Molecular mechanisms of E2F-dependent activation and pRB-mediated repression

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Summary

Alterations in transcription of genes regulated by members of the E2F family of transcription factors can be viewed as a measure of the ebb and flow in a constantly evolving battle between repressor and activator complexes. Various chromatin regulatory complexes have been linked to Rb/E2F proteins, and changes in histone modifications correlate with states of E2F-dependent transcription. E2F has traditionally been viewed in the context of cell-cycle control. However, several recent studies have revealed a new aspect of E2F function in which pRB/E2F-family proteins confer stable repression of transcription. Such repression is evident in both actively proliferating cells and in cells that have withdrawn from the cell cycle.

Key words: Rb/E2F, Cell cycle, Transcription repression, Histone acetylation, Histone methylation

Introduction

The retinoblastoma tumor suppressor protein (pRB) and its relatives, p107 and p130, are negative regulators of cell proliferation that figure prominently in most models of cell-cycle control. Cell-cycle progression is driven by mitogenic growth signals. These signals result in the synthesis of G1 cyclins, the positive-regulatory partners of cyclin-dependent kinases (CDKs), and eventually the accumulating pool of CDK activity overrides the constraining effects of CDK inhibitors. Once active, G1-specific CDKs target multiple substrates, including the pRB family. By phosphorylating pRB-family members, CDKs relieve the constraints on cell proliferation that this group of proteins maintain. Although pRB-family members are periodically inactivated during the normal cell cycle, these proteins are also inappropriately inactivated in several other situations. pRB-family proteins are targeted by DNA tumor virus proteins during oncogenic transformation, and pRB is thought to be functionally inactivated in most tumor cells, either through mutation of the *RB1* gene itself or through dysregulation of the kinases that control its activity.

Precisely how pRB-family proteins control cell proliferation is not completely understood. There is a broad range of possibilities given that pRB-family members associate with a wide variety of transcription factors and chromatin-associated complexes. Nevertheless, pRB-family members are generally believed to function through their effects on the transcription of genes regulated by the E2F proteins. E2F-binding sites are found in the promoters of many genes whose functions are needed for cell proliferation or whose products drive cell-cycle progression. The best-studied forms of E2F are heterodimeric complexes that contain one subunit encoded by the E2F family together with a subunit encoded by the DP family. Mammalian cells contain at least seven E2F-family members (Fig. 1) and two DP-family members. Although subunits E2F-1 to E2F-6 act as heterodimers with a DP subunit, the recently described E2F-7 subunit binds to DNA in a DP-independent manner. E2F-1 to E2F-5 associate with pRB-family members, whereas E2F-6 and E2F-7 appear to act independently of pRB-family proteins.

For simplicity, the E2F family is often subdivided into activator E2Fs (E2F-1, E2F-2 and E2F-3a) and repressor E2Fs (E2F-4, E2F-5 and E2F-6). This classification is based upon differences in the ability of these overexpressed proteins to activate transcription, or to drive quiescent cells into the cell cycle, as well as on the phases of the cell cycle where the E2F proteins can be shown to be present at E2F-regulated promoters. However, the distinction between these two groups is not cut and dried. For example, 'repressor' E2Fs can activate transcription when overexpressed, and 'activator' E2Fs have the potential to form complexes with repressor proteins. Nevertheless, a separation of activator and repressor E2Fs is useful in concept, and the distinction is reinforced by the pattern of interactions between E2F- and pRB-family members: activator E2Fs bind exclusively to pRB, whereas p107 and p130 interact specifically with repressor E2Fs. Curiously, E2F-4 interacts with all three pRB-family members.

Repressor E2Fs occupy promoters in G0/G1 phase, and typically these proteins are complexed with pRB-family members. The prevailing model is that a rise in CDK activity during G1, and the subsequent phosphorylation of pRB-family members, leads to the release of E2F-containing repressor complexes from E2F-regulated promoters, the binding of activator E2Fs and the expression of the E2F target genes. In this way, pRB-family members provide an important connection between CDK activation and the expression of genes that are needed for cell proliferation (reviewed by Cam and Dynlacht, 2003; Dyson, 1998; Nevins, 1998; Sherr and Roberts, 1999; Stevaux and Dyson, 2002; Trimarchi and Lees, 2002).

