Regulation of differentiation-specific genes by the *Drosophila* RB, E2F, And Myb-interacting proteins complex (dREAM)

by

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ABSTRACT OF THE DISSERTATION

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By Hangnoh Lee

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RB and E2F proteins play important roles in the regulation of cell division, cell death and development, by controlling the expression of genes involved in these processes. The mechanisms of repression by pRB have been extensively studied at cell cycle regulated promoters. However, little is known about developmentally regulated E2F/RB genes. Here I have taken advantage of the simplicity of the E2F/RB pathway in flies, and inspected the regulation of differentiation-specific target genes.

These genes are repressed by dE2F2/RBF and a recently identified RB-containing complex, dREAM, in a cell type- and cell cycle-independent manner. Two different types of activities are involved in their regulation. First, I find that dREAM employs histone deacetylase (HDAC) activities at promoter regions and that HDACs are required to maintain repression. Second, I find that the Polycomb Group (PcG) protein, Enhancer of zeste - E(Z), is involved in silencing of these genes through the di-methylation of histone H3 Lys27 at nucleosomes located downstream of the transcription start sites (TSS). While HDAC activity is also involved in the regulation of cell cycle dependent E2F transcription, E(Z) functions at differentiation-specific target genes only, indicating that the two groups of genes are regulated in a distinct manner.
The differentiation-specific genes are also regulated differently from cell cycle-related E2F targets, in a way that they do not depend on E2F-activation. E2F/RB repression is maintained throughout the cell cycle. I demonstrated that the dREAM complex is required for dE2F2 binding at differentiation-specific, but not cell cycle-regulated E2F/RB target gene promoters. Especially, dREAM complex is necessary for dE2F2/RBF binding in S-phase.

Taking together, my results demonstrate that dREAM plays a dual role in the regulation of differentiation-specific genes. dREAM complex is required for the stability of dE2F2/RBF complexes at these promoters during S-phase, and also for the repression mechanisms employed at these genes.
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Chapter I

Introduction
1. E2F/RB family of proteins are transcription factors that regulate the cell cycle

The E2F family of proteins are transcription factors. In mammals, eight different E2F genes produce activating (E2F1-3) or repressive (E2F4-8) proteins. E2F function is controlled by RB family proteins; pRB (=RB-1), p107 (=RBL-1), and p130 (=RBL-2). They are known to physically associate with E2F proteins. To bind to target DNA, E2F proteins need to form heterodimers with DP proteins. RB family members cannot bind to DNA directly, but are recruited by E2F/DP heterodimers.

E2F/RB activity is required for various cellular functions, yet the best understood property is their regulation of the cell cycle at the G1/S transition. In the traditional view of E2F/RB regulation, a repressive complex of E2F and a RB family protein is bound at the promoter regions of genes required for S phase (Figure 1). The transition into S phase is promoted by Cyclin-Dependent Kinases (CDKs). CDKs phosphorylate RB family proteins and lead to the dissociation of the repressive E2F/RB complex. CDK phosphorylation also liberates another type of E2F protein, an activating E2F, from RB proteins and allows it to bind at the promoters to transactivate the genes.
Figure 1 Cell cycle regulation of E2F and RB proteins

In the early stages of G1, the repressive complexes of E2F/RB are bound at the target promoters and suppress the transcription of the genes required for S phase entry. As cells undergo cell-cycle progression, G1 phase-specific cyclin-dependent kinases (CDKs) phosphorylate RB proteins. This phosphorylation results in the dissociation of RB proteins from E2Fs. Activator E2Fs (green), then, occupy the promoter regions and enable gene transcription.
2. Members of E2F/RB system

A. *E2F proteins in mammalian systems*

(i) **Activator E2F proteins (E2F1-3)**

In mammals, E2F proteins are the products of 8 different E2F genes. Among them, E2F1, E2F2 and E2F3 function as activators of transcription and promote cell cycle progression. E2F1 was the first identified E2F protein, a cellular factor that binds to the promoter of the adenoviral E2 gene. The viral E2 gene is stimulated by the product of adenovirus E1A gene (Imperiale et al., 1984) and is required for transforming quiescent cells into dividing tumors. Subsequently the E2F2 and E2F3 proteins were identified based on their sequence homology to E2F1 (Dyson et al., 1993; Helin et al., 1992). All three proteins have a ubiquitination site and a Cyclin A binding domain at N-terminus, which are required for the regulation of half-life and DNA binding affinity (Dynlacht et al., 1997; Marti et al., 1999). They also have DNA binding domain, a dimerization domain and a marked box domain that mediate interactions with various transcription factors. pRB binds to the transactivation domain at C-terminus (Reviewed in Dimova and Dyson, 2005; Figure 2A). Overexpression of these proteins transactivates luciferase reporter constructs with E2F binding sites (Lees et al., 1992). The *E2F3* gene is known to transcribe two different isoforms. Interestingly, the two isoforms of E2F3 have opposing functions. E2F3a belongs to the activator E2F group, while E2F3b belongs to the repressive group. E2F3a and E2F3b are transcribed using two different promoters, and their first exons are different. As a result, E2F3b lacks the N-terminal domain and this is believed to be the reason that E2Fb is constitutively expressed throughout the cell cycle (Leone et al., 2000)
Figure 2. Structures of mammalian E2F, DP and RB family of proteins

(A) Schematic structure of mammalian E2F proteins. E2F1 – 3 have conserved Cyclin A binding domain, DNA binding domain (DBD), Dimerization domain, Marked Box domain (MB) and transactivation domain. Dimerization domain is required to form a heterodimer with DP proteins.
RB binding site is within the transactivation domain. E2F 6 – 8 do not have transactivation domain, and they are not controlled by RB family of proteins. There is no dimerization domain for DP in E2F7 and 8. (B) DP proteins lack transactivation domain. (C) RB family of proteins. “A” and “B” are two conserved domains that make up the structural pocket. Both CDK inhibitor domain and CDK binding domains are required to inhibit CDK functions in growth suppression.
(ii) Repressive E2F proteins (E2F4-5)

The other five E2F proteins (E2F4-8) are repressors of transcription and cell proliferation. E2F4 and E2F5 were the first to be identified (Bernards et al., 1989). Both proteins have significant similarity to the E2F1 protein but lack the cyclin A-binding domain (Beijersbergen et al., 1994; Hijnmans et al., 1995). E2F4 and E2F5 are involved in repression of genes required for cellular proliferation. Chromatin immunoprecipitation assays demonstrated that many E2F target promoters are bound by E2F4 in G0/G1 phase, but during cell cycle progression the DNA-protein interaction is disrupted (Takahashi et al., 2000; Verona et al., 1997). Mouse embryonic fibroblasts (MEFs) from E2F4 and E2F5 double knockout mice cannot be arrested at G1 when p16, a CDK inhibitor, was over-expressed (Gaubatz et al., 2000). These observations underscore the repressive roles of E2F4/5 in cell cycle progression. The repression activity may employ co-repressors. E2F4/5 – p107/p130 complexes were shown to interact with various transcriptional repressors such as histone deacetylases (HDACs) or histone methyltransferases (HMTs) (Brehm et al., 1998).

In addition to its repressive roles, E2F4 and E2F5 may function as activators in some contexts. Overexpression of them induces S phase entry and up-regulation of a number of cell cycle genes, although the degree is less compared to the overexpression of E2F 1-3 (Lukas et al., 1996; DeGregori et al., 1997). How they function as activators needs further study.

(ii) Repressive E2F proteins with distinct repression mechanisms (E2F6, 7, 8)

E2F6 is also a repressive E2F protein with slightly different structure and mechanism of repression. It was found based on sequence homology to a conserved E2F DNA-binding domain. Overexpression of E2F6 inhibits expression of E2F-responsive reporter genes, demonstrating its role in transcriptional inhibition. (Cartwright et al., 1998; Trimarchi et al., 1998). Surprisingly,
E2F6 does not have an RB family protein-binding domain that is found in E2F1-5 proteins (Figure 2a). Instead, E2F6 was shown to interact with chromatin modifiers and Polycomb Group proteins (PcG). These proteins include RYBP (Ring 1- and YY1-binding protein 1), Bmi 1, Ring 1/2, HP (Heterochromatin Protein) 1γ, YAF2 (YY1 interactive protein = PHO), or EPC (Enhancer of Polycomb) proteins (Attwooll et al., 2005; Ogawa et al., 2002; Trimarchi et al., 2001). Based on these observations, it was suggested that E2F6 plays a role in establishing long-term repression of cell cycle genes by inducing cell cycle exit and cellular quiescence (G0) (Courel et al., 2008; Ogawa et al., 2002; Pohlers et al., 2005).

E2F7 and E2F8 are the newest members of the repressive E2F family with different DNA binding pattern (Christensen et al., 2005; Di Stefano et al., 2003; Logan et al., 2004; Logan et al., 2005; Maiti et al., 2005). Several groups have demonstrated that E2F7 and E2F8 bind to a consensus E2F-binding sequence. Ectopic expression of E2F7/8 results in suppression of E2F-driven gene transcription and inhibition of cellular proliferation, classifying E2F7 and E2F8 as transcriptional repressors. Despite their similar structure to other E2F members, both function independently of DP proteins. E2F7 and E2F8 lack the DP-dimerization domain, but contain two DNA-binding domains (DBD) instead, which enables them to form E2F7:E2F7 and E2F8:E2F8 homodimers or E2F7:E2F8 heterodimers to bind to DNA (Figure 2A).

B. DP proteins are required for DNA binding of E2Fs

All the E2Fs, except E2F7 and E2F8, heterodimerize with DP proteins for DNA binding (Bandara et al., 1993; Helin et al., 1993; Huber et al., 1993). Three DP proteins exist in mammals - DP1, DP2/3 and DP4. DP proteins have high homology to E2F1, but it lacks the C-terminal domain that is required for transactivation and/or RB family protein binding (Figure 2B). Therefore it is believed that DP1 by itself does not have direct transcriptional activity, but it becomes functional through dimerization with E2F proteins (Helin et al., 1993). Although DP1 and
DP2 are expressed differentially across tissues, their biochemical properties are similar. Both proteins are capable of binding to E2F proteins either to activate or to repress genes (Girling et al., 1994; Wu et al., 1995). The murine homolog of DP2 is often referred to as DP3, since the protein was discovered after human DP1 and DP2 (Ormondroyd et al., 1995). Human DP4 has similar primary structure to DP1 or DP2. However, its binding to E2F largely reduces the DNA-binding activity of the heterodimer. As a result, E2F/DP4 complex lacks trans-activating capability, and ectopic expression of DP4 delays G1 to S cell cycle transition (Milton et al., 2006; Qiao et al., 2007). This observation indicates distinct characteristics of DP4, which should be explored further to understand the regulatory roles of DP proteins in control of E2F activities.

C. RB family proteins regulate E2Fs.

pRB was identified as an interacting partner of E2F. Conceptually, adenoviral E1A can function in infection by suppressing another cellular factor that prevents early viral transcription (Nevins, 1981). E1A can release E2F from an E2F-containing complex that is found in uninfected cells (Bagchi et al., 1990), thereby allowing E2F to promote infection. In search of these factors, researchers have identified various sized cellular proteins that bind to viral E1A protein using immunoprecipitation (Harlow et al., 1986). One of them is a 110 kDa protein - pRB (Lee et al., 1987a; Lee et al., 1987b). Further studies identified that the interaction of pRB with viral E1A or E2F depends on the LXCXE motif, which can be found in various cellular proteins. E2Fs, cyclin D1, HDAC1, HDAC2, BRG1, as well as viral proteins such as adenovirus E1A, HPV E7, SV40 T antigen contain a LXCXE motif and interact with pRB (Brehm et al., 1998; Dowdy et al., 1993; Dunaief et al., 1994; Lee et al., 1998; Moran, 1993a, b). The physical interaction of pRB with E2F is influenced by the phosphorylation status of pRB. When hypo-phosphorylated, pRB can bind to E2F (Chellappan et al., 1991) and regulate the transcriptional potential of E2F. Hyper-phosphorylated pRB is inactive and releases free E2F.
The identification of the RB protein also led to the discovery of its two relatives, p107 (RBL1) and p130 (RBL2). p107 was discovered as an E1A co-immunoprecipitating protein. The interaction of p107 and E1A also depends on the LXCXE motif of E1A (Dyson et al., 1989). The other member of the RB family, p130, was identified by mass spectrometry analysis of the proteins precipitated by an E1A antibody (Li et al., 1993).

pRB, p107, and p130, are collectively called ‘pocket proteins’. X-ray crystallography studies revealed that pRB contains a structural pocket to which viral proteins can bind (Figure 2C) (Hannon et al., 1993; Kim and Cho, 1997). p107 and p130 have homologous sequences for the pocket domain as well. Aside from its association with viral proteins, the pocket region serves as a site for interacting with many cellular proteins, including E2F proteins. (Ferreira et al., 1998; Kim et al., 2001; Lee et al., 2002; Lee et al., 1998). Particularly, cellular or viral proteins that contain LXCXE motif are known to interact with the hydrophobic region of the pocket (Chan et al., 2001b; Lee et al., 1998). In addition to the pocket domain, p107 and p130 have binding sites for cyclin-dependent kinases that are not found in pRB (Hannon et al., 1993; Lees et al., 1992) (Figure 2C).

Each RB family protein member specifically interacts with different E2F proteins. All activating E2Fs (E2F1-3) bind to pRB, but not to p107 or p130 in vivo (Lees et al., 1993). E2F4 can bind to all three RB family proteins to form repressive complexes. E2F4 mainly interacts with p130 in cells arrested in G0 because p130 is the predominant species in G0 cells. In cycling cells E2F4 switches partners to pRB or p107 (Beijersbergen et al., 1994; Moberg et al., 1996; Vairo et al., 1995). E2F5 preferentially interacts with p130 in vivo, although it has some affinity to p107 in vitro (Hijmans et al., 1995). However, E2F5 does not bind to pRB. E2F6, E2F7 and E2F8 do not interact with RB family proteins because they lack C-terminal binding domain (Cartwright et al., 1988; Christensen et al., 2005; Di Stefano et al., 2003; Logan et al., 2004; Logan et al., 2005; Maiti et al., 2005; Trimarchi et al., 1998). The different E2F/Pocket Protein complexes in mammals are described in Figure 3.
Mammals have three activator E2Fs (E2F 1,2,3a); they are regulated by pRB. There are six repressive E2Fs (E2F 3b, 4 – 8). E2F4 can interact with all RB family of proteins, while E2F5 binds to p107 and p130, but not pRB. E2F 6 – 8 do not have binding sites for the pocket proteins. E2F6 interacts with Polycomb Group Proteins (PcG)

**Figure 3.** The E2F/RB networks in mammalian systems
D. Homologs of E2F and RB in worms, flies, and plants

E2Fs and RB family proteins are evolutionally conserved in multicellular eukaryotic systems, indicating their functional importance. Most of the widely used model organisms have E2F and RB related proteins. The E2F/RB networks in *C. elegans*, *Drosophila*, and plants are well-characterized (Figure 4).

*C. elegans* has three E2F-like genes that encode EFL-1, EFL-2, and EFL-3 proteins (Figure 4A). All of them require DP-like protein (DPL-1) for DNA binding (Figure 4A). The gene structure of *C. elegans* EFL-1 shows a strong similarity to mammalian E2F4 and it has a repressive role in transcription. Based on its sequence, EFL-2 does not appear to have a clear mammalian counterpart, yet its pro-apoptotic activity is reminiscent of mammalian E2F1. Both EFL-1 and EFL-2 are regulated by a single RB family protein, LIN-35 (Ceol and Horvitz, 2001; Lu and Horvitz, 1998; Page et al., 2001; Schertel and Conradt, 2007). EFL-3 is a mammalian E2F7/8 homolog. This protein has been shown to interact with LIN-39, which is the *C. elegans* counterpart of human Polycomb (Pc) (Winn et al., 2011).

Like worms, the *Drosophila* system has a simpler, but functionally well-conserved E2F/RB network (Figure 4B). Two *Drosophila* E2F proteins, dE2F1 and dE2F2 serve as activating or repressing E2F proteins respectively. They are regulated by two different RB family proteins, RBF1 and RBF2, and the regulation is similar to that in mammals. RBF1 interacts with both activator and repressor E2F proteins. On the other hand, RBF2 regulates only dE2F2. E2Fs heterodimerize with *Drosophila* DP protein, which is the sole homolog of mammalian DPs (Du et al., 1996a; Duronio et al., 1995; Frolov et al., 2001; Stevaux et al., 2002). There have been no equivalents of the mammalian E2F6, 7, and 8 found.

E2F and RB family proteins exist in plants as well. RBR (RB-related) is found to be the equivalent component of mammalian RB family proteins that regulates plant E2F proteins (Ach et al., 1997; Grafi et al., 1996; Magyar et al., 2000; Rossignol et al., 2002). Not surprisingly, different
plant species seem to have different number of RB members and potentially different E2F-RB combinations. For instance, maize has two different RB-related proteins ZmRBR1 and ZmRBR3, while Arabidopsis has only one AtRBR that regulates six AtE2F proteins (Figure 4D) (Lendvai et al., 2007; Mariconti et al., 2002; Sabelli et al., 2005). Interestingly, AtE2Fd,e,f were identified as DP independent E2Fs before mammalian E2F7 and E2F8 were found.

Studies of E2F and RB family of proteins in non-mammalian models are a great help in elucidating their functions. Understanding how E2F/RB functions in a network is difficult to study in the mammalian system because of the complexity and functional redundancy. Availability of simpler E2F/RB networks in model organisms provides genetically accessible means to study a complex pathway.
Figure 4. E2F/RB networks in model organisms

(A) *C. elegans* has one RB protein (LIN-35) that regulates EFL-1 (repressor) and EFL-2. The role of EFL-1 is not well understood, but is likely to be an equivalent of mammalian E2F1. EFL-3 does not bind to LIN-35, but interacts with Polycomb Group Proteins (PcG) similar to mammalian E2F6.
(B) *Drosophila system.* dE2F1 is an activator and dE2F2 is a repressor. RBF1 binds to both of dE2Fs. RBF2 only can interact with dE2F2. No equivalents for E2F 6 – 8 have been found yet. (C) *Arabidopsis* has one RB homologous protein, AtRBR. AtRBR regulates three E2F-like proteins (AtE2Fa,b,c). AtE2Fa and AtE2Fb are activators and preferentially dimerize with AtDPa. AtE2Fc is a repressor and binds to AtDPb. AtE2Fd,e,f are mammalian E2F7-8 equivalents. They do not interact with RB or DP family of proteins.
3. Cell cycle regulation of E2F and RB

A. *CDK mediated phosphorylation of RB family of proteins is important for the G1/S transition*

Phosphorylation of RB family proteins is important for their functions during the cell cycle. pRB and p107 proteins are phosphorylated as the cells go into S phase. Both pRB and p107 are hypo-phosphorylated in early G1 phase. In late G1, proliferative signals activate cyclin D and E that turn on CDK4/6 (cyclin D) and CDK2 (cyclin E). (Kitagawa et al., 1996; Zarkowska and Mittnacht, 1997). Phosphorylation of pRB as well as p107 by CDKs promotes dissociation of repressive complexes and also releases activator E2Fs from pRB. As a result, E2F1-3 are able to transactivate cell cycle-related genes. Therefore, in the traditional view of how E2F/RB regulate cell cycle progression, RB family proteins are supposed to be phosphorylated during the S phase to allow transcription of genes required for cell cycle progression.

pRB has 16 putative CDK phosphorylation sites (Ser/Thr-Pro), where 15 of them have been demonstrated to be phosphorylated by CDKs (Hirschi et al., 2010; Knudsen and Wang, 1996; Lukas et al., 1997). In their elegant study of *in vitro* pRB phosphorylation, the Mittnacht’s lab has demonstrated that different CDKs phosphorylate different residues of pRB. They found that CDK2 and CDK4 phosphorylate partially overlapping (two residues), but essentially different sites of pRB. The result suggests that distinct patterns of CDK phosphorylation may determine different functions of the protein. Supporting this idea, CDK2 is able to dissociate the large T antigen of Simian Virus 40 from pRB, while CDK4 cannot (Zarkowska and Mittnacht, 1997). A similar study has shown that CDK2 mediated phosphorylation of pRB is not enough to dissociate E2F4 from pRB; a distinct modification by CDK4 (Ser 795) is required for pRB inactivation (Connell-Crowley et al., 1998). These observations indicate that different kinases may have different roles in phosphorylating pRB, but understanding how they co-ordinate cell cycle progression requires further study. Phosphorylation of p107 is similar in many ways to that of pRB. However, SV40 large T antigen does not bind hyper-phosphorylated pRB, but does bind to
p107, suggesting viral proteins/co-factor binding differs between the two proteins (Classon and Dyson, 2001; Farkas et al., 2002).

Phosphorylation of p130 is distinct from that of pRB and p107. Only three out of 22 phosphorylation sites on p130 are conserved in pRB. In addition, p130 has at least four phosphorylation sites that are not phosphorylated by any CDKs. These observations suggest that p130 phosphorylation might be regulated in a unique way, distinct from pRB or p107 (Canhoto et al., 2000; Farkas et al., 2002; Mayol et al., 1996). One interesting finding is that the phosphorylation of p130 is required for the turn-over of p130. p130 protein is abundant in G0 phase, but the amount of the protein is reduced as cells re-enter the cell cycle. It has been shown that CDK4/6 mediate phosphorylation of p130 and mark it as a target of proteasome-mediated proteolysis (Tedesco et al., 2002). Therefore, it seems that the functions of G1/S CDKs contribute to the switch from p130/E2F5 to p107/E2F4. Additionally, it has been demonstrated that three out of four non-CDK phosphorylation sites are phosphorylated by Glycogen Synthesis Kinase (GSK)-3 (Litovchick et al., 2004). GSK-3 mediated phosphorylation increases p130 stability without disrupting the interaction with E2F proteins. Inhibition of GSK3 function by addition of growth factors (serum stimulation) reversed the phosphorylation of p130, which demonstrated the connection between growth factor signaling and cell cycle re-entry.

The phosphorylated RB family proteins become active again by de-phosphorylation in late M phase. Protein Phosphatase I (PP1) plays a role in de-phosphorylation of pRB and p107 (Alberts et al., 1993; Berndt et al., 1997; Ludlow et al., 1993). The function of PP1 is also regulated by phosphorylation. CycB-CDK1 phosphorylates PP1 (Kwon et al., 1997; Puntoni and Villa-Moruzzi, 1997). It has recently been shown that an additional phosphatase, Protein Phosphatase 2A (PP2A) is important in the de-phosphorylation of p107 and p130 (Garriga et al., 2004; Kolupaeva et al., 2008). Interestingly, unlike the PP1 that is only active from late M to early G1, PP2A is active throughout the cell cycle. Based on this idea, it was suggested that equilibrium of various CDKs and Phosphatases at a certain cell cycle phase is critical for controlling p107 and
p130 phosphorylation (Garriga et al., 2004). Hypo-phosphorylated pRB and p107 can be recycled to suppress cell cycle related genes in G1 phase.

