2F2/RBF-regulated genes. *Mol. Cell. Biol.* **24**: 9124–9136.
- Toscani, A., Mettus, R.V., Coupland, R., Simpkins, H., Litvin, J., Orth, J., Hatton, K.S., and Reddy, E.P. 1997. Arrest of spermatogenesis and defective breast development in mice lacking A-myb. *Nature* **386**: 713–717.
- Tower, J. 2004. Developmental gene amplification and origin regulation. *Annu. Rev. Genet.* (in press).
- Tsukiyama, T., Daniel, C., Tamkun, J., and Wu, C. 1995. ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. *Cell* **83**: 1021–1026.
- van Nocker, S. 2003. CAF-1 and MSII-related proteins: Linking nucleosome assembly with epigenetics. *Trends Plant Sci.* **8**: 471–473.
- Verreault, A., Kaufman, P.D., Kobayashi, R., and Stillman, B. 1996. Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. *Cell* **87**: 95–104.
- von Zelewsky, T., Palladino, F., Brunschwig, K., Tobler, H., Hajnal, A., and Muller, F. 2000. The *C. elegans* Mi-2 chromatin-remodelling proteins function in vulval cell fate determination. *Development* **127**: 5277–5284.
- White-Cooper, H., Leroy, D., MacQueen, A., and Fuller, M.T. 2000. Transcription of meiotic cell cycle and terminal differentiation genes depends on a conserved chromatin associated protein, whose nuclear localisation is regulated. *Development* **127**: 5463–5473.
- Wismar, J., Löffler, T., Habtemichael, N., Vef, O., Geissen, M., Zirwes, R., Altmeyer, W., Sass, H., and Gateff, E. 1995. The *Drosophila melanogaster* tumor suppressor gene lethal(3)malignant brain tumor encodes a proline-rich protein with a novel zinc finger. *Mech. Dev.* **53**: 141–154.
- Zwicker, J., Liu, N., Engeland, K., Lucibello, F.C., and Muller, R. 1996. Cell cycle regulation of E2F site occupation in vivo. *Science* **271**: 1595–1597.