Exactly how do E2F- and pRB-family members control gene expression? Biochemical studies show that pRB can repress transcription in at least three distinct ways when it is recruited to E2F-regulated promoters. First, pRB binds directly to the activation domain of the activator E2Fs and, in doing so, it

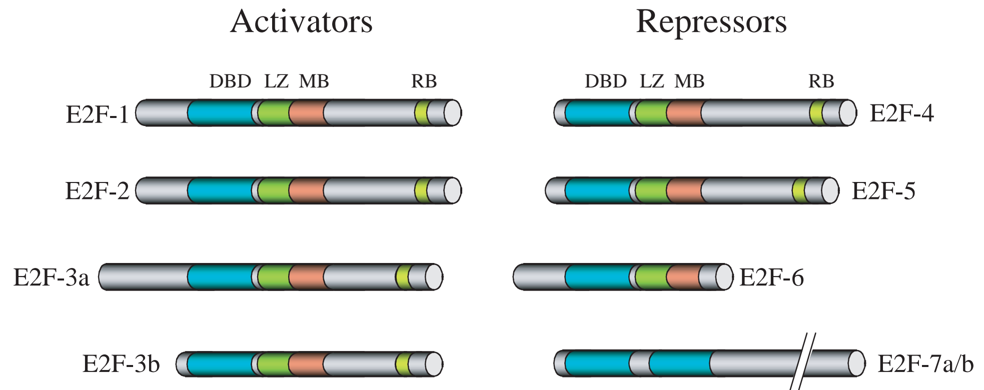

Fig. 1. Structure of E2F-family members. E2F-1–E2F-6 contain a DNA-binding domain (DBD) and a DP-dimerization domain that includes two conserved motifs: a leucine repeat sequence (LZ) and the marked box (MB). Unlike other E2Fs, E2F-7 has two distinct DBDs and does not require DP to bind to DNA (de Bruin et al., 2003; Di Stefano et al., 2003). E2F-1–E2F-5 contain a conserved C-terminal motif that mediates association with pRB-family members (RB).

blocks the activity of this domain (Flemington et al., 1993; Helin et al., 1993). Second, its recruitment to a promoter blocks the assembly of pre-initiation complexes, potentially allowing it to inhibit the activity of adjacent transcription factors (Ross et al., 1999). Third, pRB uses a protein interaction domain that is distinct from its E2F-binding site to associate with complexes that modify chromatin structure (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Meloni et al., 1999; Ross et al., 2001; Sellers et al., 1995). Similar binding sites are found in both p107 and p130 and, by forming these complexes, the pRB-family proteins serve as molecular adapters allowing chromatin-modifying enzymes to be recruited to E2F-regulated promoters. At present, there is no definitive evidence to tell us which of these three mechanisms of repression is the most important for cell-cycle control.

The basic unit of eukaryotic chromatin is a nucleosome comprising 147 bp of DNA wrapped around a histone octamer that contains two molecules of histones H2A, H2B, H3 and H4. Each histone contains a globular region, a histone-fold domain, and a less-structured N-terminal tail that protrudes outwards. These tails are targeted by a variety of post-translational modifications, including acetylation, ubiquitylation, phosphorylation, methylation and others (Lachner et al., 2003). Such changes are thought to regulate gene expression, and the status of any given nucleosome reflects the concerted action of localized modifying and de-modifying enzymes. Lysine acetylation and deacetylation are among the best-studied histone modifications. Histones are generally hyperacetylated at the promoters of actively transcribed genes but are hypoacetylated at silenced genes. Many transcription regulators recruit histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities. The acetylation of histone tails has been hypothesized to stimulate transcription by loosening DNA histone contacts, perhaps making chromatin less compact. By contrast, deacetylated chromatin is less accessible to transcription factors and is thought to represent a repressive conformation. There is a strong correlation between acetylation state and gene expression but the precise structural consequences of histone acetylation are unknown

The specific effects of histone methylation on chromatin structure are also uncertain. Unlike histone acetylation, the addition of methyl groups to lysine or arginine residues does not alter the overall charge of the nucleosome. However, these changes do appear to affect the properties of chromatin. Importantly, the methylation of different residues correlates with different transcriptional states. For example, methylated K9 of histone H3 (me-K9-H3) is concentrated in centromeric heterochromatin and in regions where transcription is repressed. In actively transcribed genes, histone H3 is not methylated on K9 but instead carries methylated K4 (me-K4-H3). It has been proposed that these collective modifications represent a 'histone code' and that, depending on the nature and position of the modifications, the combination of changes determines both the organization level of the chromatin and its transcriptional status (reviewed by Jenuwein and Allis, 2001; Lachner and Jenuwein, 2002; Strahl and Allis, 2000). As described below, recent studies indicate that E2F and pRB proteins can affect both the acetylation and methylation of histones at E2F-regulated promoters.

Interplay between HATs and HDACs at E2F-regulated promoters

Chromatin immunoprecipitation (ChIP) experiments on synchronized populations of cells reveal dynamic changes in the binding of different E2Fs, HATs and HDACs to E2F-regulated promoters and concurrent changes in histone acetylation. Most studies have found that the E2F-4–p130 repressor complexes predominate in cells in which E2F targets are repressed, and these are replaced with activator complexes containing E2F-1, E2F-2 and E2F-3 at mid to late G1 phase. The timing of replacement correlates with the dissociation of HDACs from promoters, the subsequent appearance of HATs (p300, CBP, P/CAF or Tip60), the hyperacetylation of histone H3 and histone H4, and the induction of E2F-dependent transcription (Caretti et al., 2003; Ferreira et al., 2001; Rayman et al., 2002; Takahashi et al., 2000; Taubert et al., 2004; Wells et al., 2000).