**B. CDK inhibitors regulate CDK activity, and thus E2F/RB functions**

Phosphorylation of RB family proteins by CDKs is regulated by CDK inhibitors. There are two groups of CDK inhibitors that function at the G1/S transition. The first group includes small inhibitory peptides, called Inhibitors of Kinase 4 (INK4). Four members of the INK4 family; p16INK4a, p15INK4b, p18INK4c, and p19INK4d, interact with CDK4/6 and inhibit their binding to Cyclin D. This prevents CDKs from phosphorylating RB family proteins. The second group includes CIP/KIP type CDK inhibitors such as p21CIP1 (CDK Interacting Protein), p27KIP1 and p57KIP2 (Kinase Inhibitory Protein). CIP/KIP proteins bind directly to and inhibit CycE-CDK2 and CycA-CDK2 complexes in a cyclin-dependent manner. Interestingly, CycD-CDK4 is also able to form a complex with CIP/KIP, but without losing its catalytic activity. CycD-CDK4 binds to CIP/KIP and sequesters it from CycE-CDK2. Therefore, Cyclin D accumulation plays two important roles at the transition from G1 to S. It activates CDK4 to phosphorylate RB family proteins, and then it releases CycE-CDK2 free of KIP/CIP to phosphorylate RB family proteins on additional sites (Sherr and Roberts, 1995).

Structural bases for the inhibition by these two different CDK inhibiting groups are well described by crystallographic studies. INK4 group inhibitors share an ankyrin repeat motif, which is necessary to bind to the non-catalytic site of CDK4/6. This binding induces a conformational change of the catalytic site, which prevents the binding of substrates (Cyclin D and ATP) (Russo et al., 1998). On the other hand, CIP/KIP type inhibitors bind to the active form of the Cyclin-CDK complex. These proteins have a special helical structure that mimics ATP and blocks the catalytic cleft of CDK2 (Pavletich, 1999; Russo et al., 1998).
Ectopic expression of INK4, or CIP/KIP inhibitors arrest cells in G1. Importantly, INK4-induced G1 arrest requires the functions of RB family proteins, while CIP induced arrest is not pRB dependent (Shapiro and Harper, 1999). For instance, mouse embryonic fibroblasts lacking pRB do not accumulate in G1 phase, but progresses into S phase when p16INK4a was over-expressed (Bruce et al., 2000; Medema et al., 1995). These observations suggest that p16INK4a function targets RB family protein via cyclin-CDK. This linearized regulatory pathway of p16INK4a, CyclinD-CDK4, RB and E2F reiterates how RB, and thus E2F, activities are regulated in a cell cycle dependent manner. Dr. Weinberg recapitulated the importance of this regulation and proposed that the “RB pathway” is implicated in cancer (or, often called p16-RB pathway, Figure 5) (Weinberg, 1995). The RB pathway controls cell cycle and failure of this control results tumorigenesis. Mutations in the RB pathway are found in almost every human cancer. Tumorigenic mutations can be categorized into two classes; gain-of-function mutations of proto-oncogenes such as Cdk’s and E2f’s, or loss-of-function mutations of tumor suppressor genes that encode p16 or pRB (Dimova and Dyson, 2005).
Figure 5. RB pathway

pRB pathway as suggested by Dr. Weinberg, 1995. The p16/INK4a tumor suppressor protein functions as an inhibitor of CDK4 and CDK6. p16 prevents Cyclin D from binding to CDKs. CycD/CDK triggers pRB phosphorylation, which inactivates pRB’s control over E2F proteins. Viral oncoproteins, such as human papillomavirus E7 bind to and block pRB function to drive abnormal cell proliferation.
4. Mechanisms of transcriptional regulation by RB/E2F family members

A. E2F proteins activate transcription

E2F1 – 3a serve as activators of transcription. They can promote gene transcription by recruiting both general transcription factors as well as co-activators. The transactivation domain of mammalian E2F1 modulates more than 70 transcription factors through its interaction with the TATA-box binding protein (TBP), a general transcription factor and component of the TFIID complex. Other general transcription factors, such as TFIIH, also interact with E2Fs (Pearson and Greenblatt, 1997; Ross et al., 1999).

Gene activation by E2Fs also employs remodeling of the chromatin landscape into favorable condition for transcription. Activating E2Fs are linked to histone acetylation. Increased acetylation of lysine residues of histone H3 and H4 promotes transcription. It is widely believed that histone acetylation neutralizes the positive charges of lysine residues, reducing electrostatic interaction between DNA and histones (Workman and Kingston 1998). This change makes DNA more accessible to the general transcription factors and promotes transcription. At promoter regions, histone acetylation takes place before the assembly of the Pre-initiation complex to position the complex and other transcription factors (Li et al., 2007; Workman and Kingston, 1998). Histone acetylation is catalyzed by histone acetyltransferases (HATs). E2Fs has been demonstrated to interact with various HATs, such as the p300/CBP co-activator family proteins, PCAF (P300/CBP-associated Factor), and TIP60 (Tat-Interactive Protein 60) (Martinez-Balbas et al., 2000; Morris et al., 2000; Taubert et al., 2004; Trouche and Kouzarides, 1996).
B. E2F/RB proteins repress transcription

(i) Passive repression

Researchers have also found how E2F and RB family proteins function mechanistically in gene repression. Hiebert et al. demonstrated that an interaction between RB family of proteins and E2F suppresses transactivation of E2F (Hiebert et al., 1992). The pRB protein can bind to the transactivation domain of E2Fs (1 – 3) and block E2F’s interaction with TBP and other general transcription factors. By competing with the interaction between TBP and E2Fs, RB family protein could delay the formation of the pre-initiation complex, leading to repression of target genes in a passive manner (Pearson and Greenblatt, 1997; Ross et al., 1999).

(ii) Active repression by chromatin remodeling

A repressive complex of E2F/RB also can change the chromatin landscape to suppress transcription in an active manner. Three different mechanisms are known to alter chromatin structure.

Firstly, ATP-dependent chromatin remodeling enzymes change the association of histones with DNA. Those enzymes use the energy of ATP-hydrolysis to slide nucleosomes. So, their activity changes the positioning of histone octamers along the DNA, leading to alteration of nucleosomal density (Schnitzler et al., 1998; Vignali et al., 2000).

The second mechanism is the post-translational modification of histones tails. Acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation are known to be employed to modify histones (Strahl and Allis, 2000). These modifications can be recognized by other proteins with specialized domains, such as bromodomain (acetylation) or chromodomain (methylation), and recruit effector proteins that can alter chromatin structure either actively or repressively in different contexts. In the “histone code” hypothesis, it is thought that different combinations of histone
modifications can lead to distinct transcriptional outcomes. (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Although the exact mechanisms and functions of each modification are not fully understood, some specific cases are studied in detail (Table 1 and 2). For example, histone acetylation is important for activation of transcription, while de-acetylation of histones inhibits transcription (Struhl, 1998). Another, relatively well-characterized modification is histone methylation. Histone methylation is thought to mark chromatin to provide signals for downstream events (Sims et al., 2003). Different lysine residues on histone H3 or H4 tails can be modified by mono-, di-, or tri-methylation. It is generally accepted that mono- or trimethylated lysine 4 of Histone H3 (H3K4me1, H3K4me3), monomethylated H3 lysine 9 or 27 (H3K9me1, H3K27me1), trimethylated H3 lysine 36 (H3K36me3) and dimethylated H3 lysine 79 (H3K79me2) correlate with gene activation, whereas di- or trimethylated H3 lysine 9 and 27 (H3K9me2/me3, H3K27me2/me3), or trimethylation of histone H4 lysine 20 (H4K20me3) are found in repressed chromatin (Barski et al., 2007; Steger et al., 2008).

Assembly of histone variants into chromatin is the third way to regulate transcription (Henikoff, 2005). Histone variants have a small number of amino acid changes. They are believed to tag regions of chromatin to create specialized structure (Dhillon and Kamakaka, 2000; Henikoff, 2005; Perrini et al., 2004; Santisteban et al., 2000).
Table 1. Histone Deacetylases (HDACs) and their suggested biological functions.

<table>
<thead>
<tr>
<th>Mammal</th>
<th>Functions (references)</th>
<th>Drosophila¹</th>
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</table>
| HDAC1, 2 | - Silencing leads to cell growth inhibition and deregulated transcription (Foglietti et al., 2006)  
- Loss of dHDAC1 leads a G2 arrest (Foglietti et al., 2006)  
- Found in mSin3a, NURD/Mi2/NRD, and CoRest corepressor complex (Yang and Seto, 2003)                                                       | dHDAC1      |
| HDAC6    | - Silencing leads increased tubulin acetylation levels but not associated with a deregulation of gene express (Foglietti et al., 2006 and Hubbert et al., 2002 (Human))  
- Human HDAC6 rescues neurodegeneration and provides a link between autophagy and the Ub-Proteosome system (Pandey et al., 2007)  
- In human, predominantly resides in the cytoplasm, but is also found in nucleus (De Ruijter et al., 2003)                                                                                      | dHDAC2      |
| HDAC3    | - Silencing leads to cell growth inhibition and deregulated transcription (Foglietti et al., 2006)  
- Associates to SMRT and NCoR co-repressors (Yang and Seto, 2003)                                                                                                                      | dHDAC3      |
| HDAC4, 5, 7, 9 | - No growth phenotype and no significant deregulation of gene expression (Foglietti et al., 2006)  
- HDAC4,5 and 7 interact with a myogenic transcription factor, MEF2 (McKinsey et al., 2001)                                                                                 | dHDAC4      |
| HDAC11   | - No growth phenotype and no significant deregulation of gene expression (Foglietti et al., 2006)  
- Human HDAC11 was found not to reside in any of the known HDAC complexes (Sin3, N-CoR/SMRT) (Gao et al., 2002)  
- Recent reports:  
  - HDAC11 regulates the expression of interleukin 10 and immune tolerance (Villagra et al., 2009)  
  - HDAC11 regulates oligodendrocyte-specific gene expression and cell development in OL-1 oligodendroglia cells (Liu et al., 2009) | dHDACX      |

¹- Drosophila classification is based on Foglietti et al. (2006).
Table 2. Types of Histone Methylation

<table>
<thead>
<tr>
<th>Histone Modification</th>
<th>Suggested Function</th>
<th>System</th>
<th>Enzymes</th>
<th>Reference (Enzymes)</th>
<th>Interaction with ER/GR</th>
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<tbody>
<tr>
<td>H3K9 mono</td>
<td>repression?</td>
<td>Human/Mouse</td>
<td>Su(v3)H1</td>
<td>Rea et al., 2000, Peters et al., 2001</td>
<td>Nicolas et al., 2003</td>
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<td></td>
<td>activation?</td>
<td>G9a</td>
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<td>Tachibana et al., 2002</td>
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<td></td>
<td>facultative heterochromatin</td>
<td>EuHMTase 1</td>
<td>O’Carroll et al., 2000, Peters et al., 2001</td>
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<td>Pericentric heterochromatin</td>
<td>Drosophila</td>
<td>Su(var)3-9</td>
<td>Schultz et al., 2002 (human), Yang et al., 2002 (mouse)</td>
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<td>Czermiński et al., 2001</td>
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<td>(Peters et al., 2003) - mouse</td>
<td>Sc: absent</td>
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<td>Reviewed in Sims et al 2003</td>
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<td>H3K27 mono</td>
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<td>Human/Mouse</td>
<td>Ezh2</td>
<td>Kuzmichev et al., 2002</td>
<td>Mosquera et al., 2004 (Plant)</td>
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<td>G9a</td>
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<td>Tachibana et al., 2001, Tachibana et al., 2005</td>
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<td>Repression (euchromatin)</td>
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<td>EZ2</td>
<td>Muller et al., 2002, Schwartz et al., 2006</td>
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<td>Yeast</td>
<td>absent</td>
<td>Review in Li et al., 2007</td>
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<td>Activation?</td>
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<td>(Vakoc et al., 2006) - human</td>
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<td>tri</td>
<td>repression (Polycomb)</td>
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<td>X-inactivation</td>
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<td>Gonzalo et al., 2000</td>
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<td>Inter-locking of chromatin</td>
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<td>dSET8</td>
<td>Fang et al., 2002</td>
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<td>Beisel et al., 2002</td>
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<td>di</td>
<td>repression (absent in transcribed region)</td>
<td>Yeast</td>
<td>Sp: Set9</td>
<td>Review in Li et al., 2007</td>
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<td>(Schotta et al., 2004) - mouse</td>
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E2F and RB family of proteins employ the first two mechanisms of altering chromatin structure. They are known to interact with ATP-dependent chromatin remodelers. pRB requires SWI/SNF to suppress cell cycle-related genes. It was demonstrated that the human homologs of SWI2/SNF2, BRG1 and hBRM, can bind to pRB. Introduction of mutant SWI/SNF proteins that cannot interact with pRB failed to repress S phase genes in G1 (Dunaief, 1994; Zhang et al., 2000).

E2F/RB proteins also associate with histone modifiers. There are eleven histone de-acetylases (HDACs) in mammals. Three of them interact with RB family proteins (HDAC1, 2, and 3) to help E2F/RB mediated repression (Table 1). The LXCXE motif in HDAC 1-3 proteins is crucial for the interaction with RB family proteins (Brehm et al., 1998; Ferreira et al., 1998; Luo et al., 1998; Magnaghi et al., 1998).

In addition to histone acetylation, repression by RB family proteins employs various histone methyltransferases (HMTs), or complexes that contain HMTs (Table 2). pRB is known to interact with Suv3-9, a human homologue of Drosophila SU(VAR) 3-9. This enzyme functions as a histone methyltransferase that modifies Histone H3 Lys9. Trimethylation of H3K9 positions are believed to be an important signal in formation of a heterochromatic region where genes are silenced (Schotta et al., 2002; Schotta et al., 2003). In addition to Suv3-9, pRB also interacts with another important constituent of heterochromatin, HP1 (Heterochromatin Protein 1) (Eisenberg et al., 1990). HP1 is found at the centromere and telomere regions of chromatin, where heterochromatin is well-developed, and is responsible for the packaging of chromatin. Interestingly, pRB protein employs the function of Suv3-9 and HP1 to suppress the CyclinE gene in the “euchromatic” region. It has been demonstrated that pRB associated Suv3-9 trimethylates nucleosomes at the promoter region of Cyclin E gene, which are recognized by HP1 for further repression (Nielsen et al., 2001).

Another important histone methyltransferase that is linked to E2F/RB is E(Z) (=EZH2 in mammals) (Kotake et al., 2007; Tonini et al., 2004). E(Z) is a member of Polycomb Group Proteins (PcG) that are famous for their regulation of homeotic genes and body plan. PcG has two
sub-complexes, PRC1 and PRC2 (Polycomb Repressive Complexes). PRC2 contains E(Z) that trimethylates Histone H3K27 in PcG dependent repression. This methyl mark is recognized by the PRC1 protein, PC (Polycomb). In addition to E(Z), RING ubiquitin ligases in PRC1 also interact with pRB and repress the Cyclin A and Cdc2 genes (Dahiya et al., 2001; Kotake et al., 2007). Lastly, E2F6 is known to recruit PcG proteins in a RB family protein independent manner (Ogawa et al., 2002).
5. Biological Functions of E2F/RB family proteins

A. Functions of E2F/RB

E2F/RB bind to numerous gene promoters and modulate various cellular processes (Bieda et al., 2006; Ren et al., 2002). Well-known targets include genes encoding DNA polymerase, Proliferating Cell Nuclear Antigen (PCNA), cyclins, and E2F1 itself, which have roles in the G1 to S phase progression of the cell cycle (Ren et al., 2002; Xu et al., 2007). Now we know that E2F proteins regulate a larger number of targets than initially thought. A recent ‘ChIP-on-chip’ study suggests that more than 20% of human promoters are bound by E2F proteins.

Despite the diversity of E2F members, genetic studies of E2F mutants and recent introduction of genome-wide techniques unveiled the extended transcriptional networks and biological importance of E2F proteins. It is known that E2F proteins have roles in the following processes:

- Cell cycle – G1/S transition, DNA replication, Checkpoints, Mitosis
- Apoptosis and DNA Damage response
- Differentiation and Developmental processes

B. E2F/RB proteins control the cell cycle

(i) E2F/RB regulates the cell cycle at the G1/S transition

Activating E2Fs promote cell cycle progression, especially at the G1/S transition. Their overexpression drives quiescent cells into S phase (DeGregori et al., 1997; Johnson et al., 1993; Lukas et al., 1996; Shan and Lee, 1994), and overcomes growth arrest signals such as TGF-β or CDK inhibitors (DeGregori et al., 1995; Schwarz et al., 1995). It has also been demonstrated that overexpression of each activator E2Fs (1, 2, 3a) leads to an increased cell proliferation rate
Ectopic expression of Drosophila dE2F1 induces S phase entry and cell proliferation in imaginal discs (Asano et al., 1996; Du et al., 1996b). In Arabidopsis, overexpressed AtE2F-a enables differentiated and non-dividing cells to re-enter S phase (Rossignol et al., 2002).

In contrast, loss of activator E2F function results in delayed S phase entry. Inhibition of E2F3a function have attenuated S phase (Leone et al., 1998). Moreover, combined removal of all activating E2F proteins (E2F1, 2, 3a) arrests cells in the G1 phase (DeGregori et al., 1997; Lukas et al., 1996; Wu et al., 2001). Studies in model organisms further support these observations. de2f1 mutant flies show reduced DNA synthesis, and their embryos have smaller size due to the growth retardation (Duronio et al., 1995; Frolov et al., 2001). RNAi-mediated depletion of dE2F1 in Drosophila S2 cells blocks S phase entry of the cells and causes G1 arrest (Dimova et al., 2003; Frolov et al., 2003). Likewise, micro-injection of a dominant-negative E2F inhibits cell cycle progression in Xenopus embryos (Tanaka et al., 2003).

E2F4 and E2F5 inhibit S phase entry. Mouse embryonic fibroblasts lacking E2F4 and E2F5 proliferate normally. However, the cells cannot be arrested in G1 when p16 was over-expressed (Gaubatz et al., 2000). This indicates that E2F 4/5 are required for the p16 and RB family protein-mediated control of S phase entry. Genetic analysis in Drosophila supports this idea. Slow larval growth and cell proliferation defects that are caused by dE2F1 mutation are suppressed by removing dE2F2 (Frolov et al., 2001). Together, these observations highlight the functional antagonism between activator and repressor E2Fs in S phase entry and cell cycle progression.

Consistent with their regulation of the G1/S transition, E2F/RB proteins regulate transcription of numerous target genes that are required for S phase. Genome-wide studies from many research groups gave us better understanding of cell cycle-related targets. From ChIp-on-chip analyses and expression array studies it has been determined that E2F/RB regulates various genes required for
- DNA replication: DNA polymerase \( \alpha \), PCNA, thymine kinase, thymine synthase, DNA primase, etc.
- Cell cycle control: Cyclin E, CDK2, E2F1, E2F2, E2F3, etc.
- DNA repair: Rad51, Rad54L, etc.
- Check point: CHK1, Mad2L1, Bub3, TTK, ATM, CENP-E, etc.

(Conboy et al., 2007; Giangrande et al., 2004; Ishida et al., 2001; Ren et al., 2002)

Although the transcription of S phase genes is important for the G1/S transition, there is a disconnect between transcriptional events and cell cycle progression. One example is from a study using a CDK-resistant pRB (Angus et al., 2004). While transcription of the cell cycle-related genes is shut off, levels of the proteins are not attenuated. This raises the question whether E2F-dependent transcription is necessary every cycle. It has been proposed that E2F function in transcription is essential only when cells re-enter the cell cycle from a prolonged G0 (Attwooll et al., 2004). In addition to this idea, it was found that stable expression of E2F1 protein that lacks the transactivation domain does not exhibit a prolonged G1 phase (Zhang et al., 1999). This observation suggests that transactivation by E2F proteins might be dispensable for S phase entry in some circumstances. Another study has demonstrated that some E2F target genes, such as PcnA and Ccne1 (cycE) are up-regulated in E2F1,2,3 triple knockout mouse embryonic fibroblasts despite the fact that the cells are arrested in G1 (Wu et al., 2001). Therefore, transactivation of E2F target genes and G1/S transition are not equal. How exactly the transcriptional regulation of E2F/RB is linked to the cell cycle progression warrants further study.

(ii) E2F/RB regulates the cell cycle beyond G1/S transition

One interesting finding to come out of the genome-wide studies is a potential role for E2F/RB in the control of mitosis, or the G2/M transition. The studies identified many genes that
are required for mitosis such as, Cdc2 (Cell division cycle 2 or CDK1), Cdc20, Bub1, and Cyclin B. Regulation of G2/M genes was previously described in a study of Drosophila string (cdc25) gene. String protein is the rate-limiting factor of mitosis initiation, and its expression is induced by dE2F1 (Neufeld et al., 1998). Taken together these studies indicate that the cell cycle regulation function of E2F/RB goes beyond the G1/S transition. However, how transcription of G2/M genes is delayed relative to the G1/S program is not well understood.

In addition to the induction of DNA replication related genes, E2F and RB are thought to have direct roles in DNA replication. Physical interaction has been demonstrated between E2F/RB and components of the DNA replication machinery, such as PCNA, Origin Recognition Complex (Orc), and others (Angus et al., 2004; Bosco et al., 2001). dE2F or RBF mutant flies cannot perform site-specific amplification of chorion genes in follicle cells in oogenesis (Bosco et al., 2001). This observation suggests potential roles of E2F and RB family of proteins at replication origins, yet how precisely they function is not clear.

**C. E2F/RB proteins regulate apoptosis**

E2F/RB coordinate apoptotic events. One of the first pieces of evidence comes from the observation of cells over-expressing E2F. Degregori et al. demonstrated the effects of overexpression of each E2F protein (DeGregori et al., 1997). In their study, overexpression of E2F1 induces apoptosis, while overexpression of E2F2 or E2F3 promotes the G1/S transition. Accordingly, target genes activated by each E2F were E2F-specific. E2F1 induces a different set of genes compared to E2F2 or E2F3. Indeed, regulation of apoptosis is a function that only E2F1 has. *In vivo* studies also demonstrated that ectopic expression of E2F1 but no other E2Fs promotes apoptosis. Although E2F3 can contribute to apoptosis in an E2F1-dependent manner, E2F1 is the
primary factor that functions directly in the apoptotic pathway (DeGregori et al., 1997; Wang et al., 2000).

Genetic studies further unraveled the role of E2F1 and pRB in apoptosis. E2F1 knockout mice exhibit enlarged thymus due to failure of apoptosis during thymocyte maturation (Field et al., 1996). Furthermore, the mice are more susceptible to tumor formation and show a wide spectrum of tumor phenotypes (Yamasaki et al., 1996). These findings suggest that E2F1 serves as a tumor suppressor through its apoptotic functions. In agreement with this idea, loss of pRB causes mouse embryos to die during development due to multiple problems that include increased levels of apoptosis in the central nervous system and liver (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Furthermore, mutation of E2f1 suppresses the apoptotic events in Rb-/- mice. It was demonstrated that the number of apoptosis decreases in CNS of Rb-/-;E2F1-/- mice compared to that of Rb-/- mice and that the life span was extended (Tsai et al., 1998). Altogether, these observations indicate that E2F1 functions in apoptosis, and that pRB regulates this function.