Other studies have implicated HDACs in the repression of E2F-regulated promoters in G1 phase (Brehm et al., 1998; Ferreira et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998) (reviewed by Brehm and Kouzarides, 1999; Harbour and Dean, 2000). pRB associates with HDAC activity in vivo and binds to class I HDACs (HDAC1-HDAC3) in vitro. Co-expression of HDAC1 enhances pRB-mediated repression in transient transfection experiments, and inhibition of HDAC activity with trichostatin A interferes with pRB-mediated repression at a subset of E2F-regulated promoters (Brehm et al., 1998; Zhang et al., 2000). Although HDAC proteins contain sequences that are similar to pRB-binding motifs that

have been characterized in other proteins, they might also associate indirectly with pRB through bridging molecules such as RBP1 (Lai et al., 2001). pRB has also been reported to interact with the Polycomb group protein (PcG) HPC2 (Dahiya et al., 2001). However, interactions between the endogenous HPC2 and pRB proteins have not been reproducible (see erratum for Dahiya et al., 2001) and the potential connection between these proteins awaits confirmation.

Besides covalent modifications of histone tails, chromatin structure can be affected by nucleosome-remodeling complexes, which use the energy of ATP hydrolysis to weaken the interaction between DNA and histones (reviewed by Narlikar et al., 2002). BRG1 and hBRM, which are human homologs of the yeast nucleosome-remodeling complex SWI2/SNF2, have been implicated in pRB-mediated repression. Both BRG1 and hBRM have been shown to associate with pRB-family proteins and further experiments have shown that BRG1 is needed for pRB to arrest certain cell lines (Dunaief et al., 1994; Strober et al., 1996; Zhang et al., 2000). Initially, it was thought that the functional synergy between pRB and BRG1 results from their physical association. However, a recent study has indicated that a direct physical interaction is not required for pRB-induced growth arrest and transcriptional repression of E2F target genes (Kang et al., 2004). Instead, BRG1 has been shown to bind to, and activate transcription from, the promoter of the gene encoding the CDK inhibitor p21. This effect, which does not require the reported pRB-binding domain of BRG1, appears to be important for BRG1-induced growth arrest (Hendricks et al., 2004; Kang et al., 2004).

ChIP experiments have demonstrated that the recruitment of HDAC to E2F-regulated promoters in mammalian cells depends on an intact E2F-binding site in the promoter and requires p107 or p130 (Ferreira et al., 2001; Rayman et al., 2002). In the same experiments, the recruitment of another repressor protein, mSin3B, to many of the same promoters did not require p107 or p130. It seems likely that several different repressor complexes might act at E2F-regulated genes and that different protein-protein interactions could be involved at individual targets. Curiously, several studies have struggled to find pRB at E2F-regulated promoters, and the analysis of $Rb^{-/-}$ cells has revealed that pRB is not required for HDAC1 to be recruited to E2F targets (Rayman et al., 2002). These results have led to the idea that pRB may not be the primary regulator of E2F activity in all situations but is preferentially recruited to E2F sites under specific conditions, such as cell-cycle arrest induced by the CDK inhibitor $p16^{INK4a}$ (Dahiya et al., 2001) or Ras-induced senescence (Narita et al., 2003).

A general theme to emerge from these studies is the idea that the activation of E2F-responsive genes results from the interplay between various histone modifications, such that repressor E2F complexes impose a repressive mark and activator E2Fs reverse (or override) this mark (Brehm and Kouzarides, 1999). Support for this model comes from studies in *Drosophila*, which contains just two E2F genes and provides a simpler system for investigation than mammalian cells. RNA interference (RNAi) studies showed that *Drosophila* cells lacking dE2F1, which is the activator E2F, can be driven into S phase by elevated levels of endogenous cyclin-E–CDK2 (Frolov et al., 2003). Under these conditions, an E2F repressor complexes containing the *Drosophila* homolog of pRB-family members, RBF1, is disrupted, and RBF1 is no longer present at E2F-regulated promoters. However, E2F targets remained repressed despite the lack of the RBF1 repressor. In this situation, cells initiating DNA replication do not progress normally through S phase, possibly because the levels of E2F-regulated replication proteins fall to suboptimal levels. These findings suggest that the induction of E2F-dependent transcription is a two-step process that requires not only the disruption of repressor complexes but also the positive action of an activator E2F. The activator is presumably necessary to reverse the effects of the repressor (Frolov et al., 2003).

The best candidates for E2F-recruited activators are HATs, and several different HAT activities have been linked to E2F (Table 1). CREB-binding protein (CBP), one of the first HATs to be discovered, interacts with the activation domain of E2F-1 and stimulates E2F-1-mediated activation (Trouche and Kouzarides, 1996). Two other HATs, p300 (a protein that is highly homologous to CBP) and the associated factor P/CAF, also associate with E2F proteins (Martinez-Balbaz et al., 2000; Marzio et al., 2000; Morris et al., 2000). More recently, Tip60, a HAT that is unrelated to CBP, has been found to interact with E2F-1 in overexpression experiments. The ectopic expression of E2F-1 results in the concurrent recruitment of five subunits of the Tip60 complex (Tip60, TRRAP, p400, Tip48 and Tip49) to E2F-regulated promoters (Taubert et al., 2004). Overexpression of a dominant-negative E2F-1 blocks E2F binding to target promoters, eliminating H4 acetylation and partially reducing H3 acetylation. This suggests that the histone acetylation is a consequence of E2F binding rather than a prerequisite (Taubert et al., 2004).

HATs have also been implicated in the selective activation of E2F targets. Following DNA damage, E2F-1-mediated induction of *p73*, a gene encoding a homolog of p53, correlates with the specific recruitment of P/CAF. In this setting, the binding of P/CAF seems to be especially important, because *p73* expression is not activated by E2F-1 when P/CAF is first depleted from the cells (Pediconi et al., 2003).

Correlations between histone modification and gene expression are highly suggestive; however, it is important to consider some of the limitations of the data. The finding that histone acetylation at the promoters of E2F targets coincides with the activation of transcription does not tell us whether these changes are required for induction of E2F-dependent transcription or whether they are simply a consequence of transcription. Although HATs are often found at E2F-regulated promoters, in most cases it is unclear whether the recruitment of a given HAT complex is rate limiting for E2F-dependent transcription. In addition, it is unclear whether histones are the sole, or even the primary, target of the E2F-recruited HAT/HDAC activities.

Interpretation of experiments examining E2F-HAT interplay has been complicated by the discovery that both E2F-1 and pRB can be acetylated (Chan et al., 2001; Martinez-Balbaz et al., 2000; Marzio et al., 2000; Morris et al., 2000). Acetylation of E2F-1 on three lysine residues outside its DNA-binding domain by P/CAF increases its DNA-binding activity, stabilizes the protein and stimulates its transactivation potential (Martinez-Balbaz et al., 2000; Pediconi et al., 2003). p300/CBP can also acetylate E2F-1. Conserved lysines exist in E2F-2 and E2F-3, and these proteins can serve as substrates

Table 1. Chromatin-modifying activities associated with E2F- and pRB-family members

Process	Proteins proposed to be involved	References
Repression mediated by pRB-family proteins	Sin 3	Rayman et al., 2002
	BRG1/hBRM	Dunaief et al., 1994; Hendricks et al., 2004; Kang et al., 2004; Strober et al., 1996; Zhang et al., 2000
	HPC2	Dahiya et al., 2001
	SUV39H1/Eu-HMT1/HP1	Nicolas et al., 2003; Nielsen et al., 2001; Vandel, 2001
	PRMT5	Fabbrizio et al., 2002
	RbAp46/48	Kennedy et al., 2001
	HDAC1/HDAC2/HDAC3	Brehm et al., 1998; Ferreira et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998
	SAP30	Lai et al., 2001
	RBP1	Lai et al., 1999
E2F-mediated activation	CBP/p300	Marzio et al., 2000; Morris et al., 2000; Trouche and Kouzarides, 1996
	P/CAF	Martinez-Balbaz et al., 2000
	GCN5/TRRAP	Lang et al., 2001
	Tip60	Taubert et al., 2004
	ASC-2	Kong et al., 2003
E2F-mediated repression (pRB-family independent)	RYBP/Ring1/MEL-18/mph1/Bmi1	Trimarchi et al., 2001
	Max/Mga/HP1γ/G9a/EuMT1	Ogawa et al., 2002
	Prohibitin	Wang et al., 1999

for p300/CBP, albeit to a lesser extent (Marzio et al., 2000). No acetylation of E2F-4 and E2F-5 was found in these studies. Acetylation might therefore be a feature of the 'activator' forms of E2F. Indeed, cyclin-E–CDK2-mediated phosphorylation of E2F-5 promotes its interaction with p300/CBP and stimulates transcription (Morris et al., 2000). Mutation of the acetylation sites in E2F-1 reduces its ability to activate transcription (Martinez-Balbaz et al., 2000), and the mutant protein displays reduced binding to the endogenous *P1p73* promoter following DNA damage (Pediconi et al., 2003). These findings raise the possibility that acetylation of E2F-1 is important for its recruitment to specific promoters. Interestingly, pRB-associated HDAC activity is sufficient to deacetylate E2F-1 (Martinez-Balbaz et al., 2000; Marzio et al., 2000), and the notion that this might impede the recruitment of E2F-1 is appealing. The interplay between HATs and HDACs at E2F-regulated promoters is thus probably not simply limited to their effects on histone acetylation, but might also include changes in the binding and/or activity of pRB/E2F-family members themselves.