In addition to the mammalian studies, regulation of apoptosis by E2Fs is also observed in other model systems. For example, ectopic expression of dE2F1 induces apoptosis in Drosophila eyes (Du et al., 1996b). Similarly, dE2F1 is important for DNA damage-induced apoptosis in Drosophila wings (Moon et al., 2005; Wichmann et al., 2010). In C.elegans, it has been demonstrated that EFL-2 have pro-apoptotic role in the germ line development (Schertel and Conradt, 2007).

In mammals, E2F1 may trigger apoptosis by up-regulating the ARF tumor suppressor gene (p14 in humans and p19 in mice; both are referred to as ARF hereafter). Induction of ARF led to the idea that ARF is an effector of apoptosis caused by up-regulated E2F1 activity (Hallstrom and Nevins, 2003). ARF binds to the MDM2 protein (Pomerantz et al., 1998; Zhang et al., 1998). MDM2 is an E3 ubiquitin ligase for the p53 protein that is involved in its degradation of p53. Interaction of ARF neutralizes the p53 inhibitory function of MDM2. Inhibition of MDM2 results
in accumulation of p53, which triggers p53-dependent apoptosis (Kowalik et al., 1998). ARF binds to many proteins other than MDM2, which suggests that it may induce apoptosis in a p53-independent manner. The idea that ARF may induce apoptosis without p53 is still controversial (Sherr, 2006). However, induction of ARF by E2F1 may play a role in p53-independent apoptosis.

E2F1 may also induce apoptosis in an ARF-independent manner. This speculation came from the observation that in some cells ARF overexpression causes cell cycle-arrest, then cell death (Quelle et al., 1995; Sherr, 1998). Therefore, it was suspected that there might be additional E2F1 targets that are involved in apoptosis. The APAF-1 (Apoptosis Protease-Activating Factor 1) gene was identified as one of these targets. Its promoter has an E2F1 binding site in addition to a p53 binding element. APAF-1 is a cytoplasmic protein that binds to Cytochrome C, released from mitochondria in the apoptotic process. APAF-1 and Cyt C organize the apoptosome, which cleaves pro-caspase 9 to activate it. Then the active form of caspase 9 promotes subsequent “caspase cascade” that triggers the apoptotic processes further (Moroni et al., 2001; Nahle et al., 2002).

Induction of p73, a homologue of p53, is another example of ARF independent apoptosis mediated by E2F1. ARF overexpression does not change p73 levels. Furthermore, human MDM2 only degrades p53, but not p73, suggesting that ARF does not have a role in p73 dependent apoptosis. E2F1 has been demonstrated to up-regulate p73 in a direct manner (Irwin et al., 2000). This observation could explain how E2F1 induces apoptosis in cells that have lost p53 function.

In addition to ARF, p73 and APAF-1 proteins, E2F/RB proteins are known to regulate the transcription of other pro-apoptotic genes and caspases. E2F/RB has been demonstrated to control the expression of nearly all Bcl-2 Homology 3(BH3)-only proteins that perform pro-apoptotic functions (Hershko and Ginsberg, 2004). BH3-only protein coding genes, such as PUMA, Noxa, Bim, and Hrk, are regulated by E2F1. E2F/RB also directly regulates caspase 3, 7, 8, and 9 (Hershko and Ginsberg, 2004; Nahle et al., 2002). In summary, E2F/RB regulate the apoptotic processes at several different levels.
What does trigger E2F1 dependent apoptosis? Apoptosis occurs when E2F1 is up-regulated by oncogenes or loss of pRB-regulation that may cause inappropriate cell cycle entry. As described above, mice lacking pRB display high apoptotic levels in CNS and lens. Viral oncoproteins that inhibit pRB function are known to induce apoptosis as well. Expression of adenoviral E1A, SV40 T antigen, or HPV E7 induces apoptosis in animal- and cell-based studies (Howes et al., 1994; Pan and Griep, 1995; Putzer et al., 2000; Symonds et al., 1994). Oncogenic Ras signaling or de-regulation of the Myc transcription factor also results in E2F1 dependent apoptosis. Their functions can promote E2F1 expression indirectly via induction of Cyclin D (Ras and Myc) or E2F3a (Myc) (Sears et al., 2000; Tanaka et al., 2002). Moreover, both Ras and Myc synergize with E2F1 and up-regulate transcription of Arf, to trigger ARF dependent apoptosis (Aslanian et al., 2004; Berkovich et al., 2003; Zindy et al., 1998). These observations suggest that E2F1 dependent apoptosis might be the basis for a tumor-suppressive mechanism in response to the inappropriate S phase entry.

DNA damage can also trigger E2F1 dependent apoptosis. During DNA damage, checkpoint kinase 2 (Chk2) phosphorylates Ser 364 of E2F1 to prolong E2F1 stability and induce apoptosis. However, this E2F1-dependent apoptosis does not occur in cells from ATM (Ataxia-Telangiectasia Mutated) or NBS1 (Nijmegen Breakage Syndrome) patients, suggesting Atm and Nbs1 gene products are required for the apoptosis. (Rogoff and Kowalik, 2004; Rogoff et al., 2004; Stevens et al., 2003). Indeed, ATM kinase, or ATR (ATM and Rad3-related) kinase functions are required for the phosphorylation of serine 31 of E2F1, which prevents E2F1 degradation. Once stabilized by DNA-damage induced phosphorylation, E2F1 replaces E2F4 at promoter regions of the pro-apoptotic gene p73 (Lin et al., 2001; Pediconi et al., 2003).
**D. Regulation of differentiation and developmental processes**

As judged by the phenotypes of mutant animals, E2F and RB proteins have important roles in cell differentiation and development. Mice lacking E2F1 show testicular atrophy and defects in thymocyte development (Field et al., 1996; Yamasaki et al., 1996). E2F2 knockout mice have defects in T lymphocyte homeostasis and develop autoimmunity (Murga et al., 2001). E2F3 null mice die from congestive heart failure, suggesting a critical role in cardiac development (Humbert et al., 2000). Combinational loss of activating E2Fs provides further insights into their roles in development. For example, $E2f1^{-/-};E2f2^{-/-}$ double-knockout leads to developmental problems in the nerve system and pancreatic abnormality (Iglesias et al., 2004; Jiang et al., 2007). In other instances, both E2F1 and E2F3 are required for post-natal development of mice. The double-knockout pups die earlier due to multi-organ failure (Tsai et al., 2008). These observations demonstrate that different E2F proteins may have co-operative and unique functions in the development of different organs (Cloud et al., 2002). Functions in developmental processes are not limited to the activating E2F proteins. E2F4 or E2F5 null mice have abnormalities in intestinal epithelium or cranial development (Lindeman et al., 1998; Rempel et al., 2000). E2F6 knockout mice show homeotic transformation of the axial skeletons that is very similar to the phenotype of polycomb mutant mice (Storre et al., 2002). This phenotype is believed to be the consequence of mis-regulated HOX gene expression; E2F6 represses subset of the HOX genes in polycomb complexes dependent manner (Ogawa et al., 2002).

RB family proteins are also important in development. $Rb^{-/-}$ mutant mice die between day 13 and 15 of gestation and show abnormalities in neuronal, hematopoietic, lens, and muscle differentiation (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Some of these phenotypes, including lens defect, are cell-autonomous, while others such as nervous and hematopoietic system defects are non-cell autonomous because they can be rescued by the presence of wild type cells in chimeric mice models. Conditional knockout of $Rb$ further demonstrated that hypoxia causes the
observed nervous system defects, possibly as a result of an erythroid defect (Liu et al., 2004). In addition, Wu et al reported that pRB is essential to placental development by regulating the terminal differentiation of trophoblast cells (Wu et al., 2003). Because placental defects lead to improper nutrition and gas exchange between mother and fetus, it was suggested that many of previously observed non-cell autonomous phenotypes could be due to the defect in the placenta. Indeed, conditional knockout of Rb in the embryo, but not in the placenta, increased the chance of survival. Rb null embryos with wild type placenta survived to birth and have reduced defects in the nervous system (de Bruin et al., 2003; Wu et al., 2003). The reconstitution of wild type placenta rescued the CNS and erythropoietic defects. But the pups die shortly after birth due to skeletal muscle defects as well as lens problems, indicating that these phenotypes are the direct effect of Rb loss (de Bruin et al., 2003; Wu et al., 2003). While developmental defects in p107/- or p130/- mice are minimal, double knockout mice exhibit abnormal limb development and neonatal lethality (Cobrinik et al., 1996).

In addition to a role in development, E2F/RB proteins are also important in cellular differentiation. Phenotypes of E2F4 null mice display intestinal defects that are due to failure in differentiating cells within villi (Rempel et al., 2000). E2F4 protein also functions in differentiation of human epidermal keratinocytes in vitro (Paramio et al., 2000a), and terminal differentiation of adipocytes or maintenance of differentiated neuronal cells require E2F4 function as well (Fajas et al., 2002; Persengiev et al., 1999). E2F5 is involved in differentiation of neural tissues, and mice lacking E2F5 display hydrocephalus (Lindeman et al., 1998). The roles of the newly discovered E2F members in differentiation are not well understood, but E2F7 was suggested to function in preventing squamous differentiation by repressing differentiation activators (Hazar-Rethinam et al., 2011). E2F1 generally antagonizes cell differentiation, and inhibition of E2F1 is required for differentiation in many cases. For example, suppression of E2F1 is important in adipocytes, chondrocytes, and keratinocytes (Paramio et al., 2000b; Porse et al., 2001; Scheijen et al., 2003; Wong et al., 2003). Some of these examples have demonstrated that E2F1 has an
opposing role against E2F4 to promote clonal expansion, while preventing terminal differentiation of the cells (Fajas et al., 2002; Paramio et al., 2000a). However, it should be noted that their roles are largely context-dependent. For instance, inhibition of E2F1 or overexpression of E2F4 increases the differentiation of keratinocytes, while the same treatment impairs adipogenesis. Relatively less is known about the roles of the other activating E2Fs (E2F2 and E2F3a) in cell differentiation, yet recent studies have reported that E2F3a mediates neuronal migration and differentiation through pRB (Chen et al., 2007; McClellan and Slack, 2007).

In addition to E2Fs, pocket-proteins have also been shown to affect differentiation. Studies of adipogenesis have demonstrated that 3T3 cells lacking both p107 and p130 undergo adipocyte differentiation more efficiently, suggesting these two proteins suppress cell differentiation (Classon et al., 2000). In contrast, pRB promotes differentiation, so pRB deficient cells had defects in conversion into adipocytes. These observations suggest opposing roles for RB family of proteins in cell differentiation. In other instances, pRB is required for muscle differentiation. pRB deficient myoblasts are not able to differentiate into myotube properly, because they cannot exit from the cell cycle (Novitch et al., 1996; Zacksenhaus et al., 1996). Similarly, Rb-/- embryo exhibits impaired retinal development, which is due to inappropriate exit from the cell cycle of retinal progenitor cells (Zhang et al., 2004). These results suggest that pRB has an essential function in cell differentiation in addition to its role in cell proliferation.

The importance of E2F and RB proteins in development and differentiation is also shown in model organisms. Mutations of efl-1 and dpl-1 in *C. elegans* have defects in embryonic asymmetry (Page et al., 2001). EFL-1 and LIN-35(RB) have also a role in determining vulval cell-fate determination, and thus vulva development (Ceol and Horvitz, 2001). In *Drosophila*, dE2F/dDP is necessary for the dorsoventral polarity of the oocytes (Myster et al., 2000). In addition, dE2F/RBF regulation of proliferation and apoptosis is important in proper development of eye, wing, and imaginal discs (Asano et al., 1996; Frolov et al., 2001; Moon et al., 2005; Secombe et al., 1998). dE2F and RBF function in cell differentiation as exemplified in *de2f1*
mutant flies that have abnormal photoreceptors of the eye (Brooks et al., 1996). In *Xenopus*, E2F is required for patterning of the embryonic axis. It determines ventral and posterior cell fates (Suzuki and Hemmati-Brivanlou, 2000).

E2F/RB function could affect development at multiple levels. Therefore, as described in RB null phenotypes, developmental failure in E2F/RB mutants could be an outcome of impaired proliferation of certain cells but could be equally due to problems in apoptosis during development, or imperfect cell differentiation. Mouse studies have shown that abnormal development of E2F4 null mice is at least partially due to flawed differentiation (Rempel et al., 2000). Even differentiation defects themselves could be caused either by defects in cell cycle exit or by mis-expression of differentiation specific genes. Because of its diverse function, the E2F/RB system can be involved in all the different aspects of development, and malfunction in any one of them could lead to impaired differentiation and/or development.
6. Cell cycle-independent functions of E2F and RB

Apoptosis genes should be kept repressed in healthy, proliferating cells regardless of the cell cycle stage. Similarly, differentiation specific genes need to be expressed only in specific tissues. If E2F/RB proteins regulate apoptosis and differentiation related genes, how can these genes remain repressed during S-phase when cell cycle-related genes are de-repressed and induced by E2Fs? There must be a mechanism to regulate E2F/RB activity at these genes independently of the cell cycle.

Early clues came from the identification of S phase-resisting E2F/RB complexes. These findings suggested the existence of CDK independent and cell cycle-uncoupled regulation of E2F and RB family of proteins (Moberg et al., 1996; Timchenko et al., 1999). Indeed, studies in *Drosophila* and mammals have identified groups of differentiation specific and apoptosis genes that are regulated in a cell cycle independent manner (Aslanian et al., 2004; Dimova et al., 2003; Iwanaga et al., 2006; Komori et al., 2005; Young and Longmore, 2004).

A. Regulation of differentiation specific genes

The discovery of a new class of genes that are regulated by E2F/RB in a CDK independent manner reveals novel aspects of E2F/RB regulation and forces us to revise the classical model. In microarray experiments that used *Drosophila* S2 cells, Dimova *et al.* subdivided the E2F/RB target genes into five groups based on the degree of expression change after depletion of dE2F1 (activator), dE2F2 (repressor), or dDP (both) (Table 3, Dimova *et al.*, 2003).

A difference was observed between classic E2F/RB target genes and “non-classic” target genes. The classic E2F/RB target genes (Groups A and B) are mainly controlled by dE2F1 and RBF1 and are functionally linked to cell cycle progression. In contrast, (Group D and E) were regulated exclusively by dE2F2/RBF1 and RBF2, and had no cell cycle-related functions. For
Group E/D genes, dE2F2/RBF regulation was un-coupled from the cell cycle. Chromatin immunoprecipitation revealed that dE2F2, RBF1 and RBF2 were still bound to the target promoters and repress the genes in S phase of the cell cycle, while only dE2F1 was present at classic E2F gene promoters. This observation demonstrates that the repression mediated by dE2F2 and RBF for Group E/D genes are not coupled to cell cycle progression (Figure 6). Most of the known Group E/D genes have functions in differentiation and gametogenesis, indicating that repression by dE2F2/RBF plays a role in differentiation and developmental processes (Dimova et al., 2003; Korenjak et al., 2004).

Cell cycle-independent E2F target genes were also identified in mammalian systems (Iwanaga et al., 2006). Using rat embryonic fibroblasts, E2F1 target genes that are only induced upon E2F1 over-expression were screened. The result led to the identification of genes that were not recognized from previous microarray analyses (Ishida et al., 2001; Iwanaga et al., 2006; Muller et al., 2001; Polager et al., 2002). The genes differentially expressed in E2F1 overexpressing cells not only included the traditional E2F target genes, but also a group of genes that function in development and differentiation. Interestingly, some were not induced in serum-stimulated cells. This observation suggests that at least some of the E2F1-responsive genes are not expressed during normal cell cycle progression. Although it is not clear if their repression in normal cell cycle is E2F dependent, the results show that differentiation/development related E2F target genes are regulated in a different manner from cell cycle-related genes (Iwanaga et al., 2006).
Table 3. Distinct patterns of transcriptional change upon the depletion of dE2F1, dE2F2 or DP proteins.

<table>
<thead>
<tr>
<th>Group</th>
<th>dsE2F1</th>
<th>dsE2F2</th>
<th>dsDP</th>
<th>Known Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A.</td>
<td>↓</td>
<td>-</td>
<td>↓</td>
<td>Cell cycle, Chromatin</td>
</tr>
<tr>
<td>Group B.</td>
<td>↓</td>
<td>-</td>
<td>-</td>
<td>DNA repair, Check point</td>
</tr>
<tr>
<td>Group C.</td>
<td>↓</td>
<td>↑</td>
<td>-</td>
<td>(unknown)</td>
</tr>
<tr>
<td>Group D.</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>Sex-specific</td>
</tr>
<tr>
<td>Group E.</td>
<td>-</td>
<td>↑</td>
<td>↑</td>
<td>Differentiation markers</td>
</tr>
</tbody>
</table>

dE2F1, dE2F2, dsDP: Depletion of each gene by dsRNA.
Up-arrows (↑): increased expression in dE2F or DP depleted cells.
Down-arrows (↓): decreased expression in dE2F2 or DP depleted cells.
Bar: no expression change.

Note that genes in Group A require dE2F1 to be expressed (dsE2F1=dsE2F1+dsE2F2), while Group E genes are repressed by dE2F2 (dsE2F2=dsE2F1+dsE2F2).
**Figure 6.** Cell cycle dependent vs. Cell cycle independent regulation of transcription by E2F/RB in *Drosophila*

(A) Regulation of cell cycle-related E2F/RB target genes, which is coupled to the cycle progression. Activating E2Fs induce gene transcription in S phase as RB becomes phosphorylated.

(B) Cell cycle independent control. Repressive complexes of E2F/RB are not disrupted at the G1/S transition. The genes are not induced by growth stimuli, but require tissue-specific or developmental stage-specific factors to be expressed.
B. Regulation of Apoptotic genes

Apoptosis related genes are regulated in a cell cycle independent manner as well. In experiments using human osteosarcoma cells, it was demonstrated that pro-apoptotic genes are suppressed by pRB throughout cell cycle (Young and Longmore, 2004). Binding of pRB protein is not disrupted during cell cycle progression, even in S phase. Inhibition of pRB function by E1A over-expression resulted in de-repression of apoptotic genes and cell death.

Another example is the regulation of the Arf gene. In normally growing cells, it is known that ARF expression is very low. Its expression increases in tumor cells. Transcription of Arf is induced by ectopic expression of oncogenic proteins, such as E2F1, c-MYC, and RAS (Lowe and Sherr, 2003). It has been shown that E2F3b is the key repressor of Arf in quiescent mouse embryonic fibroblasts (Aslanian et al., 2004). Interestingly, E2F3b-mediated repression does not disappear even after serum stimulation and cell cycle progression. This observation suggests that the regulation of Arf is cell cycle independent. E2F1-dependent transactivation of Arf is induced by abnormal growth signals, oncogenic signals, or de-regulated E2F activity caused by pRB defects. However, the gene is not turned on by CDK activation and pRB phosphorylation (Komori et al., 2005).
7. dREAM complex dependent repression

A. dREAM is a multi-protein repressive complex

In Drosophila, cell cycle independent repression of E2F responsive genes requires the function of a multi-protein complex, called dREAM (Drosophila Retinoblastoma, E2F and Myb-interacting proteins), or MMB (Myb-MuvB, see below) complex. Two groups independently purified this complex in search of RBF (Dr. Brehm Lab), or Mip120 (Dr. Botchan lab) associated complexes from Drosophila embryos and S2 cells, respectively (Korenjak et al., 2004; Lewis et al., 2004). The dREAM complex contains dE2F2 and RBF proteins. However, dE2F1 is not found, which implies a function for dREAM/MMB in gene repression (Korenjak et al., 2004; Lewis et al., 2004, Table 4). In addition to dE2F2 and RBFs, dREAM/MMB complex contains the Myb transcription factor and Myb-interacting proteins such as Mip40, Mip120, and Mip130/TWIT. A histone chaperon, p55/CAF1, is also found in the complex. One of the groups (Dr. Botchan’s) also purified dRpd3 and L(3)MBT as sub-stoichiometric members of the dREAM complex, but the participation of these proteins and their functions in the complex are not fully understood yet.

dREAM is a repressive complex. Drosophila polytene chromosome staining has demonstrated that dREAM components are found at transcriptionally silent regions of the chromatin, where RNA polymerase II does not bind. Knockdown of each dREAM component, except for Myb, de-represses transcription of only differentiation specific E2F/RB target genes (Group E/D genes) (Georlette et al., 2007; Lewis et al., 2004). These findings support the idea that dREAM complex function in gene repression, and specifically repression of differentiation specific genes. However, how dREAM achieves this repression is not well understood.
Table 4. Components for dREAM, DRM, or DREAM complexes

<table>
<thead>
<tr>
<th>Drosophila dREAM/MMB</th>
<th>Drosophila tMAC</th>
<th>C. elegans DRM/NuRD/SynMuvB</th>
<th>Human DREAM/LINC^H</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBF1/2(^{DM})</td>
<td>Lin-35(^{C})</td>
<td>RBL1/p107, RBL2/p130</td>
<td></td>
</tr>
<tr>
<td>dE2F2(^{DM})</td>
<td>Eff-1(^{C})</td>
<td>E2F4</td>
<td>E2F5</td>
</tr>
<tr>
<td>dDP(^{DM})</td>
<td>Dpl-1(^{C,N})</td>
<td>DP1/2</td>
<td></td>
</tr>
<tr>
<td>p55/Caf1(^{DM})</td>
<td>p55/Caf1</td>
<td>Lin-53(^{C})</td>
<td>RBP4</td>
</tr>
<tr>
<td>Mip130(^{DM})</td>
<td>Aly</td>
<td>Lin-9(^{C})</td>
<td>LIN9</td>
</tr>
<tr>
<td>Mip40(^{DM})</td>
<td>MIP40</td>
<td>Lin-37(^{C})</td>
<td>LIN37</td>
</tr>
<tr>
<td>dLin52(^{DM})</td>
<td>Lin-52(^{C})</td>
<td>Lin-52(^{C})</td>
<td>LIN52</td>
</tr>
<tr>
<td>Mip120(^{DM})</td>
<td>Tomb</td>
<td>Lin-54(^{C})</td>
<td>LIN54</td>
</tr>
<tr>
<td>Myo(^{DM})</td>
<td>n/a</td>
<td>MYBL1/2</td>
<td></td>
</tr>
<tr>
<td>Rpd3(^{M})</td>
<td>Hda-1(^{C,E,N})</td>
<td>HDAC1/2(^{E})</td>
<td></td>
</tr>
<tr>
<td>L(3)MBT(^{M})</td>
<td>Lin-61(^{E})</td>
<td>L3MBT(^{E})</td>
<td></td>
</tr>
</tbody>
</table>

Comr\(^{T}\)  Top\(^{T}\)

| Gei-4\(^{B}\) | Hpl-2 (HP1)\(^{B}\) | Let-418\(^{R,N}\) | Lin-13\(^{B}\) | Lin-15b\(^{B}\) | Lin-36\(^{B}\) | Lin-52\(^{B}\) | Lin-65\(^{B}\) | Mep-1\(^{B}\) | Met-2\(^{B}\) | Tam-1\(^{B}\) | Tra-1\(^{B}\) | Ubc-9\(^{B}\) |

D: Reported by Dr. Brehm’s Lab (Korenjak et al., 2004)
M: Reported by Dr. Botchan’s Lab (Lewis et al., 2004)
T: tMAC components whose homologues are not found in other complexes (Beall et al., 2007)
C: Physically interact in C.elegans as DRM complex (Harrison, et al., 2006)
H: Human Counterparts of dREAM complex components (Litovchick et al., 2007, Schmit et al., 2007)
E: Not in C.elegans DRM and Human DREAM/LINC (Harrison et al., 2006, Litovchick et al., 2007, Schmit et al., 2007)
B. dREAM components are homologous to C. elegans Synthetic Multi-vulvar Class B gene products

The identification of the dREAM/MMB complex revealed striking overlap between dREAM components and the products of *C. elegans* Synthetic Multi-vulvar Class B genes (synMuv B). In worms, multi-vulvar phenotype is caused by oncogenic RAS mutation. Genetic studies have demonstrated that “synthetic” multi-vulvar (synMuv) phenotype appears when RAS function is not controlled properly. The genes in synMuv Class A and B are known to suppress RAS function in a redundant manner, therefore loss-of-function mutations from each class synergistically induce synMuv phenotype (Ferguson and Horvitz, 1989). An additional study has proposed the existence of new negative regulators of Ras signaling pathway, synMuv Class C, whose functions are redundant to the previous two classes (Ceol and Horvitz, 2004). The functions of Class A gene products are not well-understood. SynMuv Class C genes encode proteins that assemble Tip60-like histone acetyltransferase complex (Ceol and Horvitz, 2004). As shown in Table 4, Class B genes include all members of dREAM complex (Ceol and Horvitz, 2001; Lu and Horvitz, 1998). The fact that loss of Class B genes results in abnormal vulvar development indicates a role of the dREAM complex in differentiation and developmental processes.

C. dREAM components are important for the site specific gene amplification in Drosophila follicle cells

dREAM components have been demonstrated to function in endoreduplication, a site-specific DNA replication of *chorion* genes in *Drosophila* (Beall et al., 2002). Amplification of the *chorion* loci is important for eggshell formation and occurs in the ovarian follicle cells. At these loci, DNA is repeatedly amplified in a restricted region, without spreading to the whole genome or following mitotic events. This process enables the follicle cells to produce a large amount of transcripts required to build the eggshell (Orr-Weaver, 1991). Two DNA elements are known to be
important for this site-specific DNA amplification; an enhancer sequence ACE3 and an Origin recognition complex (Orc) binding site, Ori-B (Calvi and Spradling, 1999).

Further study of these two elements identified a group of DNA binding proteins that include Myb, Mip40, Mip120, Mip130 and p55. Consistent with their importance in endoreduplication, mip mutant flies have a thin eggshell and are female sterile. Immunostaining of their follicle cells demonstrated that bromodeoxyuridine (BrdU) is incorporated throughout genome, indicating loss of control of site-specific DNA replication (Beall et al., 2004; Beall et al., 2007). This phenotype is similar to that of de2f2, rbf1, and dp mutant follicle cells where DNA replication takes place throughout nucleus (Cayirlioglu et al., 2001). These studies suggest that dE2F2, RBF1, and Mip proteins may function together in controlling endoreduplication in follicle cells.

The function of Myb in this context is not fully understood. myb mutant flies are not viable. This could be due to the loss of an important regulator for the G2/M and maybe for the G1/S, transcription; or it could be due to the genomic instability resulting from loss of Myb (Manak et al., 2002; Okada et al., 2002). myb mutant clones from genetic mosaic flies showed that DNA amplification is mis-regulated in a different manner from mip mutants. In this case, pre-replication Complex binds to Ori-B in a site-specific manner, but amplification does not occur, loss of BrdU incorporation was observed (Beall et al., 2002). This suggests that the function of Myb and Mip proteins are required at different time points in DNA replication. Remarkably, myb and mip double mutant flies are viable, suggesting that Mip and Myb may have opposing functions (Beall et al., 2004; Beall et al., 2007).

**D. dREAM complex homologs in mammals and worms**

dREAM complex homologs have been found in *C.elegans* and Human (Harrison et al., 2006; Litovchick et al., 2007), indicating that the complex is evolutionally conserved. DRM (DP,
Rb and MuvB) contains a subgroup of synMuvB gene products. It was identified in search of LIN-37 (= Mip40 in Drosophila) interacting proteins. DRM complex contains LIN-35 (RB homolog), EFL-1 (a repressive E2F), DPL-1 (DP protein), and homologs of Myb-interacting proteins LIN-9 (Mip130), LIN-37 (Mip40), and LIN-54 (Mip120). Equivalents of p55/CAF1 and LIN-42 were also found, but HDA-1 (dRpd3) and LIN-61 (L(3)MBT), counterparts have not been identified. Myb is also not part of the complex, as worms do not have Myb-like proteins. DRM complex is required to suppress multi-vulvar phenotype and is suggested to be a negative regulator of transcription (Ceol et al., 2006; Harrison et al., 2006). Remarkably, mutations in DRM display different phenotypes that did not appear in mutations of the NuRD-like complex that belongs to the same synMuvB class. While disruption of DRM was not sufficient to induce synthetic multi-vulvar phenotype without a second mutation in synMuv Class A genes, any mutation in NuRD-like complex members exhibited weak and partial multi-vulvar phenotype (Harrison et al., 2006). This finding is important because it suggests that DRM is a distinct complex from NuRD despite the fact that the components of both DRM and NuRD are in the same class of synMuv genes and that they share members (Table 4).

Human homologs of dREAM, or DRM complexes have been identified by two groups. Using human glioblastoma cells (T98G), a p130 containing complex was purified and termed DREAM (DP, RB-like, E2F, And MuvB, Litovchick et al., 2007). Composition of human DREAM complex is very similar to fly dREAM complex. However, it should be noted that human B-Myb was not detected in p130 in co-immunoprecipitation assays, and neither HDACs nor human L(3)MBT were associated with the complex. Furthermore, while p107 may recruit the DREAM complex in the absence of p130, pRB is not part of the complex. The DREAM complex was shown to repress cell cycle-dependent genes in quiescent cells (Litovchick et al., 2007).

In a second study a hLIN37 (=Drosophila Mip40) containing complex was purified and named LINC (LIN Complex). LINC shares many characteristics with DREAM (Schmit 2007 et al., Table 4). However, it was discovered that there is a subunit switch from E2F4/p130 to
B-Myb/p107 during cell cycle re-entry, and thus there appear to be two different complexes, one containing E2F4/p130 and one Myb/p107. LINC binds to a number of E2F-responsive promoters in quiescent cells. Interestingly, the study suggested that LINC functions in transcriptional activation. The complex associates with G2/M genes in S phase, and disruption of LINC by RNAi knockdown results in reduction of G2/M gene expression (Schmit et al., 2007). However, it should be noted that in these experiments only LIN but not E2F/RB proteins were depleted. Therefore, it is possible that the activation function might be restricted to the Myb-containing version of LINC, but not to the E2F4-containing DREAM/LINC complex. Further studies are required to fully understand the function of DREAM/LINC complexes in the regulation of cell cycle genes. It will also be interesting to determine if they have any functions in controlling differentiation specific genes in proliferating cells as it has been demonstrated in Drosophila.

**E. dREAM complex and chromatin remodeling**

Although it is not clear how dREAM complex represses its target genes, the idea that modification of chromatin is part of the repression mechanism is favored for several reasons. As described previously, RB family proteins are known to interact with various chromatin remodelers that includes ATP-dependent chromatin remodelers (SWI/SNF, NURD, ISWI complexes), Histone deacetylases (HDACs), Histone methyltransferases (Su(Var) 3-9, Su(Var) 4-20), and DNA methyltransferases (DNMT).

Additionally, a member of dREAM complex, the p55 protein, is a histone chaperon that helps the assembly of nucleosomes during chromatin rearrangement by recognizing Histone H4 tails (Murzina et al., 2008). p55/CAF1 is found in various chromatin modifying complexes, such as histone acetyltransferase HAT1, NuRD (nucleosome remodeling histone deacetylase complex), NURF (nucleosome remodeling factor), Sin3 complex that contains HDAC, CAF-1 (chromatin assembly factor 1) and Polycomb Repressive Complex 2 (PRC2) that function in the regulation of
Hox genes (Loyola and Almouzni, 2004). Consistent with this idea, an *in vitro* binding assay demonstrated that dREAM complexes specifically associate with unmodified histone H4 tails but fail to bind hyper-acetylated tails (Korenjak et al., 2004).

dRpd3 and L(3)MBT were identified as sub-stoichiometric components in the dREAM complex by Dr. Botchan's group. dRpd3, or dHDAC1, has histone deacetylase activity which is required for many transcriptional repression events (Kadosh and Struhl, 1998; Kennedy et al., 2001). L(3)MBT (Lethal-3-Malignant Brain Tumor), is thought to be functioning in locking chromatin structure to compact nucleosomal arrays (Trojer et al., 2007). Therefore, their association with the dREAM complex implies a potential function of the complex in chromatin remodeling. Remarkably, both belong to the synMuv Class B genes in *C. elegans*, suggesting their function is linked to that of the dREAM complex (Ceol and Horvitz, 2001; Ferguson and Horvitz, 1989). Supporting this observation, inhibition of dRpd3 (= dHDAC1) shows inappropriate BrdU incorporation in *Drosophila* follicle cells similar to what is observed in e2f2, rbf1, or mip mutant flies (Lewis et al., 2004).

Despite these observations, it is not established whether dREAM-mediated repression employs chromatin remodeling. Although dREAM complex contains p55/CAF1 as a subunit, RNAi depletions of various chromatin remodeling complexes that contains p55/CAF1 failed to de-repress the expression of Group D/E genes, except for p55/CAF1 itself (Taylor-Harding et al., 2004). Moreover, it is not clear if dRpd3 and L(3)MBT are essential components of the dREAM complex. Human and worm dREAM homologues do not contain them (Harrison et al., 2006; Litovchick et al., 2007; Schmit et al., 2007). It is possible that they are only in the genetic pathway (synMuvB), and do not interact physically with the complex. Further study will be needed to describe their potential functions in the dREAM complex.
F. Different compositions of the dREAM complex may have different functions

Accumulating evidence supports the idea that the dREAM/MMB complex may have different compositions to perform different functions in different cellular contexts. Genome-wide study of dREAM complex binding in tissue cultured cells revealed that dREAM complexes, including partial complexes, with different compositions are found at more than 1500 gene promoter regions in Drosophila (Georlette et al., 2007). dE2F2 is essential for repression at many of these promoters, while Myb is necessary at others to activate genes. Furthermore, dE2F2 and Myb behave in a mutually exclusive manner to position dREAM complex at different promoters. In many cases, the two proteins are not found together, suggesting subunit-switch between dE2F2 and Myb is important to direct dREAM/MMB complex at different targets (Georlette et al., 2007), similar to what has been observed in humans (Schmit et al., 2007). This idea is confirmed by a genetic study done in Drosophila, which demonstrated that switching the repressive dE2F2 into Myb protein is important for the expression of Polo gene in Drosophila wing discs (Wen et al., 2008). Therefore, it seems that dREAM/MMB complex regulates not only differentiation specific genes, but cell cycle related genes. For these reasons, the most recent literature about dREAM/MMB complex distinguishes the two different compositions; dREAM complex that contains only dE2F2, or MMB complex that only contains Myb (Litovchick et al., 2011; Tschop et al., 2011). Switching of E2F and Myb seems to require a kinase function that phosphorylates one of the DREAM components, LIN-52. A study in human DREAM complex demonstrated that DYRK1A kinase protein promotes DREAM complex subunit switching, and thus different transcriptional activity of DREAM complex (Litovchick et al., 2011).

Another example of subunit switching comes from the discovery of the tMAC complex. Biochemical purification of a Mip40-containing complex from Drosophila testis tissue identified a complex related to dREAM/MMB (Beall et al., 2007). The tMAC complex contains Mip40 and
p55/CAF1 that are also found in the dREAM complex. However, instead of Mip120 and Mip130, tMAC contains Tombola (Tomb) and Always early (Aly), which are homologues of Mip120 and Mip130 respectively. The complex is required for male fertility, especially for the differentiation of spermatids. Remarkably, it lacks both dE2F2 and Myb proteins. Therefore, it is likely that Mip proteins serve as a scaffold for various complexes, providing tissue specific functions in different settings (Beall et al., 2007).
8. Questions

Evading growth suppression is one of the hallmarks of cancer cells (Hanahan and Weinberg, 2000, 2011). Since the identification of the retinoblastoma gene in 1986, RB family proteins have been extensively studied for their function in tumor suppression as well as their regulation of target proteins like E2Fs. Most of the work has focused on identifying the various members in the E2F/RB family and their functions in modulating cell cycle-dependent processes such as checkpoint, DNA repair, and mitosis events. In more recent years, emerging roles for E2F/RB proteins have been unveiled – roles in the control of cell differentiation and development. However, E2F and RB functions in these processes are far less well understood. The present thesis focuses on the functional and mechanistic analysis of how E2F/RB family proteins repress transcription of differentiation specific genes in a cell cycle independent manner. Particularly, I investigated how the genes are negatively regulated by the dREAM complex in the Drosophila system.

A. How is the repression of differentiation specific genes different from the repression of cell cycle genes?

The repression of the differentiation specific E2F target genes in proliferating cells is distinct because it is transmitted to daughter cells after each cell division. How can the repressed state be maintained throughout cell division? How is the repression of differentiation specific genes different from that of cell cycle genes? To answer these questions, I investigated if there is an epigenetic mechanism that represses these genes. Specifically, histone modifications, such as histone deacetylation or histone methylation were examined to decipher the specific chromatin signature that differentiation specific genes may have. I also asked if the dREAM complex has a role chromatin remodeling (Chapter II).
**B. How can E2F/RB complexes be maintained at differentiation specific gene promoters in S phase?**

The second important question regarding the repression of differentiation specific genes is how these genes can be repressed with high CDK activity in S phase. Dimova et al. has demonstrated that dE2F2 and RBF proteins are maintained at the promoters of these genes in S phase synchronized cells, and the target genes are not expressed. How can this be achieved when dE2F2/RBF complexes are disrupted at the promoter regions of cell cycle-related genes? I hypothesized that the assembly of the dREAM complex is required for the maintenance of E2F and RB in S phase at differentiation specific target genes. To test this idea, I investigated how dE2F2/RBF binding is affected in dREAM component depleted cells. I also asked if the RBF1 protein is phosphorylated by CDKs in S phase at the dREAM target gene promoters (Chapter III).

**C. How can dE2F2, but not dE2F1, be recruited to the promoters of differentiation specific genes?**

The differentiation specific genes are regulated only by the repressive complex of dE2F2 and RBF proteins. Interestingly, it has been demonstrated that knockdown of dE2F2 does not allow dE2F1 to bind at the differentiation specific gene promoters. What is the mechanism of dE2F1 exclusion from these promoter regions? I investigated if the proteins in dREAM complex inhibit the recruitment of dE2F1 at the promoters. To test this possibility, dREAM components dE2F, RBF, or Mip proteins were selectively depleted using RNAi and dE2F1 binding to the promoters was monitored (Chapter III).
9. References (Chapter I)


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Chapter II

Drosophila RB proteins repress differentiation-specific genes via two different mechanisms
1. Acknowledgements (Chapter II)

The work present in Chapter II has been published in the journal Molecular and Cellular Biology.


As the first author of the paper, I performed the majority of the work presented in this chapter. However, some of the results are produced in collaboration with several colleagues. The northern blot analyses that demonstrated the change of E2F target gene expression in various conditions are performed by Dr. Dessislava Dimova and Anna Martinez (Fig 9C, 14D, 15B and 15F). Dr. Dimova also contributed the dRpd3 ChIP data for Fig 10A, which I confirmed with independent ChIP assays combined with real time qPCR in Fig 10B. The S2 cell lines stably expressing HA-tagged E(Z) proteins are established by Dr. Katsuhito Ohno. Linda Ragusano helped me in establishing the conditions for the ChIP assays, which includes but not limited to cell fixation, sonication, antibody titration, and Real Time PCR. modENCODE consortia kindly allow me to mention their result of the genome wide dRing binding before their publication.
2. Abstract

The RB and E2F proteins play important roles in the regulation of cell division, cell death, and development by controlling the expression of genes involved in these processes. The mechanisms of repression by the retinoblastoma protein (pRB) have been extensively studied at cell cycle-regulated promoters. However, little is known about developmentally regulated E2F/RB genes. Here, we have taken advantage of the simplicity of the E2F/RB pathway in flies to inspect the regulation of differentiation-specific target genes. These genes are repressed by dE2F2/RBF and a recently identified RB-containing complex, dREAM/MMB, in a cell type- and cell cycle-independent manner. Our studies indicate that the mechanism of repression differs from that of cell cycle-regulated genes. We find that two different activities are involved in their regulation and that in proliferating cells, both are required to maintain repression. First, dE2F2/RBF and dREAM/MMB employ histone deacetylase (HDAC) activities at promoter regions. Remarkably, we have also uncovered an unconventional mechanism of repression by the Polycomb group (PcG) protein Enhancer of zeste [E(Z)], which is involved in silencing of these genes through the dimethylation of histone H3 Lys27 at nucleosomes located downstream of the transcription start sites (TSS).
3. Introduction

The retinoblastoma protein (pRB) is a critical regulator of cell division, cell death, and differentiation in metazoans, and its activity is altered in most human tumors (9, 22, 47, 48, 60). The best understood property of pRB is its ability to modulate the action of the E2F family of transcription factors and to regulate cell cycle progression (11, 13, 56). pRB and the related proteins p107 and p130, collectively referred to as "pocket proteins," or RB family proteins (RB), bind to the heterodimeric E2F/DP factors and provide a module of transcriptional regulation that couples the expression of many genes with cell cycle progression. In quiescent cells, E2F and pocket proteins form repressive complexes that prevent the transcription of genes required for S-phase entry. This repression is then relieved at the G1-to-S transition by the activity of cyclin-dependent kinases (Cdk). At the promoters of cell cycle-regulated genes, repressive E2F/RB complexes are replaced by activating E2Fs, and this allows for the coordinated expression of many genes required for cell division (13, 56).

The biological activities of pRB extend beyond cell cycle regulation. Work in the past several years has greatly expanded the spectrum of genes regulated by E2F and RB. In addition to genes required for DNA replication and cell cycle progression, these now include a number of genes involved in sex determination, differentiation, and development (6, 12, 25, 36, 40, 50, 61, 62, 64). While pRB-dependent control of differentiation has been implicated in tumor suppression, the regulation of differentiation by pRB remains poorly understood (7, 27, 31). Despite extensive studies of the mechanism of repression by pRB at cell cycle target genes, little is known about the means by which differentiation-specific targets are regulated. This is especially intriguing because some of these targets remain repressed in many different cell and tissue types, including proliferating cells (12, 52).
Repression by pRB has been linked to BRG1, which is a component of the human Swi/Snf complex, and to histone deacetylases (HDACs), histone methyltransferases, DNA methyltransferase (DNMT), and other corepressors (11, 18, 34). The large number of putative corepressors suggests that there might be multiple repression mechanisms and that different cofactors might be employed at different promoters and in distinct cellular states. For instance, the histone methyltransferase Suv39h is required for histone H3 lysine 9 (H3K9) methylation and subsequent repression of S-phase gene promoters in differentiating cells but not in cycling cells (1). Similar results were obtained in senescent cells (37). These findings strongly suggest that the mechanism of permanent silencing which is triggered upon differentiation is distinct from the transient repression mechanism in cycling cells. The stable repression of differentiation-specific genes differs in one aspect from the stable repression of cell cycle genes: it must also be maintained in proliferating cells during S phase.

The complexity of the E2F/RB pathway in mammals, as exemplified by a large number of distinct yet interrelated E2F/RB complexes, has made the study of the mechanisms of action of RB less than straightforward. Simpler organisms, such as Drosophila melanogaster and Caenorhabditis elegans, are increasingly being recognized as valuable tools for understanding various aspects of E2F/RB biology. This is due in large part to the high level of conservation and relative simplicity of the pathway. In Drosophila, there are two RB homologues, RBF1 and RBF2, and only two E2F family members, dE2F1, the activator, and dE2F2, the repressor. They form heterodimers with one DP homologue, dDP (11, 53, 59).

Analysis of the E2F/RB transcriptional program in flies has revealed that there are several types of E2F/RB regulation. The regulation of genes with periodic expression is dependent on dE2F1 activation and on varying degrees of RBF1- and RBF2-mediated repression (groups A, B, and C). In contrast, other genes (groups D and E) have little or no dependence on dE2F1 activation
and are repressed by dE2F2/RBF1 and -2 (12). Group D/E genes have functions in differentiation and development and exhibit gender- and tissue type-specific expression patterns. The repression of these genes by dE2F2/RBFs is maintained in actively proliferating cells (12, 52).

Recent studies have identified a novel RB-associated complex in flies (dREAM/MMB), worms (DRM), and humans (DREAM/LINC). In actively proliferating cells, dREAM/MMB is required for the repression of group D/E genes but not for cell cycle-regulated E2F/RB target genes (21; E. J. Kwon, B. Taylor-Harding, D. K. Dimova, and N. J. Dyson, unpublished observations). In flies, the complex is comprised of dE2F2, dMyb, and Myb-interacting proteins (Mip) homologous to the C. elegans synthetic multivulva class B (synMuvB) gene products. One group found that the complex also contained dRpd3/HDAC1 and L(3)MBT, whereas these proteins were absent in other preparations (21; E. J. Kwon et al., unpublished). The human (DREAM/LINC) and worm (DRM) complexes are similar in composition, but in humans, the complex contains either E2F4 or Myb but not both, and in the worm, there is no Myb component (20, 24, 28, 30, 32). The evolutionary conservation suggests that this complex may have important roles in the development of multicellular organisms, but regulatory details may differ depending on its precise composition.

The mechanism(s) of action of dREAM/MMB remains uncertain. In this study, we have investigated the role of the dREAM/MMB complex in RB-mediated repression at developmentally regulated genes by examining the chromatin modifications at these genes and their dependence on E2F/RB/dREAM/MMB. We find that two distinct mechanisms of repression are employed, one of which involves HDAC activity and histone deacetylation of nucleosomes at promoter regions and the other the activity of a Polycomb group protein, Enhancer of zeste [E(Z)], and the dimethylation of histone H3 lysine 27 (H3K27me2) at nucleosomes located downstream from the transcription start site.
4. Materials and Methods

Cell culture and RNAi.