E2F-dependent transcription seems to involve several different HAT activities. For example, Lang et al. failed to detect interaction between P/CAF and E2F-1 (Lang et al., 2001). Instead, they found that E2F-1 and E2F-4 bind to GCN5, a P/CAF-related HAT, and its cofactor TRRAP, a component of the SAGA chromatin-remodeling complex. These proteins cooperate to activate an E2F-dependent reporter in transient transfection experiments. Analysis of promoter occupancy of several cell-cycle-regulated genes, including *cyclin A2*, *cdc2*, *cyclin B2* and *E2f-1*, in synchronized NIH3T3 mouse fibroblasts revealed a dynamic recruitment of HATs (Caretti et al., 2003). Following the release of repressive complexes from the promoter, activator E2F appears to recruit transiently a P/CAF complex. This coincides with a burst of histone acetylation and is followed by an increase in the binding of NF-Y, a histone-like CCAAT-binding trimer. In turn, the binding of NF-Y was followed by arrival of p300/CBP, a change that correlates with transcriptional activation. These studies paint a complicated picture in which E2F-dependent transcription involves a cascade of HDAC and HAT activities. A key goal for future studies will be to determine which of these complexes are rate limiting at endogenous E2F-regulated genes and when.

pRB-mediated repression and histone methylation

Studies of pRB-mediated repression took a surprising twist with the discovery that pRB interacts with the histone methyltransferase SUV39H1 (Nielsen et al., 2001; Vandel et al., 2001). SUV39H1 specifically methylates K9 of histone H3 (Rea et al., 2000) and this creates a high-affinity binding site for the chromodomain-containing protein HP1 (Bannister et al., 2001; Lachner et al., 2001). HP1 was originally identified as a component of pericentric heterochromatin in *Drosophila* and is proposed to drive gene silencing by producing a heterochromatin-like state (reviewed by Grewal and Elgin, 2002; Li et al., 2002). ChIP experiments showed that DNA adjacent to the transcription start site of the *cyclin E* gene, one of the best-studied targets of E2F, is specifically associated with both me-K9-H3 and HP1. This association is not seen further upstream of the promoter. Most importantly, there is no association of me-K9-H3 in this region in cells lacking pRB, SUV39H1 or its close relative SUV39H2. Because pRB and SUV39H1 cooperate to repress transcription in transient transfection assays, and *cyclin E* expression is elevated in both $Rb^{-/-}$ and $Suv39h1^{-/-}Suv39h2^{-/-}$ double-knockout cells, pRB has been proposed to repress the *cyclin E* promoter, at least in part, by targeting a histone methyltransferase.

As mentioned above, pRB has generally proven to be difficult to detect on E2F-regulated promoters in proliferating cells by ChIP (Narita et al., 2003; Takahashi et al., 2000; Wells et al., 2000). One potential explanation is suggested by the long-term nature of histone methylation. Possibly, pRB might only need to be transiently present at the promoter and, once methylation has been initiated, this modification may be maintained by SUV39H1 and HP1. An analogous 'hit and run'

scenario has recently been shown to occur when a KAP1 repressor complex containing SETDB1, which is the me-K9-H3 HMT, is transiently targeted to an integrated reporter. Although KAP1 is only present for a short time, its presence leads to the enrichment of me-K9-H3 and HP1, and results in long-term silencing of the reporter (Ayyanathan et al., 2003).

Further support for a link between histone methylation and the RB pathway comes from studies in *Caenorhabditis elegans* in which *hpl-2*, a homolog of *HP1*, genetically interacts with *lin35* (the gene encoding the worm homolog of pRB). Couteau et al. propose that the targeted recruitment of HPL-2 by LIN35 contributes to the repression of vulval specification genes (Couteau et al., 2002).

Other types of histone methylation might also be important at E2F-regulated promoters. PRMT5, a type II arginine methyltransferase, is also implicated in the regulation of the *cyclin E* promoter (Fabbrizio et al., 2002). PRMT5 is a component of a large complex that includes E2F-4 and Rbp46/48 but contains neither SUV39H1 nor HP1. ChIP experiments revealed that PRMT5 is present at the *cyclin E* promoter in the vicinity of the transcription start site and that methylation of R3 of histone H4, a target of PRMT5, specifically occurs in the same region. Fabbrizio et al. have shown that H4 arginine methylation interferes with the acetylation of nearby lysine residues (Fabbrizio et al., 2002). Overexpression of PRMT5 is sufficient to repress *cyclin E* and can inhibit cell proliferation.

In many respects, the idea that *cyclin E* expression is regulated by histone methylation was unexpected (Nielsen et al., 2001). *Cyclin E* expression fluctuates through the cell cycle. It is repressed in quiescent cells, activated at the G1/S transition and is then rapidly downregulated as cells move through S phase. The processes that regulate the *cyclin E* promoter should thus be dynamic and readily reversible. By contrast, me-K9-H3 and the recruitment of HP1 have been implicated in the stable repression of transcription and epigenetic regulation that persists through many cell cycles. Unlike histone acetylation, histone methylation is thought to be relatively stable, and no de-methylation enzymes have been identified to date.

There are several potential explanations for this apparent paradox. Besides hypothetical direct de-methylation, there are other ways to reverse methylation, such as histone replacement or histone clipping (reviewed by Bannister et al., 2002). Alternatively, the effects of histone methylation might be reversed by phosphorylation of adjacent residues (Fischle et al., 2003) and a de-methylation might not be necessary for cell-cycle regulation.

The initial studies examined asynchronously dividing cells, and the me-K9-H3 signal detected at the *cyclin E* promoter could have come primarily from a sub-population of non-dividing cells (Nielsen et al., 2001). However, using a cell line in which inducible expression of E1A allows cells to be synchronously driven into the cell cycle, Ghosh and Harter found evidence for rapid changes in H3 methylation at two E2F-regulated promoters: those of *cyclin A* and *cdc6* (Ghosh and Harter, 2003). ChIP experiments showed that both are bound by E2F-4–p130 complexes and enriched with me-K9-H3 in quiescent cells. Following E1A induction, gene expression correlates with the loss of E2F-4, p130 and me-K9-H3 and the appearance of E2F-1 and association of K9-acetylated histone H3. Intriguingly, E1A appears transiently at both promoters and its presence precedes their activation, which suggests that it triggers these changes. HP1 was not detected at the *cyclin A* or *cdc6* promoters in quiescent cells.

Acetylation and methylation of K9-H3 are two mutually exclusive modifications, and one function of K9-H3 methylation at E2F-regulated promoters might be to prevent untimely activation of the gene by sporadic HAT activity. Fine mapping of the pattern of acetylation within the *cyclin E* promoter indicated that HDAC targets a single nucleosome positioned next to the transcription start site (Morrison et al., 2002). It would be interesting to know whether the me-K9-H3 mark is similarly restricted.

Nicholas et al. recently studied the interplay between acetylation and methylation at another E2F-responsive gene, *dihydrofolate reductase* (*DHFR*) (Nicolas et al., 2003). They found that, in quiescent cells, the histone H3 associated with the *DHFR* promoter is hypoacetylated on both K9 and K14 but is methylated on K9. Serum stimulation decreases the level of K9 methylation and concurrently increased acetylation of both K9 and K14 (Nicolas et al., 2003). *DHFR* is regulated by the two pRB relatives, p107 and p130. Both interact with SUV39H1 and with the histone H3-K9-specific eukaryotic methyltransferase (Eu-HMT1) when overexpressed. Further experiments are needed to determine whether the recruitment of either of these methyltransferases is crucial for the regulation of the *DHFR* promoter. SUV39H1 interacts with HDAC1, HDAC2 and HDAC3, and SUV39H1-mediated repression of a heterologous promoter is blocked when cells are treated with the HDAC inhibitor trichostatin A (Vaute et al., 2002). This is consistent with the idea that lysine residues need to be deacetylated in order to be methylated.

This growing collection of evidence points to a role for histone methylation in the repression of E2F-regulated promoters by pocket proteins. It is clear that histone methylation can be found at E2F target promoters, but further studies are needed to delineate the importance of these changes in E2F regulation. Does K9-H3 methylation occur at all E2F-regulated promoters, or only some, and is it present at every cell cycle or only under certain conditions? Is histone methylation more or less important than histone acetylation in E2F regulation, and which HMTs are important for E2F function? Finally, if K9-H3 methylation is reversed in a cell-cycle-dependent manner, how is this achieved?

Stable repression by pRB-family members

Since me-K9-H3 is best known as a marker of heterochromatin, one could view its presence at E2F-regulated promoters as a strong hint that E2F targets are permanently repressed under some circumstances. Indeed multiple, independent lines of evidence now show that pRB/E2F-family proteins do mediate stable repression of transcription. These effects are either independent of cell-cycle position or resistant to cell-cycle progression, and they reveal an aspect of pRB/E2F regulation that was not previously appreciated (Fig. 2).

Narita et al. have described the formation of punctate DNA foci, which are reminiscent of heterochromatin-like structures, in senescent human cells (Narita et al., 2003). The appearance of these foci depends upon an intact $p16^{INK4a}$/RB pathway and coincides with an increase in the levels of me-K9-H3 and the

recruitment of pRB and HP1γ to E2F-regulated promoters. E2F-1 fails to activate transcription in these cells, which suggests that senescence might involve the silencing of the E2F transcriptional program. Whether these changes are restricted to E2F-regulated promoters is unclear, as is whether pRB remains at the promoters permanently. Interestingly, Sage et al. found that the acute loss of pRB in senescent cells allows re-entry into the cell cycle but these cells ultimately re-establish senescence after several passages (Sage et al., 2003).