Drosophila melanogaster SL2 cells were cultured at 24.5°C in Schneider's insect medium (Invitrogen/GIBCO) supplemented with 10% fetal bovine serum (FBS; HyClone). RNA interference (RNAi) was performed as previously described (51). Sodium butyrate (NaB; Sigma) treatment and sample collection for Northern and Western blot analysis were performed as previously described (54). Stable cell lines expressing either an N-terminal [FLAG-HA-E(Z)] or a C-terminal [E(Z)-HA-FLAG] FLAG and hemagglutinin (HA)-tagged E(Z) protein under the inducible metallothionein promoter were generated using Cellfectin (Invitrogen). Cells were transfected according to the manufacturer's instructions with the PmtFHEZ or PmtEZHF construct, and stable transfectants were selected for 3 to 4 weeks in medium containing 0.2 mg/ml hygromycin B (Roche). E(Z)-expressing cell lines were incubated with copper sulfate (200 µM) for 24 h to induce expression. Induction resulted in a 5-fold increase in E(Z) protein levels.

Plasmid construction.

The full-length open reading frame (ORF) of E(z) was first assembled in pBluescript II KS+ (Stratagene) with the E(z) 3’ untranscribed region (UTR) to generate pBSEZ-3’UTR. The E(z) ORF was amplified with PfuTurbo DNA polymerase (Stratagene) using primers NTEZXBA and EZXHOCT and cDNA synthesized from total RNA of Sg4 cells, which are derived from S2 cells, or wing discs of third-instar larvae as the template. The E(z) 3’UTR was amplified using primers NTEZ3UTRXHO and EZ3UTRKPNCT and genomic DNA as the template. The PCR products were inserted in the XbaI-KpnI sites of pBluescript II KS+. To generate the copper sulfate-inducible FLAG-HA-tagged expression construct PmtFHEZ (tag at the N terminus) or PmtEZHF (tag at the C terminus), a fragment containing the E(z) ORF was amplified with
PfuTurbo DNA polymerase using NTEZECOV and EZECOVCT as primers and pBSEZ-3'UTR as the template and inserted in the EcoRV sites of PmtFH or PmtHF, respectively (kind gifts from T. Kusch). Oligonucleotide sequences are as follows: NTEZXBA,
5'-GGCTCTAGAAATAAGCACTAAAGTGCCGCCCAGT-3'; EZXHOCT,
5'-GACCTCGAGTCAAAACATTTTCATTTGCAGCTCTATGCCCA-3'; NTEZ3UTRXHO,
5'-TGTTCTCGAGCGAGTCTACTTGAAAAATCGTATCAT-3'; EZ3UTRKPNCT,
5'-CTCGATATCAACAATTTCCATTTCCACGCT-3'; NTEZECOV,
5'-GAAGATATCATGAATAAGCACTAAAGTGCCG-3'; and EZECOVCT,
5'-CTCGATATCAACAATTTCCATTTCCACGCT-3'.

**RNA isolation and Northern blotting.**

Total RNA was isolated using Trizol (Invitrogen) reagent. Northern blotting using riboprobe was performed as previously described (12).

**Western blotting and immunoprecipitation.**

Western blotting was performed using standard techniques, and the following antibodies were used: dE2F2 (rabbit polyclonal), RBF1 (mouse monoclonal DX3), RBF2 (mouse monoclonal DR6), anti-FLAG (Rockland), p55CAF1 (Abcam ab1766), dRpd3/HDAC1 (Abcam ab1767), anti-HA (Covance), anti-E(z), and anti-Pc (rabbit polyclonal; gift from T. Kahn). For immunoprecipitation assays, cells were lysed in radioimmunoprecipitation (RIPA) buffer (51) and immunoprecipitated with anti-RBF1 (DX5 mouse monoclonal), anti-RBF2 (DR3 mouse monoclonal or rabbit polyclonal), or nonspecific (anti-β-tubulin) antibodies. Ethidium bromide (EtBr) was added (200 µg/ml), and lysates were incubated for 30 min on ice. Precipitates were removed by 5 min of centrifugation, and the resulting lysate was used in immunoprecipitation experiments. The EtBr concentration was maintained during the washing steps.
Chromatin immunoprecipitation assays.

Chromatin immunoprecipitation (ChIP) was performed as previously described (12, 19), with the following modifications. Chromatin was sheared to an average size of 300 bp using a Bioruptor (Diagenode), and immunoprecipitated DNA was analyzed by quantitative real-time PCR (LightCycler 1.5; Roche) using the standard curve method. The results are represented as the ratio of the amount of a specific sequence over the amount of a nonspecific (RP49 promoter or bxdPRE) sequence. Each immunoprecipitation was performed at least three times, and the standard deviation was calculated. Antibodies used for ChIP are as follows: anti-histone H3 (Abcam ab1791), anti-H3K9me2 (Upstate 07-441), anti-H3K9me3 (Abcam ab8898 and Upstate 07-442), anti-H3K27me2 (Upstate 07-452), anti-H3K27me3 (Abcam ab6002 and Upstate 07-449), anti-H4K20me1 (Upstate 07-440), anti-H4K20me2 (Upstate 07-367), anti-H4K20me3 (Upstate 07-463), anti-acetyl H3 (Upstate 06-599), anti-acetyl H3K27 (Abcam ab4729-25), and anti-acetyl H4 (Upstate 06-866). Primers were designed to amplify between 100 and 150 bp of the sequences. Primer sequences are available upon request.
5. Results

A. Low histone acetylation levels at developmentally regulated E2F/RB target gene promoters.

Prior studies have shown that a novel RB-associated complex, dREAM/Myb-MuvB (MMB), is required for the repression of developmentally regulated but not for cell cycle-regulated E2F target genes (21, 28, 30; E. J. Kwon et al., unpublished). However, no known transcriptional repression activity has been associated with the complex.

RBF proteins are known to physically interact with dRpd3/HDAC1, and dRpd3 was found to copurify with the dREAM/MMB complex in one study (30, 54). Additionally, Mip130, one of the dREAM/MMB components, was found to preferentially bind in vitro to nonacetylated histone H4 tails (28). Therefore, we used chromatin immunoprecipitation (ChIP) to examine the histone acetylation at the promoters of several of the 37 class D/E genes previously identified in SL2 cells (12).

The acetylation signals for both histone H3 and H4 in normal SL2 cells were extremely low, comparable to the levels at a Polycomb-repressed region—the regulatory region of the bithoraxoid gene (Fig. 7, bxdPRE).
Figure 7 Changes in histone acetylation levels at group D/E gene promoters in cells depleted of dREAM/MMB components.

(A) Western blot analysis of whole-cell extracts from SL2 cells incubated with double-stranded RNA (dsRNA) targeting dE2F2, p55CAF1, or white (control). β-Tubulin served as loading control. (B) Northern blot analysis of total RNA extracted from cells incubated with dsRNA targeting Mip40, Mip120, or white. (C and D) ChIP assay was performed with antibodies recognizing panacetylated histone H3 (left panels) or panacetylated histone H4 (right panels) in cells incubated with dsRNA targeting white (control), dE2F2, p55CAF1, Mip40, or Mip120. The amount of coprecipitated DNA was determined by quantitative real-time PCR. Results are normalized to those for a nonspecific sequence (promoter of RP49) and represent the averages of the results of three independent experiments. The promoter region of CG7628 (a non-E2F-regulated gene) and sequences surrounding the regulatory region of the bithoraxoid gene (bxdPRE) were used as negative controls.
We used RNA interference (RNAi) to selectively remove components of the dREAM/MMB complex and examined the acetylation levels. The removal of dE2F2, p55/CAF1, Mip40, or Mip120 resulted in an increase in histone H3 and H4 acetylation at group D/E genes but not at the bxdpRE. We also examined the promoter of the CG7628 gene. This gene is not an E2F/RB target, as we cannot detect binding of dE2F2 or any other dREAM/MMB component at its promoter. Nonetheless, its expression is increased in dE2F2-depleted cells. We found that the low histone acetylation levels at the promoter of CG7628 did not change upon dE2F2 or dREAM/MMB disruption. These findings indicate that the increased acetylation levels observed at the other promoters are not simply the consequence of increased transcription but, rather, a direct result of disrupting dE2F2/RBF and dREAM/MMB function.
B. **HDAC activity is required for the repression of developmentally regulated genes.**

We asked whether HDAC activity, dRpd3 activity in particular, was important for the repressed state of group D/E genes. Previous studies have shown that HDAC inhibition did not lead to the derepression of several E2F target genes (54). Given our findings that group D/E gene promoters are deacetylated upon dREAM/MMB disruption, we wanted to further investigate this issue.

We used RNAi to deplete the putative HDAC1-dRpd3 and examined histone acetylation. The histone acetylation levels at the promoters did not change significantly in dRpd3-depleted cells (Fig. 8B). We considered the possibility that the presence of dREAM/MMB at group D/E promoters may prevent histone acetylation and that, therefore, no increase in acetylation would be observed in cells lacking dRpd3. However, removing dRpd3 and two dREAM/MMB subunits simultaneously did not result in any further increase in acetylation levels (Fig. 8B). Our results suggest that dRpd3 is not important for the repression of these genes and other HDACs might be recruited; alternatively, multiple HDAC proteins can function at these genes. To account for possible functional redundancy among the different HDACs, we treated cells with the general HDAC inhibitor sodium butyrate (NaB). The inhibition of all HDAC activity resulted in an increase in the acetylation of both histone H3 and H4 at group D/E gene promoters (Fig. 9B) that correlated with the derepression of these genes (Fig. 9C). HDAC inhibition leads to a decrease in the number of S-phase cells (54). However, this change in cell cycle distribution should have no effect on group D/E genes, as they are regulated in a cell cycle-independent manner (12). In contrast, cell cycle-regulated E2F targets are affected, and this can be seen in the decrease in the levels of DNA primase (Fig. 9C, DNAprim). We note that the different group D/E genes exhibit distinct sensitivities toward HDACs. As previously observed, CG8399, an atypical member of the group of developmentally regulated genes, was derepressed in both dRpd3-depleted and sodium
butyrate-treated cells (54). The Arp53D mRNA was modestly but reproducibly increased in cells lacking dRpd3 (Fig. 9C) and strongly increased when all HDAC activity was inhibited. In contrast, CG3505 and CG17142 were not affected by dRpd3 depletion but were derepressed in sodium butyrate-treated cells. Our results are consistent with those of studies performed by Foglietti and colleagues, who examined genome-wide changes in gene expression in cells lacking various HDAC proteins and in cells treated with the HDAC inhibitor trichostatin A (17).
Figure 8 Effect of dRpd3 depletion on histone acetylation at group D/E gene promoters.

(A) Western blot analysis of whole-cell extracts from SL2 cells incubated with double-stranded RNA (dsRNA) targeting dRpd3 or Mip40 and Mip120. (B) ChIP assay was performed with panacetylated histone H3 (left panel) or histone H4 (right panel) antibodies in cells incubated with dsRNA targeting white (control), dRpd3, Mip40 and Mip120 (cotreated), or Mip40, Mip120, and dRpd3 (cotreated). Results are normalized to those for RP49 promoter sequences.
Figure 9  Inhibition of HDAC activity affects acetylation levels and repression of group D/E genes.

(A) Western blot analysis of whole-cell extracts from cells treated with either PBS (control) or sodium butyrate (NaB) for 16 h. Blots were probed with antibodies recognizing acetylated histone H3 and β-tubulin. (B) ChIP assay was performed with antibodies recognizing panacetylated histone H3 (left panels) or panacetylated histone H4 (right panels) in cells treated with PBS (control) or
sodium butyrate (NaB). (C) Northern blot analysis using probes to DNA primase (DNAprim; a cell cycle-regulated E2F target gene), and several group D/E genes, Arp53D, CG3505, CG17142, and CG8399. Note that CG3505 and CG17142 generate two transcripts of different sizes. Total RNA was extracted from cells incubated with double-stranded RNA (dsRNA) targeting dRpd3 or with NaB or control (white dsRNA, PBS).
Given the strong evidence for physical interactions between dRpd3, RBFs, and dREAM/MMB, we asked whether we could detect dRpd3 at any of the group D/E gene promoters. Consistent with the expression data, we have been unable to detect dRpd3 at the promoters of CG3505 or CG17142, suggesting that at these genes, RBFs may employ other HDAC activities. In contrast, dRpd3 was bound to a specific region of the Arp53D promoter, and this binding was dependent on dE2F2 (Fig. 10). While we cannot completely exclude the possibilities that dRpd3 may bind to promoter-distal regulatory regions not included in our ChIP analyses and that the lack of derepression seen in dRpd3-depleted cells may be due to functional redundancy, we propose that different HDACs might be important at different group D/E genes. Our results clearly show that deacetylation of histones by HDACs is important to maintain the repression of developmentally regulated dE2F/RBF target genes.
Figure 10 dRpd3 binding at the Arp53D promoter region.

(A) ChIP assay performed with anti-RPD3 or nonspecific antibodies (Ab). Coprecipitated DNA was analyzed for the presence of promoter sequences of DNA polymerase α (DNApolα), RP49, or two regions of the Arp53D promoter (distal and proximal regions as determined by the presence of putative E2F binding sites and described for panel D). (B) ChIP results from three independent experiments were determined by quantitative real-time PCR. (C) dRpd3 binding is dE2F2 dependent. ChIP assay was performed with anti-RPD3 antibodies in white (control) or dE2F2-depleted cells. ds, double stranded. (D) Structure of Arp53D promoter region. D, distal region; P, proximal region; TSS, transcription start site. The numbers depict the distance from the TSS.
**C. Histone methylation patterns at developmentally regulated E2F/RB target gene promoters.**

A number of researchers have shown that RB family members interact with the histone methyltransferase SUV39H1 and that repression at cell cycle-regulated genes involves the methylation of histone H3 lysine 9 (H3K9) (38, 39, 58). To our surprise, we detected no H3K9 trimethylation at any of the group D/E promoters examined (Fig. 11A). Similarly, H3K9 dimethylation was absent at all but two gene promoters (Fig. 11B, CG12767 and CG8399), and the methylation at these two genes was not dependent on E2F/RB (data not shown). Our negative results are not due to poor sensitivity of the assay, as we readily detect H3K9 tri- and dimethylation at two regulatory regions (Fig. 11G and H, respectively) which have been previously identified as being methylated (8). Thus, methylation of histone H3K9 does not play a role in the repression of developmentally regulated E2F/RB targets in SL2 cells.
Figure 11  Histone methylation at group D/E gene promoters.

ChIP assay was performed with anti-H3K9me3 (A), anti-H3K9me2 (B), anti-H3K27me2 (C), anti-H4K20me3 (D), anti-H4K20me2 (E), anti-H4K20me1 (F), anti-H3K9me3 (G) anti-H3K9me2 (H), or nonspecific antibodies. Immunoprecipitated DNA was quantified by quantitative real-time PCR. Results are normalized to those for RP49 sequences and represent the averages of the results of three independent experiments. The promoter sequences of Spn5 (H3K9me3) (G) or CG11165 (H3K9me2) (H) served as positive controls for the assay. Ab, antibody.
The lethal 3 malignant brain tumor protein, L(3)MBT, is a transcriptional repressor that affects the repression of several but not all group D/E genes (30). L(3)MBT was also found to copurify with the MMB complex. Its human counterpart, L3MBTL1, associates with pRB and negatively regulates the expression of cyc E, a cell cycle-regulated E2F/RB target (26, 57). Interestingly, L3MBTL1 is believed to function by compacting nucleosomal arrays, and this compaction was dependent on mono- and dimethylation of histone H4 lysine 20 (H4K20) (42, 57). We decided therefore to examine histone H4 lysine 20 methylation at group D/E promoters using antibodies that recognize H4K20me1, H4K20me2, and H4K20me3. None of the promoters examined exhibited detectable histone H4K20 methylation (Fig. 11D, E, and F). The antibodies we have utilized have been successfully used for the detection of histone H4K20 methylation by ChIP by many groups. However, while H4K20 heterochromatic regions have been found in flies (15, 45), we have been unable to identify corresponding gene regions in SL2 cells to use as positive controls in our assays. Therefore, we cannot completely rule out the possibility that H4K20 methylation plays a role at group D/E genes, but our results suggest that L(3)MBT may regulate some of these genes either indirectly or through a mechanism that does not involve histone H4K20 methylation.

The idea that dE2F2/RBF repression is sustained in dividing cells and is used to generate developmentally regulated patterns of expression at group D/E genes draws a parallel with the function of the Polycomb group proteins. Furthermore, there are several reports in the literature linking the Polycomb and Rb pathways (3, 10, 29, 35, 55). For this reason, we considered the methylation of histone H3 lysine 27 as a potential means to keep these genes repressed. We looked at histone H3 lysine 27 (H3K27) trimethylation in putative E2F-dependent promoter regions that we identified previously in microarray studies (12, 52) by examining the data from a genome-wide study of H3K27 trimethylation in fly tissue culture cells (46). We also compared the distribution of H3K27 trimethylation with that of dE2F2 using recent genome-wide binding studies (21). We
found that none of the putative dE2F2 target genes exhibited any significant H3K27 trimethylation. Next, we examined H3K27 dimethylation using ChIP. We did not detect H3K27 dimethylation at most gene promoters, with two exceptions, CG8399 and CG17142 (Fig. 11C). Previous studies have shown that the atypical group D gene CG8399 might be regulated by multiple repression complexes, including Esc/E(Z), which is responsible for histone H3K27 methylation (54). Collectively, our results indicate that histone H3K9, H3K27, and H4K20 methylation may not be employed in the repression of group D/E gene promoters, although we cannot exclude the possibilities that regulatory regions other than the proximal promoters might be controlled through histone methylation or that this regulation might be cell type specific.
D. Histone H3K27 dimethylation plays a role in the repression of group D/E genes.

We were curious as to why only CG17142 among the D/E gene promoters exhibited histone H3K27 methylation and none of the others did. We inspected the CG17142 sequence to see whether something sets it apart from other group D/E gene promoters. The regions we amplified in ChIP experiments are sequences in which we previously identified putative E2F binding sites and where we had detected E2F and RBF binding (12). At the time of our first analysis, little was known about CG17142, including its promoter. Recently, CG17142 has been identified as the gene pyrexia, and its transcription start site has been mapped (16). Upon close examination, we determined that the region we have been analyzing is located downstream of the transcription start site. Interestingly, a recent genome-wide study in human cells found that histone H3K27 dimethylation is associated with silent genes and is found downstream of the transcription start sites of these genes (2). Therefore, we decided to examine the regions downstream of the transcription start sites of group D/E genes for H3K27 dimethylation. As antibodies directed against H3K27 methylation have some level of cross-reactivity, we used both anti-H3K27me2 and anti-H3K27me3 antibodies. We found that, indeed, these regions were enriched in H3K27 dimethylation but not H3K27 trimethylation (Fig. 12). In contrast, the highly expressed, cell cycle-regulated E2F/RB targets (A/B group genes) did not exhibit any histone H3K27 methylation (data not shown).
Figure 12 Histone H3K27me2 at coding regions of group D/E genes.

(A) ChIP assay was performed with anti-H3K27me2 antibodies. Sequences representing regions ~200 to ~300 bp downstream from the TSS of group D/E genes were amplified. Results were quantified and normalized to those for RP49 promoter sequences. (B) ChIP assay was performed as described for panel A but with anti-H3K27me3 antibodies. The lack of amplification indicates that the results obtained in the experiments whose results are shown in panel A are specific and not due to antibody cross-reactivity. Sequences surrounding the regulatory region of bithoraxoid (bxoPRE) served as the positive control for H3K27me3. Ab, antibody.
The role of histone H3K27me2 is not well studied or understood. It has been observed that in Drosophila polytene chromosomes, H3K27me2 is broadly associated with pericentric chromatin, as well as euchromatin (14). The results of our studies with E2F/RB target genes in Drosophila tissue culture cells are similar to the observations made of human cells (2) and suggest that H3K27me2 is a modification that is present downstream of the transcription start site of repressed genes. We wanted to determine whether the observed modification is relevant to the regulation of group D/E genes. Several lines of evidence support the idea that histone H3K27me2 is involved in the repression of E group genes by E2F/RB proteins. First, H3K27me2 is significantly reduced when dE2F/RBF and dREAM/MMB functions are disrupted (Fig. 13). Group D/E genes are redundantly regulated by RBF1 and RBF2; the depletion of either protein had no effect, but the removal of both RBFs or dE2F2 decreased H3K27me2 levels (Fig. 13B). Disruption of the dREAM/MMB complex by simultaneous depletion of Mip40 and Mip120 also resulted in reduced histone H3K27 methylation (Fig. 13C).
Figure 13 Removal of dE2F2/RBFs or dREAM/MBM reduces histone H3K27me2 levels at group D/E genes.

(A) Western blot analysis of whole-cell extracts from SL2 cells incubated with double-stranded (ds) RNA targeting RBF1, RBF2, or RBF1 and RBF2 (cotreated). Blots were probed with anti-RBF1, anti-RBF2, and anti-β-tubulin antibodies. (B) ChIP assay was performed with anti-H3K27me2 antibodies in cells depleted by RNAi of luciferase (Luc; control), RBF1, RBF2, RBF1 and RBF2, or dE2F2. (C) ChIP assay was performed with anti-H3K27me2 antibodies in cells depleted of luciferase (control) or Mip40 and Mip120. Results are normalized to those for RP49 promoter sequences and represent the averages of the results of three independent experiments.
Second, the loss of the H3K27 methyltransferase E(Z) resulted in the loss of H3K27me2 at group D/E genes (Fig. 14C). In contrast, the removal of another histone methyltransferase, G9a, had no effect. These findings are consistent with the idea that E(Z) is the sole H3K27 methyltransferase in Drosophila and that E(Z) is required to maintain the methylation state through multiple cell divisions.

Third, because the human EZH2 has been shown to be downstream of the E2F/RB pathway (4), we checked whether the levels of E(Z) are affected in E2F/RBF/dREAM/MMB-depleted cells. The E(Z) protein levels were reduced in dE2F1-depleted cells, confirming that, similar to mammals, E(z) is an E2F-regulated gene in flies (data not shown). However, the protein levels were not changed in cells lacking dE2F2, RBFs, or dREAM/MMB subunits (Fig. 14E), indicating that the reduction in H3K27me2 levels in these cells is not an indirect consequence of reducing E(Z) protein levels.

Fourth, we asked whether the loss of histone H3K27me2 will lead to the derepression of group D/E genes and whether E(Z) is required for the repressed state. We used RNAi to deplete E(Z) protein and examined group D/E gene expression by Northern blotting. We detected elevated levels of group D/E genes in E(Z)-depleted cells, indicating that repression was disrupted (Fig. 14D).

Finally, we wanted to establish a physical link between E(Z) and dE2F/RBF. We used cell lines stably expressing an inducible FLAG-HA-tagged E(Z) protein to ask if RBF proteins interact with E(Z). We immunoprecipitated either endogenous RBF1 or RBF2 and asked if we could detect E(Z). E(Z) specifically coimmunoprecipitated with both RBF proteins (Fig. 14F), and we obtained similar results with both N- and C-terminally tagged E(Z) proteins. Furthermore, the binding of E(Z) to both RBF proteins was not DNA dependent, as it was not disrupted by pretreatment with ethidium bromide (Fig. 14F; compare EtBr + and –), indicating a direct interaction between the proteins.
Figure 14 E(Z) is required for the repression of group D/E genes.