Stable repression of E2F-regulated promoters has also been observed in *Drosophila*. Genome-wide studies revealed that E2F targets can be divided into several distinct classes. Some of these targets display the cell-cycle-regulated patterns that are traditionally associated with E2F regulation, whereas others are kept stably repressed in actively proliferating cells by dE2F2-RBF repressor complexes (Dimova et al., 2003). ChIP experiments on synchronized cells showed that these complexes are continually present at the promoters of repressed target genes and that these E2F targets are expressed in a variety of developmentally regulated, tissue- and sex-specific patterns. Why dE2F-RBF repression is relieved at some promoters during cell-cycle progression but not at others is unknown.

Changes in the inducibility of E2F target genes have also been revealed in studies of C/EBPα, a liver-specific member of the CCAAT-enhancer-binding protein (C/EBP) family. C/EBPα plays an important role in differentiation and control of cell proliferation, and its overexpression induces cell growth arrest that is resistant to viral oncoproteins (Slomiany et al., 2000). More-recent studies have found that C/EBPα is a component of an E2F-4–pRB-containing complex that binds to and represses some E2F-regulated promoters. Interestingly, the size and composition of this complex differs between young and aged rats, the older animals containing a larger complex that appears to prevent cell-cycle entry (Iakova et al., 2003). C/EBPα mutants that fail to interact with E2F fail to support adipocyte and granulocyte differentiation, possibly because they fail to repress an E2F-dependent transcriptional program (Porse et al., 2001). In another study, Klappacher et al. found that the Ets repressor METS regulates growth arrest during terminal macrophage differentiation by cooperating with pRB-family members (Klappacher et al., 2002). Induced expression of METS blocks growth-factor-dependent DNA synthesis and prevents the expression of Myc in a manner dependent on p107 and p130.

Another form of irreversible E2F repression may be provided by E2F-6. Although E2F-6 lacks a pRB-binding domain, it represses transcription through an association with PcG-family proteins including Ring1- and YY1-binding protein (RYBP), Ring1, MEL-18, mph1 and the oncoprotein Bmi1 (Trimarchi et al., 2001) (reviewed by Trimarchi and Lees, 2002). *E2f-6*-deficient mice exhibit homeotic transformations of the axial skeleton that are reminiscent of defects observed in PcG-deficient mice (Storre et al., 2002). These phenotypes are consistent with the idea that E2F-6 recruits specific PcG complexes to a subset of *Hox* promoters and contributes to stable silencing of these genes. E2F-6-containing complexes isolated from HeLa cells contain Max, Mga, HP1γ and two histone methyltransferases specific for K9 of histone H3: G9a and Eu-HMT1 (Ogawa et al., 2002). Mga forms a heterodimer with Max, recognizes E boxes and acts antagonistically to Myc in transformation assays (Hurlin et al., 1999). ChIP experiments indicate that that E2F-6, Max and HP1γ can be found at E2F targets in quiescent cells. However, it is unclear whether E2F-6 has an important role in the repression of cell-cycle targets; the analysis of *E2f-6*$^{-/-}$ mice, or MEFs, has yet to reveal any defects in the control of cell proliferation (Storre et al., 2002).

The idea that pRB-family members are involved in processes that permanently repress E2F-dependent transcription is appealing because it might explain why pRB-family members are so important for cell-cycle exit and tumor suppression. The proteins that mediate the long-term repression of E2F-regulated genes have not yet been identified, and it is unclear whether stable repression of E2F-dependent transcription involves modifications that are the same as those involved in the reversible repression associated with cell-cycle transitions or a