(A) Western blot analysis of whole-cell extracts from cells treated with double-stranded RNA (dsRNA) targeting white (control) or E(Z) and probed with anti-E(Z) or anti-β-tubulin antibodies.

(B) Northern blot analysis of total RNA isolated from cells treated with dsRNA targeting white (control) or G9a and probed for G9a and β-tubulin.

(C) ChIP assay was performed with anti-histone H3K27me2 antibodies in cells depleted of luciferase (Luc; control), E(z), or G9a.

(D) E(Z) is
required to maintain the repression of group D/E genes. Northern blot analysis using probes to several group D/E genes and β-tubulin (loading control). Total RNA was isolated from cells treated with dsRNA against white (control), dE2F2, and E(z). (E) E(Z) protein levels are not affected in cells lacking dREAM/MMB components. Western blot analysis of whole-cell extracts from cells treated with dsRNA targeting luciferase (control), dE2F2, RBF1, RBF2, RBF1 and RBF2 (cotreated), or Mip40 and Mip120 (cotreated). Blots were probed with anti-E(Z) and anti-β-tubulin (loading control) antibodies. (F) E(Z) coimmunoprecipitates with both RBF1 and RBF2. Anti-RBF1 or anti-RBF2 antibodies were used in immunoprecipitations with extracts from cells expressing Flag-HA-tagged E(Z). Prior to immunoprecipitation, extracts were incubated with 200 µg/ml ethidium bromide (+) or without EtBr (−). Immune complexes were analyzed for the presence of coprecipitated HA-E(Z); one part was subjected to blotting with anti-RBF antibodies. WCE, whole-cell extract (1/200 of input was loaded); β-tubulin, nonspecific antibody control.
E. A mechanism for repression of differentiation-specific dE2F/RBF target genes.

We wanted to investigate the mechanism by which E(Z) may maintain the repression of group D/E genes. The Polycomb group repression mechanism is based on two principal types of multiprotein complexes, Polycomb group repressor complex 1 (PRC1) and Polycomb group repressor complex 2 (PRC2). E(Z) is part of the PRC2 complex, which functions as a histone methyltransferase. The repressive action of PRC2 involves histone H3K27 methylation, which is in turn recognized by the PRC1 complex. Polycomb protein (PC), a component of PRC1, binds specifically to methylated histone H3K27. It has been shown that dE2F2/RBF localize to nontranscribed regions on polytene chromosomes, but these regions do not overlap the regions of binding of PC (28). We also examined genome-wide binding data for PC and dRING, another PRC1 component (8, 41, 46), and found that they do not localize at sequences surrounding group D/E genes. The lack of binding suggests that PRC1 may not be involved in the regulation of dE2F/RBF target genes. However, it has been observed that histone H3K27 methylation and Polycomb group (PcG) protein binding do not always overlap (41, 46). We therefore asked whether there is a functional requirement for PRC1 at group D/E genes. We disrupted PRC1 function by depleting the PC component by RNAi (Fig. 15A) and examined gene expression (Fig. 15B). We found that the removal of Pc did not lead to derepression, while dREAM/MMB disruption (CAF1/p55) readily resulted in increased expression levels. Taken together, these findings indicate that PRC1 is not involved in the repression of group D/E genes by E(Z).
Figure 15 dREAM/MMB represses group D/E genes by two independent mechanisms.

(A) Western blot analysis of whole-cell extracts from cells treated with double-stranded RNA (dsRNA) targeting white (control) or Pc. The blot was probed with anti-PC or anti-β-tubulin antibodies. (B) Northern blot analysis using probes to group D/E genes or β-tubulin. Total RNA was isolated from white-, p55CAF1-, or Pc-depleted cells. (C) Changes in H3K27 acetylation levels at coding regions of group D/E genes in dE2F2- or E(Z)-depleted cells. ChIP assay was performed with anti-acetyl H3K27 antibodies in SL2 cells treated with dsRNA targeting white (control), dE2F2, or E(Z). (D) Histone H3K27me2 at coding regions of group D/E genes is not affected by HDAC inhibition. ChIP assay was performed with H3K27me2 antibodies on cells treated with PBS or NaB. (E) Histone acetylation at promoter regions of group D/E genes is not affected by the depletion of E(Z). ChIP assay was performed with panacetylated histone H3 (top panel) or panacetylated histone H4 (bottom panel) antibodies. Note that the panacetylated histone H3 antibody used in this experiment does not target the H3K27 site. (The antibody is raised against the peptide consisting of the first 20 amino acids of histone H3). (F) Northern blot analysis using probes to group D/E genes. Total RNA was isolated from cells incubated with dsRNA targeting white (control), dE2F2, E(Z), dRpd3, or both dRpd3 and E(Z) (top panel) or treated with E(Z) dsRNA or NaB or cotreated with E(Z) dsRNA and NaB (bottom panel).
We next wanted to establish the relationship between histone deacetylation and methylation and whether they cooperate or act independently in the regulation of group D/E genes. We sought to determine what effect, if any, E(Z) had on histone acetylation levels. We noted that upon E(Z) depletion, histone H3K27 dimethylation levels went down, but H3K27 acetylation levels rose at nucleosomes located downstream of the transcription start sites (TSS), suggesting that the two modifications may play opposing roles in the transcriptional regulation of these genes (Fig. 15C). In contrast, the low histone H3 and H4 acetylation levels at the upstream promoter regions did not change upon the removal of E(Z) (Fig. 15E). Likewise, an increase in histone acetylation levels at promoters did not affect histone methylation downstream of the TSS; histone H3K27 dimethylation remained unchanged in sodium butyrate-treated cells (Fig. 15D). These data indicate that the histone modifications at the two regions are independent of each other and reflect two distinct mechanisms of repression at group D/E genes: deacetylation of histone H3 and H4 at promoter regions and lysine 27 dimethylation of histone H3 at nucleosomes located downstream of the TSS.

Both mechanisms are required to maintain the repressed state, since either inhibiting HDAC activity or removing E(Z) (Fig. 9, 14, and 15) results in increased mRNA levels, similar to what is observed in dE2F2-depleted cells. Removing both, however, did not result in a further increase [Fig. 15F, dsRPD3+dsE(Z) and dsE(Z)+NaB], indicating that there are no synergistic or additive effects of inhibiting both histone deacetylation and histone methylation.
6. Discussion

The complexity of the E2F/RB pathway in mammals, both in terms of the number of different transcriptional complexes and the number of putative target genes, has made the study of RB action less than straightforward. One major issue with studies in mammals is that it is not clear which target genes are directly regulated by pRB, and this has limited the scope of investigation.

The mechanism of RB-mediated repression has been studied extensively, and the main conclusion to emerge from these studies is that the gene expression of E2F/RB target genes is linked to dynamic changes in chromatin modifications. RB is believed to recruit components of different chromatin-modifying complexes with histone deacetylase (HDAC) activities, histone methyltransferase (HMT) activities, DNA methyltransferase (DNMT) activities, and ATP-dependent chromatin remodeling (18, 34). Until recently, most of the work on RB employed overexpression approaches and reporter constructs. The use of mutant cell lines and RNAi techniques has began to provide further insights into the mechanisms of repression at several cell cycle-regulated genes. It is clear that histone acetylation and deacetylation play a role in the regulation of many E2F targets transcribed at the G1/S transition, and histone methylation has been implicated in some of these. The mechanism of repression appears to differ depending on the cellular state.

Here, we have taken advantage of the recent characterization of E2F and RB transcriptional programs in Drosophila. Specifically, the discovery of a group of genes that are regulated by RBF proteins in a cell cycle-independent manner and are involved in development has enabled us to examine the mechanisms of repression employed by RB in regulating the expression of differentiation-specific genes. We have determined that two different types of chromatin
modifications contribute to the repression of developmentally regulated RBF target genes and that they are present at two distinct locations.

The deacetylation of histone tails at the promoters of group D/E genes appears to be a prominent feature. While the promoters of highly transcribed, cell cycle-regulated RBF targets are highly acetylated, group D/E gene promoters exhibit very low acetylation levels. These low levels were dependent on the presence of dREAM/MMB. The inhibition of HDAC activity and the concomitant increase in histone acetylation levels resulted in increased expression of the genes comparable to that when dREAM/MMB was disrupted. These findings are in agreement with previous observations that dREAM/MMB components bind preferentially to nonacetylated histone tails (28). We note that another study, by Taylor-Harding and colleagues, found that in cells depleted of various HDAC proteins or treated with HDAC inhibitors, the expression of selected dE2F/RBF targets did not change (54). However, in our hands, the inhibition of all HDAC activity resulted in the derepression of all group D/E genes examined and an increase in histone acetylation at their promoters. Although we cannot currently explain the differences between the two results, we note that our findings are in agreement with those of a genome-wide study of HDAC-dependent gene expression changes in Drosophila (17). One possible explanation for the observed differences might be that the requirement for HDAC activity varies between genes and depends on growth conditions, similar to what has been found in mammalian cells (33, 49, 65; reviewed in references 5, 18, and 23).

The nature of the HDAC proteins involved in the repression is less clear-cut. In both Drosophila and mammals, dRpd3/HDAC1 appears to be part of the dREAM/MMB complex in some conditions but not in others (28, 30, 32, 43). We find that dRpd3 is not generally required for the repression of group D/E genes. This suggests that other HDAC proteins may be important for the regulation of these genes or that there is functional compensation among different HDACs.
Nonetheless, we find that dRpd3 is required for the repression of the Arp53D gene and that its binding to the promoter was mediated by dE2F2. Taken together, our results indicate that HDAC activity is an important component of several RB functions. However, its importance in the maintenance of RB-mediated repression is likely to differ between different promoters, cell types, and cellular states.

We did not detect repressive histone methylation marks at the promoters of group D/E genes. Studies with cell cycle-regulated E2F target genes indicate that histone methylation is not important in cycling cells but is important in differentiated cells (1). As our studies were performed in actively proliferating cells, we cannot exclude the possibility that the promoters of group D/E genes have methylated histones in nondividing cells. Furthermore, these genes remain repressed in many different tissue types and repression in different cell types may involve histone methylation. However, this clearly cannot be a mechanism of repression in actively proliferating cells, as we have shown that the differentiation-specific genes remain silent in S phase in SL2 cells (12).

We have determined that all of the group D/E genes examined exhibited histone H3K27me2 downstream of the TSS. Histone H3K27 methylation is linked to the mechanism of repression by Polycomb group proteins, and histone H3K27me3 is thought to play a prominent role in the silencing of genes and large chromatin domains. In Drosophila, it has been suggested that histone H3K27me2 is broadly distributed in euchromatin (14), whereas in humans, this modification is present at repressed genes (2). Our results strongly suggest that histone H3K27me2 is not as ubiquitous as it is believed to be. We did not detect histone H3K27me2 at any promoter examined; rather, this modification was specifically present downstream of the TSS of developmentally regulated E2F/RB target genes but not at other genes examined. Furthermore, E(Z), the histone H3K27 methyltransferase, was required to maintain the modification and for the repression of these genes. Our findings, taken together with the observations of human cells,
suggest that dimethylation of histone H3K27 at nucleosomes located downstream of the TSS represents a new and unexplored mechanism of repression.

At present, we do not know how the repression of group D/E genes by H3K27me2 is achieved. Our results indicate that PRC1 and histone ubiquitination are not involved in the regulation of dE2F/RBF target genes, as PC and dRING (the histone ubiquitin ligase component of PRC1) do not localize to group D/E genes and disruption of PRC1 function had no effect on the expression of the genes. This conclusion is further supported by the finding that Pc and histone H3K27 dimethylation do not colocalize on polytene chromosomes (41). It is possible that the repression involves, at least in part, the prevention of histone H3K27 acetylation, as the removal of E(Z) or dE2F2 resulted in a concomitant increase in acetylation. Alternatively or in addition to this, the dimethylated histone H3K27 may serve as a docking site for other repressive proteins. We found that H3K27me2 did not affect histone H3 and H4 acetylation at promoters and vice versa, and yet, the presence of both modifications was required to maintain repression.

The lack of a requirement for PRC1, which contains DNA binding activities, suggests that PRC2 might be recruited to these genes by dE2F2. p55/CAF1 is a component of both the dREAM/MMB and the PRC2 complex, and we found that E(Z) can bind to both RBF proteins. However, we have been unable to find E(Z) by ChIP at group D/E genes. Similarly, work in mammalian cells has failed to detect PRC2 binding at pRB-regulated genes (3), and E(Z) binding has not been detected at all histone H3K27-methylated regions (46). It is possible that PRC2 binding is transient. Alternatively, the binding might be outside the range of the sequences surveyed. In the case of group D/E genes, E(Z) activity may be required to maintain the methylation during the S phase, when newly synthesized histones are assembled onto DNA.
Several recent studies in mammalian cells link the repression by RB family proteins to PcG proteins (3, 10, 29, 55). While our studies confirm the link between the Polycomb and Rb pathways, they paint a picture substantially different than what has been observed at cell cycle-regulated genes in mammals. First, we find that repression involves histone H3K27 dimethylation rather than trimethylation and that it is not targeted at upstream promoter regions. Second, we find that while E(Z) is functionally required, PRC1 does not play a role in the repression of these genes. Third, we find that E(Z) functions together with HDAC activities rather than competing with them as has been shown at the cyclin A gene promoter in mammals (55). It is tempting to speculate that the different mechanisms of repression of group D/E genes are dictated by the need to shut down their transcription at a time (S phase) when RB-mediated repression is disrupted at cell cycle-regulated targets.

The role of the recently identified E2F/RB- and Myb-containing protein complex dREAM/MMB (flies), DRM (worms), or DREAM/LINC (humans) in various RB functions is not well understood. While in flies it appears to be required for the repression of developmentally regulated but not cell cycle-regulated E2F target genes, studies in human cells indicate that it may regulate the expression of genes transcribed in the G1/S and/or G2 phases of the cell cycle (21, 28, 30, 32, 44). Similarly, genome-wide binding studies in flies indicate that components of the complex can be found at cell cycle-regulated promoters (21, 54). Additionally, recent studies in flies demonstrated that the expression of Polo kinase, a gene expressed in G2, was controlled in a switch-like manner by dREAM/MMB and suggested a role for the complex in the epigenetic regulation of gene expression (63). Thus, the complex appears to play a critical role in different E2F/RB functions. Our results are consistent with this idea. We find that dREAM/MMB plays a role in both methods of repression at group D/E genes: the deacetylation of histones at promoters, a mechanism shared with cell cycle-regulated genes, and the dimethylation of histone H3K27, a feature unique to differentiation-specific E2F/RB targets. It is possible that
dREAM/MMB/DREAM components serve as a scaffold to assemble distinct activities at different promoters and in different cellular states, thereby mediating the epigenetic regulation of RB target genes.
7. References (Chapter II)


Chapter III

A dual Role for the dREAM/MMB complex in the regulation of differentiation-specific E2F/RB target genes
1. Acknowledgements (Chapter III)

This work represents a manuscript that is in preparation for publication. Most of the work in this chapter is carried out by me. However, some results are produced through collaborations. The generation of RBF mutants and the establishment of RBF1 wt, RBF1 S/T-A (R1A) and RBF1 S/T-E (R1E) expressing cells used in this chapter was performed by lab members, including Linda Ragusano, Jagdip Gil, Bridget Huang, Kent Horvarth, Yekaterina Voskoboynik, and Anna Martinez. The ChIP assay that demonstrated dE2F2 and RBF1 binding in R1E cell lines is performed in collaboration with Anna Martinez. Cell cycle and FACS analyses and Northern Blot analysis were performed in collaboration with Dr. Dimova.
2. Abstract

E2F and RB proteins regulate the expression of genes involved in cell cycle progression, apoptosis, differentiation, development. Recent studies indicate that they function as part of an evolutionary conserved multi-protein complex, termed dREAM/DREAM/LINC. Here we characterize the role of the Drosophila complex, dREAM, in the regulation of differentiation-specific E2F target genes in actively proliferating cells. These genes are regulated differently from cell cycle-related E2F targets, they do not depend on E2F-activation and E2F/RB repression is maintained throughout the cell cycle. In proliferating cells, their repression is dependent on dREAM. We find that dREAM plays a dual role in their regulation. First, it is required for the stability of the repressive dE2F2/RBF complexes at their promoters during S-phase. Second, we find that dREAM is indispensable for both transcriptional repression mechanisms employed at these genes.
3. Introduction

Both cell division and exit from active proliferation cycles are integral parts of the development of multi-cellular organisms, and are orchestrated by transcription factors that regulate spatiotemporal expression of specific sets of target genes. The E2F and RB families of transcription factors are critical regulators of these processes and E2F/RB activity is altered in many human tumors (5, 7, 9, 32, 40).

The E2F/RB pathway was first studied in the context of G1/S control (6, 9, 37). E2F proteins can be subdivided into repressors and activators of transcription. RB family members, also known as pocket-proteins, negatively regulate E2F activity in two ways. Binding to activator E2Fs inhibits E2F transactivation. When bound to repressor E2Fs they recruit transcriptional co-repressors such as histone deacetylases (HDACs) or histone methyltransferases (HMTs) to inhibit expression of genes required for cell cycle progression (11). Cyclin-dependent kinases (CDKs) phosphorylate pocket-proteins during cell cycle progression thereby disrupting E2F-RB interactions. This results in both relief of E2F/RB mediated repression as well as release of transcriptionally active E2F that triggers entry into S-phase.

It is now clear that E2F/RB functions extend beyond controlling S-phase entry. Biological activities for the pathway are inferred from both functional studies in mammals and other model organisms and through the identification of a vast network of target genes (2, 9, 19, 34, 39). These include control of the G1/S and G2/M transitions of the cell cycle, checkpoint control, DNA repair and recombination, apoptosis, differentiation and development. How are all these diverse activities regulated? One answer may lie in the composite nature of the E2F/RB network – there are eight E2F factors in mammals, five of which can associate with three different pocket-proteins. Individual E2F/RB complexes could perform distinct tasks and regulate different sets of genes in response to varying signals. This idea is supported by several lines of evidence, (9) and references therein.
Another answer might be provided by the recent discovery of native E2F-RB containing complexes from several different organisms (14, 17, 20, 23, 25). These complexes, called dREAM (Drosophila RBF, dE2F2 and dMyb interacting proteins) or MMB (Myb-Muv B) in flies, DREAM or LINC in humans, and DRM in worms, contain a repressive E2F, pocket-proteins, the Myb transcription factor, three Myb-interacting proteins (Mip40, Mip 120 and Mip130) and p55CAF1 (RbAp46/48). Interestingly all of the components of the complex except Myb are related to the C. elegans synMuv class B genes that negatively regulate vulva development. The human complexes appears to be comprised of either Myb of E2F4, but not both, and are referred to as MMB (Myb) or DREAM (E2F4) (24, 38). The switch between Myb and E2F4 is a regulated event (24). While dMyb and dE2F2 were initially purified as part of the same complex in flies, they do regulate different sets of genes and behave in a mutually exclusive manner to position the dREAM complex at different promoters (15).

Accumulating evidence supports the idea that the dREAM/MMB complexes may have different compositions to perform distinct functions in different cellular contexts. The complex is required to repress differentiation-specific, but not cell cycle regulated, E2F target genes in proliferating cells (15, 20, 21, 23, 36), in quiescent cells it represses cell cycle-specific targets; it is required for the activation of G2/M-specific genes (probably the MMB-Myb containing complex) (18, 27, 29, 31), and it regulates site-specific DNA replication in Drosophila follicle cells (1). Additionally, genome-wide studies have implicated dREAM/DRM in the regulation of a wide range of genes and complexes of different compositions have been found at a large number of sites throughout the genome (15, 25, 35).

We have taken advantage of the relative simplicity of the Drosophila system to study gene regulation by E2F/RB and dREAM. Specifically, we have examined the regulation of a set of E2F targets (Group D/E genes) that have functions in differentiation and development and exhibit gender- and tissue type specific expression patterns. These genes are repressed by dE2F2/RBF in proliferating cells and the repression is dependent on dREAM (15, 20, 21, 23, 36). Transcriptional
regulation of these genes differs from that of classic, cell cycle E2F target genes in several ways. We have explored whether the dREAM complex is responsible for the distinct type of regulation at these genes. We find that dREAM is required to both maintain two distinct types of repression mechanisms, as well as for the stability of dE2F2/RBF complexes in S-phase.
4. Materials and Methods

Cell culture. Drosophila melanogaster SL2 cells were cultured at 24.5°C in Schneider’s insect medium (Invitrogen/GIBCO) supplemented with 10% FBS (HyClone). RNAi treatment was performed as previously described (33). Cell cycle synchronization was performed using a double hydroxyurea (Sigma)/aphidicolin (Sigma) block and release as previously described (10). For cell cycle synchronization of RBF1–expressing cell lines, RBF1 wt or R1A expression was induced for 24 h during the arrest, and prior to harvesting the cells. To determine the number of cells in S-phase, cell proliferation labeling reagent (BrdU - Amersham) was added to the media for 6 h. Cells were fixed and stained with anti-BrdU antibody (Becton Dickinson) and a fluorescein-labeled anti-mouse IgG secondary antibody (Vector Laboratories). FACS analysis was performed with the Beckman Coulter FC500 Analyzer and data analyzed with CXP Analysis Software (Beckman Coulter).

RBF1 mutant Cell lines.

Previous work with RBF1 was done with a protein of 797 aa, yet genomic sequencing predicts an additional 48 aa at the C-terminus. To generate a clone containing the complete coding region we obtained pOT2-RBF1 (cDNA clone LD45859) from Open Biosystems. A SacI/SpeI fragment from pOT2-RBF1 was ligated to a SacI/SpeI digested pMK33-RBF1(36). The resulting plasmid contained the full coding sequence of RBF1 tagged with the FLAG peptide at the N-terminus under the control of the inducible metallothionin promoter and was used to generate stable cell lines expressing wild type RBF1 protein (R1wt). To generate mutations, full length RBF1, a BamHI/SpeI fragment of pMK33-RBF1, was sub-cloned into pBlueskript II SK+ (Stratagene). pBSK-RBF1 was mutagenized using the QuickChange Multisite Kit (Stratagene) in a two-step process. All mutations were confirmed by sequencing. Mutated RBF1 was re-inserted into
pMK33 as a BamHI/SpeI fragment to generate pMK33-RBF1A (phosphomutant) or pMK33-RBF1E (phospho-mimic). Stable cell lines expressing either FLAG-tagged RBF1 wild type (R1wt) or RBF1 mutant (R1A or R1E) proteins under the inducible metallothionein promoter were generated using Effectene (Quiagen). Cells were transfected per manufacturer’s instructions and stable transfectants were selected for 3- to 4-weeks in media containing 0.2 mg/ml hygromycin B (Roche). RBF1-expressing cell lines were incubated with copper sulfate (200µM) for 24 h to induce expression.

**Chromatin Immunoprecipitation Assays.** Chromatin immunoprecipitation was performed as previously described (10, 12) with the following modifications. Chromatin was sheared using the Bioruptor (Diagenode) to an average size of 300 bp and immunoprecipitated DNA was analyzed by quantitative real-time PCR (Roche, LightCycler 1.5) using the standard-curve method. Results are represented as the ratio of specific sequence over a non-specific (RP49 promoter or bxdPRE) sequence. Each immunoprecipitation was performed at least three times and sdev was calculated. Antibodies used for ChIP are as follows: anti-dE2F2 (rabbit polyclonal), anti-dE2F1 (rabbit polyclonal), anti –Flag (Rockland, Sigma), anti-RBF1 (mouse monoclonals DX2 + DX5), anti-H3K27me2 (Upstate 07-452), anti-Acetyl H3 (Upstate 06-599), anti-H3K27ac (Abcam ab4729-25), anti- AcetylH4 (Upstate 06-866). Primers were designed to amplify between 100-150bp of sequence, primer sequences are available upon request.

**RNA isolation, reverse transcription-PCR and Northern blotting.** Total RNA was isolated using Trizol (Invitrogen) reagent. Northern blotting using riboprobes was performed as previously described (10). A two-step RT-PCR using 0.3 µg of total RNA was performed as follows. cDNA was prepared by reverse transcription with random primers using the High Capacity cDNA kit (Applied Biosystems) as per manufacturers instructions. Analysis of cDNA was performed with PCR using GoTaq (Promega). Primer sets used were RP49 forward
(5'-TCCAAGAAGCGCAAGGAG-3'), reverse (5'-ATTCCGACCACGTACCAAGA-3'); MIP40
forward (5'ACAGCTGGATTTGCTGTTT-3'), reverse (5'-CCTACGTAATGCGCCTTT-3').
5. Results

A. The dREAM complex is required for dE2F2 binding at differentiation-specific, but not cell cycle regulated E2F/RB target gene promoters.

In actively proliferating cells the dREAM/MMB complex is required for the repression of differentiation-specific (Group D/E), but not for cell cycle E2F/RB target genes (15, 20, 21, 23, 36). Several features set these two groups of target genes apart (10). Group D/E genes remain repressed during S-phase. In contrast dE2F2/RBF-mediated repression of cell cycle target genes is relieved during S-phase and they are transcriptionally activated by dE2F1. Accordingly, dE2F2/RBF complexes are displaced from cell cycle but not from Group D/E gene promoters during S-phase. Group D/E genes are not regulated by the activator dE2F1, and dE2F1 does not bind to their promoters. We have previously shown that dREAM/MMB plays a role in two different mechanisms of repression of Group D/E genes – the de-acetylation of upstream promoter regions and the di-methylation of histone H3 K27 at sequences downstream of the transcription start site (22). While the first mechanism of repression is shared with cell cycle regulated genes, the second is unique for Group D/E genes. We next wanted to ask whether dREAM/MMB is responsible for other unique features of E2F/RB regulation at Group D/E genes. Specifically we asked whether the differential binding of dE2F1 is due to dREAM/MMB.

dE2F1 does not bind to Group D/E gene promoters even in the absence of dE2F2 (10) indicating that there is no competition between the E2Fs for binding. We reasoned that dREAM could function to prevent dE2F1 binding at these promoters. Alternatively, both E2Fs are unable to bind to these promoters on their own, and dREAM is required for the binding of dE2F2. To test these hypotheses, we disrupted the dREAM/MMB complex by depleting the Mip40 subunit via RNAi (Fig. 16D) and assayed the binding of both E2F proteins. We reasoned that if the first model was correct we would observe dE2F1 binding at Group D/E gene promoters in the absence
of dREAM. However, if the second model was true we would observe that dE2F2 binding is compromised.

dE2F1 was present at cell cycle regulated gene promoters (Fig. 16A - DNA pol α and PCNA) in both control and dREAM-depleted cells; and absent at Group E gene promoters (Fig. 16A – Arp53D, CG2887, CG3505) in all conditions assayed. These results indicate that neither dE2F2 nor the dREAM complex is preventing dE2F1 from binding at Group D/E gene promoters.

We next asked whether dREAM/MMB might be required to recruit dE2F2. We observed that while the binding of dE2F2 at the DNA pol α and PCNA promoters is largely unaffected in Mip40-depleted cells, binding was reduced at Group E gene promoters (Fig. 16 B). Removal of Mip40 results in a slight reduction in dE2F2 protein levels (Fig. 16 C). When normalized to protein levels, the binding of dE2F2 was unchanged in Mip-40 depleted cells at cell cycle regulated promoters, but impaired at Group E gene promoters (Fig. 16 E). These results show that dREAM/MMB is required to recruit dE2F2 at differentiation-specific, but not at cell cycle regulated E2F target gene promoters.
Figure 16 E2F binding at target gene promoters in dREAM-disrupted cells.

A., B. Chromatin immunoprecipitation (ChIP) performed with antibodies recognizing dE2F1 (A) or dE2F2 (B) in cells incubated with dsRNA targeting white (control), dE2F2 or Mip40. The amount of co-precipitated DNA was quantified by Real Time qPCR (qRT PCR). Results are normalized to a nonspecific sequence (promoter of RP49) and represent the average of three independent experiments. Promoter sequences analyzed are DNA polα and PCNA (cell cycle regulated genes) and Arp53D, CG2887 and CG3505 (Group E genes). C. Western blot analysis of whole cell extracts from SL2 cells incubated with dsRNA targeting white (control), dE2F2 or
Mip40. Numbers at the bottom represent quantified dE2F2 protein levels. **D.** RT-PCR analysis of mRNA levels from cells incubated with dsRNA targeting white (control), dE2F2 or Mip40. **E.** ChIP results in B. are corrected for total dE2F2 protein levels.
**B. dREAM complex is required for dE2F2/RBF binding only in S-phase.**

Another feature that sets Group D/E genes apart from traditional targets is the fact that the E2F/RB repressive complexes remain bound at the promoters during S-phase. We wondered if dREAM/MMB played a role in this stability. We first asked whether dREAM/MMB was required for dE2F2 binding during S-phase. To obtain S-phase cells, we arrested cells by a double hydroxyurea (HU)/aphidicolin block and harvested 3 h after release from the block. This resulted in approximately 75% BrdU positive cells (Fig. 17A and B). We then examined the binding of dE2F2 in both control and Mip40-depleted cells. As previously observed (10), in S phase dE2F2 is not present at cell cycle regulated promoters such as PCNA and DNA pol α, but remains bound to Group D/E gene promoters (Fig. 17E black bars). Similar to what we observed in asynchronously growing cells, the binding of dE2F2 was greatly reduced in cells lacking Mip40 (Fig. 17E).

We next asked whether dREAM/MMB functioned specifically in S-phase or whether it was required for the binding of dE2F2 in general. To address this question we obtained a population of cells devoid of S-phase cells. To this end we depleted the activator dE2F1, which leads to a severe reduction of BrdU positive cells and an accumulation of cells in G1 phase (Fig. 18A and B), (10, 12, 13) and assayed dE2F2 binding. As expected we observed an increase in dE2F2 binding in dE2F1- and dE2F1+Mip40-depleted compared to asynchronously growing cells at cell cycle regulated promoters (Fig. 18C). At Group D/E gene promoters we find that while dE2F2 binding was reduced in cells lacking Mip40, it was unaffected in dE2F1+Mip40-depleted cells. These results indicate that dREAM plays no role in dE2F2 binding outside of S-phase and that the observed reduction in dE2F2 binding in asynchronous cells was due to its impaired binding during S phase.

Collectively these results demonstrate that at Group D/E gene promoters dREAM/MMB is required for the stability of dE2F/RBF repressive complexes in S phase.
Figure 17 dREAM is required for dE2F2 binding at Group E gene promoters during S-phase.

A. Cell cycle profiles of asynchronously growing SL2 cells or cells synchronized in S-phase and treated with dsRNA targeting either white (control) or Mip40. B. Percent cells in G1, S and G2/M phase of the cell cycle as determined by 2D FACS analysis. Numbers under graph are the numbers of S-phase cells. C. Western blot analysis of whole cell extracts from SL2 cells incubated with dsRNA targeting white (control), dE2F2 or Mip40. D. RT-PCR analysis of mRNA levels from cells incubated with dsRNA targeting white (control), dE2F2 or Mip40. E. ChIP assay performed with antibodies recognizing dE2F2 in cells synchronized in S-phase and incubated with dsRNA targeting white (control), dE2F2 or Mip40.
Figure 18 dREAM is not required for dE2F2 binding outside of S-phase.

A. Cell cycle profiles of cells incubated with dsRNA targeting white (control), Mip40, dE2F1, and dE2F1 and Mip40 (co-treated). B. Percent cells in G1, S and G2/M phase of the cell cycle as determined by 2D FACS analysis. C. ChIP assay performed with antibodies recognizing dE2F2 in cells incubated with dsRNA targeting white (control), Mip40, dE2F1, and dE2F1 and Mip40 (co-treated).
C. **Phospho—mimicking RBF mutants are defective in binding to both cell cycle regulated and differentiation-specific E2F/RB target gene promoters.**

The stability of E2F/RB complexes at Group D/E genes in S-phase suggests that RB proteins might be protected from CDK phosphorylation at these promoters. Alternatively, other factors may contribute to the stability of phosphorylated E2F/RB complexes. To begin to address these possibilities, as well as the role of the dREAM/MMB complex, we generated RBF1 mutants. We mutated the 7 putative CDK phosphorylation sites (Fig. 19 A) to either Alanine (S/T – A) to generate phospho-mutant or to Glutamate (S/T- E) to generate phospho-mimic and created stable cell lines with inducible expression of either wild type (R1wt), phospho-mutant (R1A) or phospho-mimic (R1E) RBF1 proteins. We then asked whether phospho-mimic RBF proteins can bind to E2F target gene promoters. As expected, R1E proteins exhibited reduced binding at cell cycle-regulated gene promoters (Fig. 19 E left panels). Interestingly, binding was reduced at Group D/E gene promoters as well (Fig. 19E right panels). The reduced RBF1 binding was not due to dE2F2, dE2F2 binding was similar in R1wt and R1E cell lines. These results strongly suggest that phosphorylation of RBF1 disrupts binding at both types of target gene promoters, and indicate that dREAM/MMB may function to protect RB proteins from being phosphorylated at Group D/E gene promoters.
**Figure 19** Phospho-mimic RBF1 mutant cannot bind at both cell cycle regulated and Group E gene promoters.

A. Schematic representation of the 7 putative CDK phosphorylation sites of RBF1. B. Western blot analysis of whole cell extracts from SL2 cells, RBF1 wt (wt), RBF1 S/T-A phospho-mutant (A) and RBF1 S/T-E phospho-mimic (E) probed with anti-RBF1 and anti-FLAG antibodies. C.
Co-immunoprecipitation analysis of dE2F2 (upper panels) or dDP (lower panels) interactions with RBF1 mutants. D. ChIP assay using antibodies that recognize dE2F2 or non-specific antibodies in cell lines expressing either RBF1 wt (R1 wt) or RBF1 S/T-E phospho-mimic (R1E). Left panels are cell cycle regulated gene promoters, right panels – Group E gene promoters. E. ChIP assay using antibodies that recognize RBF1 or non-specific antibodies in cell lines expressing either RBF1 wt (R1 wt) or RBF1 S/T-E phospho-mimic (R1E). Left panels are cell cycle regulated gene promoters, right panels – Group E gene promoters.
**D. Un-phosphorylatable RBF1 suppresses dE2F2 binding defects in dREAM disrupted cells.**

If the dREAM/MMB complex functions to protect RB proteins from being phosphorylated by CDKs, we hypothesize that cells expressing a phospho-mutant RBF1 that cannot be phosphorylated (R1A), will no longer require dREAM/MMB for stable binding of E2F/RB at Group D/E gene promoters. Expression of an R1A mutant for a prolonged period of time results in accumulation of cells in G1. We induced R1 wt and R1A expression for 24 h to minimize the effects on the cell cycle and assayed dE2F2 binding in control and Mip40-depleted cells (Fig. 20). In cells expressing R1wt, dE2F2 binding was reduced in cells lacking Mip 40. The binding of dE2F2 in Mip40-depleted cells was partially restored when the R1A mutant protein was expressed.

As the expression of un-phosphorylatable RBF1 causes a reduction in S-phase cells (Fig. 21 C) and dREAM is only required for E2F/RB binding in S-phase it is possible that some of the observed effects are an indirect consequence of cell cycle stage. To address this, we synchronized cell in S-phase as described above and induced expression of RBF1 wt or R1A 24h prior to collecting. This resulted in a population enriched in S-phase cells (35-50% vs. asynchronously growing cells ~ 10%). R1A expressing cells exhibited only a slight reduction in S-phase population as compared to R1 wt cells (Fig. 21 A). We assayed dE2F2 binding and observed that while it was greatly reduced in Mip40-depleted R1 wt cells, there was little change in Mip40-depleted R1A expressing cells (Fig. 21 B).

Taken together these results are consistent with the idea that a major means by which dREAM/MMB maintains the stability of dE2F2/RBF complexes in S-phase at Group D/E gene promoters is by protecting RB proteins from phosphorylation.
Figure 20 Phospho-mutant RBF1 suppresses dE2F2 binding defects in dREAM disrupted cells.

A. Western blot analysis of whole cell extracts from cells expressing RBF1 wt (R1 wt) or RBF1 phospho-mutant (R1A) and incubated with dsRNA targeting white (control) or Mip40; last lane is from regular SL2 cells. B. RT-PCR an analysis of mRNA levels from cells expressing RBF1 wt (R1 wt) or RBF1 phospho-mutant (R1A) and incubated with dsRNA targeting white (control) or Mip40. C. Percent of cells in S-phase of cells expressing RBF1 wt (R1 wt) or RBF1 phospho-mutant (R1A) and incubated with dsRNA targeting white (control) or Mip40; cells incubated with dsRNA targeting white (control) or dE2F1 are shown as comparison. D. ChIP assay using antibodies that recognize dE2F2 in cells expressing RBF1 wt (R1 wt) or RBF1 phospho-mutant (R1A) and incubated with dsRNA targeting white (control) or Mip40.
Figure 21 Phospho-mutant RBF1 restores dE2F2 binding in S-phase enriched cells lacking dREAM.

A. Cells were treated with hydroxyurea/aphidicolin (double arrest-release), expression of RBF1 wt or R1A mutant was induced 24h prior to collection. Percent of cells in S-phase of cells expressing RBF1 wt (R1 wt) or RBF1 phospho-mutant (R1A) and incubated with dsRNA targeting white (control) or Mip40. B. ChIP assay using antibodies that recognize dE2F2 in S-phase enriched cells, expressing RBF1 wt (R1 wt) or RBF1 phospho-mutant (R1A) and incubated with dsRNA targeting white (control) or Mip40.
E. Repression is compromised in both wild type and phospho-mutant RBF1 expressing cells upon dREAM disruption.

We have previously shown that dREAM/MMB is required to maintain repression of Group D/E genes via two different mechanisms (22). We wanted to know whether dREAM/MMB functions solely by maintaining E2F/RB repressive complexes at the promoters or whether it also potentiates RB’s repressive functions. To address this issue we asked whether dE2F2/RBF1 could maintain repression of Group D/E genes in the absence of dREAM/MMB. We depleted Mip40 from cells expressing either wild type or the phospho-mutant RBF1 protein. If dREAM/MMB is not required for RB-mediated repression, we expected to see little or no changes even in the absence of Mip40, as binding of dE2F2/RBF is restored in R1A expressing cells.

We examined both repressive mechanisms – histone de-acetylation of promoter regions and histone H3 K27 di-methylation of sequences downstream of the transcription start site. As previously observed, histone H3 and H4 acetylation levels rose in cells lacking Mip40, and there was no difference between R1wt and R1A expressing cells (Fig. 22 A and B). While the histone H3 K27 di-methylation levels varied in the RBF1 –expressing cell lines, the reduction di-methylation observed in Mip-40 depleted cells was similarly unaffected in R1A expressing cells (Fig. 22 C). Most importantly, mRNA levels of Group E genes rose in Mip40-depleted cells in both cell lines (Fig. 22 D). This means that the genes were de-repressed in cells lacking dREAM in R1wt - and in R1A-expressing cells. Taken together these results show that dREAM/MMB is not dispensable for repression. Even when dE2F2/RBF1 complexes can be stably maintained at these promoters, the repression mechanisms are compromised in the absence of a functional complex.
Figure 22 dREAM is required for the repression of Group E genes in both R1wt and R1A expressing cells.

A. and B. ChIP assay performed with antibodies recognizing pan-acetylated histone H3 (A) or pan-acetylated histone H4 (B) in cells expressing RBF1 wt (R1 wt) or phospho-mutant (R1A) and incubated with dsRNA targeting white (control) or Mip40.  C. ChIP assay performed with antibodies recognizing histone H2 K27 di-methylation (H3K27me2) in cells expressing RBF1 wt (R1 wt) or phospho-mutant (R1A) and incubated with dsRNA targeting white (control) or Mip40.  D. Northern blot analysis of total RNA isolated from cells expressing RBF1 wt (R1 wt) or phospho-mutant (R1A) and incubated with dsRNA targeting white (control) or Mip40.
6. Discussion

The identification of native pocket-protein associated complexes in flies, worms and humans called dREAM/MMB/DRM/LINC indicates that such complexes play important roles in RB functions. The mechanisms of action of the complex are not very well understood. The complex has been shown to regulate the expression of G1/S, G2/M, as well as differentiation-specific E2F target genes, and to potentiate RBs tumor suppressive functions. It has been reported to repress as well as activate transcription, regulate site-specific DNA replication, and has been located at a large number of genomic sites. These observations indicate that dREAM/MMB/DRM/LINC plays a vital role in RB functions but also raise the question of how it can support all these diverse E2F/RB activities.

In this study we have explored the means by which the Drosophila complex – dREAM, represses differentiation-specific E2F/RB targets in actively proliferating cells. Differentiation-specific target genes (Group D/E) differ in their regulation from classic, cell cycle regulated targets and we find that the function of dREAM is required for some of the unique features of this gene regulation. Specifically, dREAM is required for the stability of dE2F2/RBF complexes at Group E gene promoters during S-phase. Our results indicate that it functions at least in part by protecting RBF1 from phosphorylation. In addition, the complex is also essential to maintain both mechanisms of repression at these genes.

A. Lack of dE2F1 binding at Group E gene promoters.

One of the distinctive features of Group E gene regulation is the lack of dependence on E2F activation – the activator dE2F1 does not bind to their promoters or regulate their expression. This poses the question of how dE2F2-specificity is achieved at these promoters. While a comparison of
E2F binding sites did not reveal any major differences between sites found at Group E and cell cycle gene promoters, dE2F1 is unable to bind to Group E gene promoters even in the absence of dE2F2 (10). This finding suggests that either dE2F1 is inherently incapable of binding to these promoters or that some factor(s) other the dE2F2 prevents it from binding. It is also possible that dE2F2 is not capable of binding without the assistance of another factor. One candidate for such a factor is the dREAM complex. It could either assist dE2F2 or prevent dE2F1 from binding. We find that disruption of dREAM did not lead to dE2F1 binding, but disrupted dE2F2 binding, suggesting that neither E2F is capable of binding to these promoters without assistance. However, upon close examination, we discovered that dREAM is dispensable for dE2F2 binding outside of S-phase, demonstrating that dE2F2 can bind to Group E gene promoters without the assistance of dREAM. These results indicate that dE2F1 is not capable of binding at these promoters either due to some subtle differences in E2F binding sites or possibly due to differences in chromatin structure.

**B. Stability of dE2F2/RBF complexes in S-phase.**

In S-phase, dE2F/RBF complexes are replaced by dE2F1 at cell cycle regulated promoters, yet remain bound and functional at Group E gene promoters. Several observations in mammals also indicate that E2F/pocket-protein complexes exist irrespective of cell cycle stage and can function in a CDK-independent manner (4, 9 and references therein, 41, 42, 44). It is well established that E2F/RB complexes are disrupted at cell cycle genes at the G1/S transition (reviewed in 6, 9, 34, 37). Are E2F/RB complexes then protected from CDK phosphorylation in some cases or is the regulation of E2F/pocket-protein interactions even more complex? Structural studies of pRB have suggested that the C-terminus of pRB functions as a molecular sensor that recognizes CDK mediated phosphorylation (30, 43). When pRB is hypo-phosphorylated, its C-terminal region stabilizes the interaction with E2F1, phosphorylation by CDKs, causes a conformational change,
and this intra-molecular interaction is thought to inhibit the interaction between pRB and E2F1(16, 30). However, it is not clear whether this is true for other RB family members or that all E2Fs interact in the same manner with pocket proteins. Furthermore, it has been shown that pRB has two distinct E2F binding sites, one being specific for E2F1 and linked to its ability to regulate E2F1-dependent apoptosis. This indicates that pRB interacts with individual E2F proteins in different ways and that regulation of distinct E2F functions are physically separable (8). Studies in Drosophila also indicate that G1 CDKs are not sufficient to disrupt repressive E2F/RB complexes at cell cycle genes (13).

In the case of differentiation-specific target genes in flies we find a simple explanation of the stability of E2F/RB complexes in S-phase – dREAM. In the absence of a functional dREAM complex, dE2F2 and RBFs exhibit reduced binding in S-phase. But how does assembly into dREAM ensure stability of E2F/RB complexes? The simplest model is that the phosphorylation sites are blocked, inaccessible to CDKs. Alternatively, dREAM, possibly in association with additional factors, may modify RBF1. For instance, pRB is known to be acetylated during differentiation of monoblastoid cells and keratocytes (3, 26, 28). Additional post-translational modifications of the protein could either prevent phosphorylation or induce a conformational change to promote stability of phosphorylated E2F/RB complexes. It will be interesting to investigate if RBF proteins bound in the dREAM complex have modifications other than phosphorylation.

Our results show that expression of a phospho-mimic RBF mutant results in impaired binding at both cell cycle regulated and differentiation-specific (Group E) gene promoters. Conversely a mutant RBF1 that cannot be phosphorylated is sufficient to bypass the need for a functional dREAM at Group E gene promoters; E2F/RB complexes are stable in S-phase even in the absence of dREAM. These findings strongly suggest that the complex functions, at least in part, by protecting RBF1 at these promoters from being phosphorylated by CDKs. While, they do not exclude the possibility of additional modifications of RBF1, the striking results obtained with a
phospho-mutant RBF1 indicate that a major means by which E2F/RB complexes remain functional despite high CDK activity is by being protected from phosphorylation.