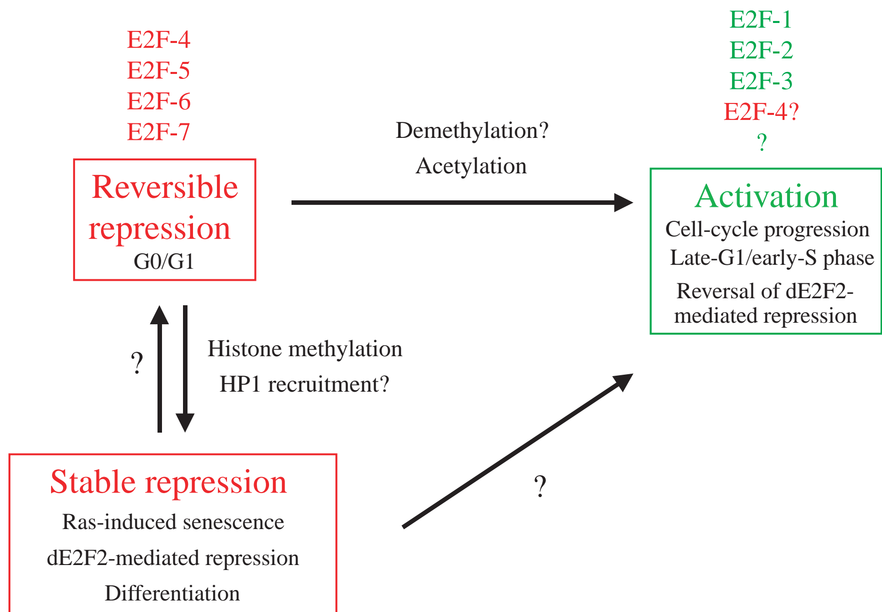

Fig. 2. pRB- and E2F-family members are involved in two distinct types of regulation. The conventional view of E2F is as a cell-cycle oscillator in which complexes of pRB- and E2F-family members provide transient repression that is readily reversed each cell cycle. E2F-binding sites are occupied by repressor E2Fs in G0/G1 and these are replaced by activator E2Fs in late-G1/early-S phase. This transition is driven by CDKs and probably involves the phosphorylation of RB-family proteins and the disruption of repressor complexes. E2F-4 can be found at E2F-regulated promoters in S phase, but it is unclear whether it acts as an activator. In addition to transient repression of cell-cycle genes, pRB-family members are involved in the stable repression of transcription. Stable repression by E2F-RB complexes has been observed in actively proliferating cells, independent of cell-cycle position, and in cells that have withdrawn from the cell cycle. The mechanisms involved in the initiation, maintenance and, potentially, the reversal of stable RB/E2F-mediated repression are largely unknown.

fundamentally different process. Several different models are possible. One possibility is suggested by experiments showing that pRB forms complexes with the DNA methyltransferase DNMT1 (Robertson et al., 2000). Perhaps DNA methylation provides a long-term mark that permanently silences E2F target genes. However, DNA methylation has not been detected at E2F reporters, even in experiments in which co-expression of both pRB and DNMT1 repressed E2F-dependent transcription (Pradhan and Kim, 2002; Robertson et al., 2000).

Another possibility is suggested by the large size of the heterochromatin-like regions observed in senescent cells. The distinction between transient repression and permanent repression might lie in the extent of the region containing me-K9-H3. It is easy to imagine that small regions of me-K9-H3 could represent a reversible mark, whereas more-extensive changes in chromatin structure give an irreversible effect. Using an antibody raised against a branched me-K9-H3 peptide that is thought to mimic a feature of heterochromatin, Ait-Si-Ali et al. reported an increase in the presence of this epitope at the *DHFR*, *cyclin E*, *B-Myb* and *cyclin D1* promoters when cells undergo terminal differentiation (Ait-Si-Ali et al., 2004). HP1 was initially reported to be present at the *cyclin E* promoter in cycling cells (Nielsen et al., 2001). Although others have failed to confirm this point using other E2F-regulated targets (Ghosh and Harter, 2003; Narita et al., 2003), Ait-Si-Ali et al. were able to detect HP1 at the promoters of S-phase-regulated genes in differentiated cells (Ait-Si-Ali et al., 2004). Because HP1 binds to methylated histones and is able, in turn, to recruit other SUV39H-containing complexes, it might allow spreading of repressive chromatin from E2F-regulated promoters in what would presumably become a pRB-independent process. The mechanisms that limit the spreading of heterochromatin are still poorly understood. We speculate that the recruitment of proteins like HP1 may be the critical switch between localized reversible repression that can occur at E2F-regulated promoters in cycling cells and the irreversible repression of E2F targets seen in senescent cells.

Conclusions and perspectives

The widespread use of ChIP methodologies and the general availability of reagents that detect specifically modified chromatin have dramatically improved our picture of the molecular events that accompany E2F-dependent activation and RB-family-mediated repression. Several different histone modifications and chromatin regulatory complexes can be found at various E2F-regulated promoters, and their appearance loosely correlates with activation or repression. This new information brings a new set of challenges and questions. Which of the many molecular changes that occur at E2F-regulated promoters are rate limiting for gene expression, and which are most important for the control of cell proliferation? Does the same process control all E2F-regulated promoters or are different types of regulation used at subsets of genes? The recent evidence that E2F- and pRB-family members are involved in the stable repression of transcription indicates that E2F-pRB complexes are able to mediate different types of transcriptional regulation under different circumstances. How many different types of E2F-mediated repression are there? What determines the reversibility of E2F-mediated repression, and what are the signals that control this switch? If the large number of protein complexes that physically associate with pRB is any guide, we can expect to find that there is not one mechanism of E2F regulation but multiple varieties. If this is the case, then the precise mechanism might not depend on the E2F-binding site itself but on the context in which it is found.

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