**C. What is the role of the dREAM complex in the regulation of differentiation-specific E2F/RB target genes?**

The initial identification and characterization of the dREAM/MMB complexes has led to the speculation that dREAM is a repression complex that functions by affecting chromatin structure (20, 23). The complex is not stably associated with any enzymatic activity, yet we have demonstrated that Group D/E genes are repressed via two distinct mechanisms in a dREAM-dependent manner (22). Is the observed dependence on dREAM the result of its ability to affect chromatin structure or is it an indirect consequence of its role in dE2F2/RBF binding? We favor the idea that dREAM plays a direct role in the repression of Group E genes for two reasons. Binding of dE2F2/RBF1 complexes does not require dREAM outside of S-phase. Only 10-12% of asynchronously growing SL2 cells are in S-phase, yet disruption of dREAM leads to the same level de-repression as the removal of dE2F2 or RBFs. Moreover, while the expression of phospho-mutant RBF1 restores the binding of dE2F2/RBF1 in S-phase, it does not restore the repression in cells lacking dREAM. A dual role for dREAM in the regulation of these genes is also more consistent with its reported involvement in other RB functions. It would suggest that the complex is capable of potentiating diverse RB activities.
7. References (Chapter III)


Chapter IV

Discussion
1. dREAM mediated repression of differentiation specific genes employs chromatin remodeling

In the present study, I have investigated how the repression of differentiation specific E2F/RB and dREAM complex target genes is different from cell cycle-related genes. I demonstrated that the promoter regions of differentiation specific genes are associated with de-acetylated histones. The function of histone deacetylases (HDAC) is necessary for this de-acetylated state in a dREAM dependent manner. The repression also requires the histone methyltransferase E(Z) as a co-repressor. Gene coding regions of differentiation specific genes have di-methylated histones H3 at Lysine 27 (H3K27me2) in an E(Z) dependent manner. When the dREAM complex is disrupted, nucleosomes downstream of transcription start site (TSS) loose H3K27me2 and H3K27 becomes acetylated. De-repression of the genes was observed when either HDAC or E(Z) function is inhibited, which suggests that the function of both co-repressors in dREAM mediated repression is important (Chapter II and Figure 23)
Figure 23 Suggested model of dREAM mediated repression

Two different modifications at two different regions exist to repress differentiation specific dREAM target genes. HDACs are required to deacetylate nucleosomes at the promoter region. A histone methyltransferase, E(Z), catalyzes dimethylation of nucleosomes downstream of the Transcription Start Site. Both histone modifications are dREAM dependent and required for the full repression of the genes. In addition, dREAM complex may have an additional function in excluding activators of transcription, such as histone acetyltransferases.
A. *dREAM complex may represses genes at the initiation step of transcription.*

Histone acetylation is important in assembling the pre-initiation complex for transcription, and also in recruiting gene-specific transcription factors (Li et al., 2007; Workman and Kingston, 1998). It is known that histone acetylation regulates initiation and/or elongation steps (Spain and Govind, 2011; Zhao et al., 2005). My study showed that differentiation specific genes that are repressed by dREAM have de-acetylated nucleosomes at their promoters (Chapter II). Although both initiation and elongation could be affected, it is likely that dREAM complex inhibits transcription at the pre-initiation complex formation step. Polytenic chromosome staining has demonstrated that distribution of Mip130 does not overlap with RNA polymerase II (Korenjak et al., 2004). Furthermore, genome-wide ChIP-on-chip data of RNA polymerase II indicated RNA polymerase is not poised for activation at dREAM target gene promoters (Muse et al., 2007).

If dREAM functions to block pre-initiation complex formation, why does knockdown of E(Z) lead to de-repression? Depletion of E(Z) did not induce histone acetylation at the promoters, but resulted in increased gene expression. One possible explanation for this is that E(Z) function may prevent transcription initiation from alternative or cryptic transcription start sites. It is also possible that depletion of E(Z) resulted in an increase of a type of histone acetylation at the promoter regions, which cannot be detected by the antibodies used in this study. Further work should be performed to understand how precisely the dREAM complex represses transcription.
B. H3K27me2 and transcriptional repression

One of the important findings in my thesis research is the identification of H3K27 di-methylation in dREAM-mediated repression, which is not well appreciated from previous studies. One of the early studies that investigated the role of H3K27me2 was a polytene chromosome staining study performed by Dr. Paro’s lab, which wanted to identify histone modifications that co-localize with polycomb (PC) protein binding. They demonstrated that distribution of PC significantly overlaps with H3K27me3, but not with H3K27me2 (Ringrose et al., 2004). The result is reminiscent of Mip130 staining of polytene chromosome, which indicates that PC does not co-localize with Mip130 (Korenjak et al., 2004). These two findings are consistent with my results and suggest that PC or PRC1 function is not necessary for dREAM-mediated H3K27 di-methylation.

A potentially controversial point is if this H3K27 di-methylation is not dREAM-specific but rather more general, non-specific, phenomenon. It was suggested that H3K27me2 is universally observed throughout the genome of Drosophila (Ebert et al., 2004; Peters et al., 2003). Immunostaining of Drosophila Polytenes chromosomes was performed to support the idea, but the study used a polyclonal antibody. It demonstrated that H3K27me2 is found in euchromatic regions and covers about 40% to 60% of the whole genome (Ebert et al., 2004). Additionally, a quantitative mass spectrometry analysis performed by another group suggested that the relative abundance of H3K27me2 is about 50% (Peters et al., 2003; Robin et al., 2007). It has also been shown by a Chip-on-chip study that H3K27me2 displayed broad peaks that cover about 40% of the whole fly genome (Pirrotta lab, personal communication). Therefore, H3K27me2 might not necessarily be dREAM-specific but could also be a signature of repressed genes or even a subset of active genes. However, it should be noted that the polyclonal antibody that these groups used exhibits cross-reactivity of a considerable degree to H3K27me1 and H3K27me3 (Peters et al., 2003). For this reason, caution should be taken in interpreting the studies using this specific
antibody. Indeed, an immunostaining study using a monoclonal H3K27me2 antibody, with low cross-reactivity displayed less than 20% occupancy over the genome (Ringrose et al., 2004). Furthermore, more recent mass spectrometry-based quantification of histone H3 have demonstrated less than 20% of histone H3 is dimethylated at Lys 27, albeit in a different species (human, mouse, tetrahymena, yeast and Arabidopsis) (Garcia et al., 2007; Johnson et al., 2004). These recent data are more supportive of our hypothesis that H3K27 di-methylation is not a widespread phenomenon but could be dREAM-specific.

Consistent with this idea, genome-wide profiling of various histone modifications throughout the human genome using high-throughput ChIP-Seq techniques suggested that H3K27me2 is not a non-specific histone mark, but specific for silent genes (Barski et al., 2007). In this study, ChIP-Seq data was correlated with the expression levels that were published by other groups (Su et al., 2004). Analyses of the two data sets unraveled that the levels of H3K27me2 are high in silenced genes. For those genes, H3K27 di-methylated histones were found downstream of transcription start sites. This pattern of methylation is in sharp contrast to H3K27 tri-methylation which peaks at promoter regions. Similar conclusion was drawn by another genome-wide study, where they demonstrated a correlation of H3K27me2/me3 in repressed genes and H3K27me1 in active genes (Rosenfeld et al., 2009).

My observations are consistent with these recent studies, suggesting that H3K27me2 is a repressive histone mark (Chapter II). It is not clear if H3K27me2 is entirely specific for dREAM mediated repression, or if H3K27me2 can be found at other repressed genes as well. My results indicate that the differentiation specific E2F/RB target genes have H3K27 di-methylated nucleosomes, which is a dREAM complex specific phenomenon. It is likely that dREAM complex dependent repression is at least one of the mechanisms of H3K27me2 dependent repression. Further studies will be needed to elucidate what the specific role of H3K27me2 is and how it is different from H3K27me3.
C. Role of H3K27me2 in dREAM mediated repression

What is the role of H3K27me2 in repression and how does it function? I have demonstrated that the function of E(Z) is required for the methylation, and gene repression. In Drosophila, E(Z) is a polycomb group protein and the only histone methyltransferase that is responsible for all three types of histone H3K27 methylation (mono-, di-, and tri-methylation) (Ebert et al., 2004; Ohno et al., 2008). It is relatively well supported that E(Z) in Polycomb Repressive Complex 2 (PRC2) tri-methylates nucleosomes, and this methylation is important in recruiting PRC1 proteins for repression (Min et al., 2003; Schwartz and Pirrotta, 2007). However in my study, dREAM dependent repression was associated with H3K27me2 but not with H3K27me3, and PRC1 function was not required for the repression. Therefore, dREAM dependent repression seems to be mediated by either E(Z) or PRC2 only. The latter is more likely because in vitro methyl transfer assay has demonstrated that E(Z) cannot methylate recombinant histone H3 by itself, but requires the other members in PRC2 complex, such as SUZ12, ESC and p55/CAF1 (Czermin et al., 2002).

H3K27me2 may inhibit recruitment of activators of transcription. I have demonstrated that one potential function of H3K27me2 is to prevent H3K27 from being acetylated. H3K27Ac was first recognized in yeast to be highly enriched at promoter regions of actively transcribed genes (Suka et al., 2001; Wang et al., 2008). There is evidence in Polycomb group protein studies, that H3K27Ac antagonizes Polycomb mediated H3K27me3 (Schwartz et al., 2010; Tie et al., 2009). It was suggested that CREB binding protein (CBP) is the histone acetyltransferase (HAT) responsible for the acetylation of H3K27. Interestingly, overexpression of CBP results in increased H3K27Ac of nucleosomes that were polycomb targets, and causes in the fly a Polycomb like mutant phenotype. Conversely, knockdown of CBP leads to higher H3K27me3 levels (Tie et al., 2009). These observations suggest that H3K27me3 represses polycomb target genes at least in part by
blocking the activation effect of H3K27Ac. Since CBP functions to counteracting E(Z) protein, it will be interesting to see if CBP activity is blocked in dREAM mediated repression.

Another possibility is that H3K27me2 provides docking sites for other co-repressors.

Compared to other post-translational modifications of histones, methylation adds a relatively small sized group to histones that does not disrupt nucleosomal structure (Berger, 2007). Histone methylation is believed to provide docking sites for regulatory proteins. Methyl lysine marks can be read by proteins that contain Chromodomain, Tudor domain, WD40 repeats, PHD fingers, or MBT domain. (Sims and Reinberg, 2006; Volkel and Angrand, 2007). My results have demonstrated that a chromodomain protein, PC (polycomb) in PRC1, is not required for the dREAM mediated repression. Then, what protein(s) do read H3K27me2? While it is not clear whether L(3)MBT, a MBT domain protein, is a member of dREAM complex, L(3)MBT belongs to the same genetic pathway (synMuvB class). The protein has been shown to recognize H4K20me1/me2 (Trojer et al., 2007) to make chromatin compact. My study found that nucleosomal density of the gene coding regions of differentiation specific E2F/RB target genes is higher than actively transcribed genes (Chapter II). Therefore, it will be interesting to examine if L(3)MBT can read H3K27me2 and maintain the compact chromatin structure. In addition, investigation of other reader proteins will be also important because RNAi depletion of L(3)MBT de-represses only a part of genes repressed by the dREAM complex (Lewis et al., 2004)
2. **dREAM mediated repression is maintained in S phase**

In Drosophila S2 cells, the repressive complexes of dE2F2 and RBFs remain bound at differentiation specific gene promoters (Group E) in S phase when CDK activity disrupts their interaction at cell cycle-related gene promoters (Group A, Dimova et al., 2003). There are two possibilities to explain this stability. First, RBF proteins could be phosphorylated but maintained as a dE2F2/RBF complex by special means. Second, RBF proteins could be protected from the phosphorylation. Are RBF proteins phosphorylated, or not phosphorylated at those promoters?

Structural studies of RB family proteins suggest why phosphorylation disrupts their interaction with E2F proteins (Rubin et al., 2005; Xiao et al., 2003). It was demonstrated that the C-terminus of pRB functions as a molecular sensor that recognizes CDK mediated phosphorylation. When pRB is hypo-phosphorylated, its C-terminal region to binds to E2F/DP and stabilizes the interaction. When pRB is phosphorylated by CDKs, the C-terminal sensor region recognizes the phosphorylation sites and induces a conformational change. This intramolecular interaction is thought to inhibit pRB function and releases E2F/DP (Harbour et al., 1999; Rubin et al., 2005). This observation suggests that the phosphorylation of pRB may prevent E2F binding, which might also be true for the other RB family members. However, it should be noted that the structural bases of other RB family proteins, p107 and p130, have not been solved yet, and they may behave in a different manner under CDK activities.

My results also suggest that, in S phase Drosophila cells, RBF1 proteins at the differentiation specific gene promoters may not be phosphorylated, and assembly of dREAM complex is necessary to maintain the RBF proteins at the promoters (Chapter III). I have demonstrated that expression of phospho-mimic RBF protein (R1E) reduces RBF binding at all E2F target gene promoters including dREAM targets. In addition to my study, Pilkinton et al. previously found that CDK4 function is important in switching from E2F4/RB to B-MYB to activate G2/M genes in human cells (Pilkinton et al., 2007a). They also demonstrated that release of human LIN-9 (=
Mip130 in Drosophila) from human LINC complex requires CDK4 mediated phosphorylation (Sandoval et al., 2009). Altogether, these results suggest that there is a mechanism that involves the dREAM complex, which maintains RB family proteins hypo-phosphorylated in S phase. But, how is this achieved?

The simplest model might be that the interaction of RB with dREAM components protects the phosphorylation sites. Alternatively, dREAM components, possibly in association with additional factors, may modify RB, so that it cannot be phosphorylated. Potential candidates could be protein acetyltransferases. RB is known to be acetylated during differentiation of monoblastoids, muscle cell lines, and keratinocytes (Chan et al., 2001a; Nguyen et al., 2004; Pickard et al., 2010). Therefore, it will be interesting to investigate if RB protein bound in dREAM complex has any other post-translational modifications.
3. Potential Functions of the dREAM complex

Initial studies of the dREAM complex have demonstrated that its function is necessary to repress differentiation specific genes that are repressed in a cell cycle independent manner (Korenjak et al., 2004; Lewis et al., 2004). However, genome-wide ChIP-on-chip studies have revealed that dREAM, or its homologous complex can be found at far more promoter regions than initially expected (Beall et al., 2007; Litovchick et al., 2007; Schmit et al., 2007). Although less than 100 genes display de-repression when the dREAM complex is disrupted, more than 1500 promoters exhibit full or partial dREAM complex binding at their promoters (Georlette et al., 2007). It is not clear why the dREAM complex is needed at so many promoters, and what functions the complex may have.

A. Regulation of G2/M transition of the cell cycle

Assembly of dREAM complex could be important in the regulation of the G2/M transition. Repression of G2/M genes by E2F/RB complexes has been reported by various studies, but how E2F/RB regulates these genes is not clear. The study of the human LINC complex suggested that B-MYB containing DREAM/LINC function is necessary to turn on G2/M genes (Schmit et al., 2007). This idea is further supported by the studies of human LIN-9, a DREAM/LINC member. LIN-9 has been shown to form a complex with MYB, which might be the DREAM/LINC component, which activates transcription of G2/M genes, such as Cyclin A, Cyclin B and CDK1, and induces progression into mitosis (Knight et al., 2009; Osterloh et al., 2007; Pilkinton et al., 2007b).
It has been demonstrated that the promoter regions of G2/M genes are occupied by E2F4 containing DREAM/LINC complex in G0 phase (Schmit et al., 2007). As the cell cycle progresses, E2F4 binding is reduced and MYB binding increases in S phase. These observations suggest that E2F4 containing repressive complexes disappear even before the entry into S phase. However, it is also possible that the observation is due to the synchronization in late S phase. Indeed, a time course study of MYB binding by the another group has shown that the binding of MYB appears from late S phase on, but is not observed in early/mid S phase (Sandoval et al., 2009). In this study, G2/M genes are not expressed until late S to early G2, suggesting there is a mechanism that represses them in mid S phase.

My study could provide a clue to understand how DREAM/LINC is able to regulate G2/M genes in S phase. My data suggests that Drosophila RBF protein is unphosphorylated even in S phase, when the protein is repressing target genes as part of dREAM complex. The same mechanism that represses the differentiation specific target genes in S phase may be required to maintain E2F/RB complexes at G2/M gene promoters until late S/G2. Thus, it will be interesting to see if depletion of DREAM/LINC members causes abnormal de-repression of G2/M genes in early S phase. If E2F4 containing complexes suppress the genes in S phase, then once again, whether assembly of DREAM/LINC complex can prevent E2F/RB from dissociation in S phase will be an important question to answer.

B. Does dREAM have a function in E2F target specificity?

dREAM complex may have a role in specific target binding of dE2F proteins. Differentiation specific E2F/RB target genes are repressed throughout cell cycle, and therefore dE2F1 does not bind at those promoters. This observation provides a simple competitive model of
dE2F1 exclusion at the promoters pre-occupied by dREAM complex prevents dE2F1 from binding. However, this model is only partially correct because RNAi depletion of dE2F2 still fails to recruit dE2F1 (Dimova et al., 2003, and Chapter III). How can dE2F1 be excluded from those promoters? Or, how is dE2F2 selectively recruited? Because the differentiation specific genes are regulated by dREAM complex, one possibility is that dREAM components contribute to the E2F target specificity.

However, this idea is not supported by my study. Knockdown of a dREAM component, Mip40, still fails to recruit dE2F1 at the promoters (Chapter III). This observation suggests that dREAM components do not exclude dE2F1. Furthermore, assembly of dREAM complex seems not to have direct role in recruiting dE2F2 at the promoters. Disruption of dREAM complex did not change dE2F2 binding in non-S phase cells (Chapter III). However, depletion of dE2F2 or Mip40 might not be enough to displace the whole complex from the promoters, and thus the remaining components could influence dE2F1 or dE2F2 binding. This concern should be addressed in future studies to investigate if assembly of dREAM complex has a role in dE2F target specificity.

C. Regulation of DNA replication

dREAM complex contains Myb and Myb-interacting proteins that are important for endoreduplication of chorion genes in *Drosophila* follicle cells (Beall et al., 2002; Lewis et al., 2004). Mip proteins co-localize with the Origin recognition complex (Orc) proteins at the sites of DNA replication. Absence of any Mip protein results in non-specific abnormal DNA replication
pattern, which is similarly observed in $de2f2$, $rbf1$, or $dp$ mutant follicle cells (Cayirlioglu et al., 2001). Then, how does dREAM complex function?

Genetic and biochemical studies have demonstrated that E2F1, or dE2F1 in *Drosophila*, is important for the initiation of DNA endoreduplication (Mendoza-Maldonado et al., 2010; Royzman et al., 1999). E2F1 and Orc1/2 physically interact via pRB, and pRB negatively regulates their function (Ahlander et al., 2008; Bosco et al., 2001). Indeed, dE2F1 mutant that lacks C-terminal RBF1 binding domain exhibits increased level of DNA replication, while transcription levels were unaffected (Royzman et al., 1999). It was suggested that RB phosphorylation is necessary for E2F1 dependent initiation of DNA replication (Mendoza-Maldonado et al., 2010). If that is the case, dREAM complex may antagonize DNA replication by maintaining RBF in S phase (Chapter III), in order to prevent non-site specific DNA replication. It is also possible that dREAM may inhibit DNA replication by excluding dE2F1 from the promoters (discussed above). Further study will be needed to investigate these possibilities.

**D. Gender specific functions**

dREAM complex may have gender specific functions. As described in Chapter I, dREAM complex is necessary for eggshell formation. Therefore lack of dREAM components results in female sterility (Beall et al., 2004; Beall et al., 2002). On the other hand, a homologous tMAC complex is required for the male fertility (Beall et al., 2007). These observations suggest that dREAM or its relative complexes perform sex specific functions. Indeed, many of the differentiation specific dREAM target genes are expressed in gender specific manner and some are gamete-specific differentiation markers (Dimova et al., 2003; Korenjak et al., 2004).
Disruption of the dREAM complex or depletion of dE2F2 induces mis-expression of these genes. For example, ovaries from de2f2 and drbf1 mutant flies abnormally express male specific genes (Stevaux et al., 2005). Similarly, knockdown of Mip130 de-represses male specific genes (Korenjak et al., 2004). These results suggest that the function of dREAM is necessary to repress genes in a specific gender. How the dREAM complex can distinguish each gender is not clear. Therefore, it will be interesting to investigate if the complex associates with sex-specific transcription factors.

My study has suggested a way that these gender specific genes can be regulated in a manner that they are not expressed anywhere else but in a specific tissue. The dREAM complex suppresses gene expression with the aid of histone acetyltransferases (HDACs) and a histone methyltransferase, E(Z) in actively proliferating SL2 cells. It will be interesting to determine whether the same mechanisms are used in other cell types as well. The repression might be disrupted by subunit switching in the dREAM complex in gender specific tissues, as is exemplified in the tMAC complex (Chapter I).

**E. Regulation of S phase genes in G0 cells**

Studies in human glioblastoma cell lines have demonstrated that the DREAM/LINC complex occupies S phase gene promoters in quiescent cells (Litovchick et al., 2007; Schmit et al., 2007). Knockdown of LIN-9, E2F4, or p130 caused de-repression of these E2F targets. Once serum is added, DREAM/LINC target genes are induced as cells enter S phase. These observations suggest that human DREAM/LINC has a repressive role in regulating cell cycle-regulated genes in G0 phase and potentially in all non-dividing, differentiated cells. Gene ontology analysis of DREAM-bound promoters revealed that DREAM/LINC does not
significantly bind at promoters of development or differentiation specific genes in the same condition (Litovchick et al., 2007).

My studies could not test if S phase genes are suppressed in quiescent cells in flies. *Drosophila* SL2 cells do not exit the cell cycle in serum starvation or contact inhibition conditions that are conventionally used in mammalian cell culture. However, it is likely that dREAM complex may function in flies to suppress S phase genes as well. ChIP-on-chip study revealed an unexpected number (>1500) of dREAM complex bound promoters throughout the *Drosophila* genome, which includes S phase genes (Georlette et al., 2007). Because depletion of dREAM components does not show obvious de-repression of the S phase genes in proliferating cells, it is not clear if they are really suppressed by the dREAM complex. It is possible that dREAM complex only binds there, so it can readily block cell cycle-related genes when cells enter a non-dividing phase during differentiation or quiescence. In order to access a possible role for dREAM in the regulation of cell cycle genes *mip* mutant flies have to be examined for possible de-repression of these genes in differentiated cells. In any case the reason for dREAMs binding at so many promoters should be addressed.

**F. Does dREAM complex have a role in Cancer?**

Study of human DREAM complex function in tumor cells will be particularly interesting, because tumor cells often have de-regulated CDK activity. As a result, pRB function should be compromised. However, unlike the tumor suppressive role that is usually observed, the functions of pRB sometimes are oncogenic in the same context (Williams et al., 2006). In a human colon cancer cell lines, pRB is required for the viability and proliferation of the tumor cells. RB promoted proliferation of cells harboring mutant *Ras*, at least in part by suppressing p107
expression. Importantly, it was able to inhibit p107 transcription despite the high CDK activity in these cells, suggesting that it functions in a CDK-independent manner (Williams et al., 2006). This raises the question of how pRB functions in such high CDK conditions.

My studies suggest one way that pRB can function in cancer cells with high CDK activity. The finding that pRB represses p107 in high CDK activity is reminiscent of RBF1’s ability to repress its target genes in S phase (high CDK activity conditions). Is pRB function mediated by assembly of human DREAM complex, similar to dreams role in RBF1 functions in flies? pRB was not found in human DREAM or LINC complex (Litovchick et al., 2007; Schmit et al., 2007). However, pRB is known to interact with DREAM components, such as human LIN-9 (Gagrica et al., 2004). It will be interesting to see if assembly of DREAM complex is required for the “oncogenic” role of pRB in these tumor cells.
4. References (Chapter IV)


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