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REVIEW

The Rb/E2F pathway: expanding roles and emerging paradigms

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The retinoblastoma gene was identified over a decade ago as the first tumor suppressor. Although the gene was initially cloned as a result of its frequent mutation in the rare pediatric eye tumor, retinoblastoma (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987), it is now thought to play a fundamental role in cellular regulation and is the target of tumorigenic mutations in many cell types. The retinoblastoma gene encodes a 928–amino acid phosphoprotein, Rb, which arrests cells in the G_1 phase (Weinberg 1995). Rb is phosphorylated and dephosphorylated during the cell cycle; the hyperphosphorylated (inactive) form predominates in proliferating cells, whereas the hypophosphorylated (active) form is generally more abundant in quiescent or differentiating cells (Chen et al. 1989). As a demonstration its tumor suppressor activity, Rb was reintroduced into Rb-deficient tumor cells and it blocked several features of the malignant phenotype (Huang et al. 1988). Mutations affecting the retinoblastoma gene are frequently encountered, not only in retinoblastoma but also in other cancers such as osteosarcoma, small cell lung cancer, prostate cancer, and breast cancer (Friend et al. 1986; Fung et al. 1987; Harbour et al. 1988; Lee et al. 1988; T'Ang et al. 1988; Bookstein et al. 1990). Indeed, children with hereditary retinoblastoma have ≥30-fold increased risk of developing a second, nonocular malignancy, especially bone and soft tissue sarcomas in adolescence and cutaneous melanomas in adulthood (Eng et al. 1993; Moll et al. 1997). These second neoplasms occur almost exclusively in patients who have germ-line mutations in the retinoblastoma gene. As a further indication of its fundamental role in tumor suppression, Rb can be functionally inactivated by constitutive hyperphosphorylation in tumors that do not have mutations in the retinoblastoma gene (Sherr 1996). In addition, DNA tumor viruses express oncoproteins, such as adenovirus E1A, SV40 large tumor antigen, and human papillomavirus (HPV) E7, that bind and inactivate Rb; these proteins are required for the viruses to transform cells (DeCaprio et al. 1988; Whyte et al. 1988; Dyson et al. 1989).

Rb function depends, at least in part, on interactions with the E2F family of DNA-binding transcription factors (E2F) (Chellappan et al. 1991; Dyson 1998; Nevins 1998). E2F sites are found in the promoters of many genes that are important for cell cycle progression, and Rb appears to repress transcription of these genes through its interaction with E2F (Blake and Azizkhan 1989; Thalmeier et al. 1989; Dalton 1992; Ohtani et al. 1995). Recent findings in the Rb/E2F field are clarifying how this pathway regulates the transition from G_1 to S phase at the molecular level. Other emerging results show that this pathway also regulates other parts of the cell cycle and that it may even have roles beyond cell cycle control. In this article, we review the current understanding of the mechanism of action of Rb and its roles in cell cycle regulation, apoptosis, and development. We refer readers to recent reviews by Bartek et al. (1997), Dyson (1998), Lipinski and Jacks (1999), and Sherr and Roberts (1999) for additional in-depth analysis of the field and for historical perspectives.

Rb structure

Rb contains several functional domains. Domains A and B are highly conserved from humans to plants, and they interact with each other along an extended interdomain interface to form the central "pocket" (Chow and Dean 1996; Lee et al. 1998), which is critical to the tumor-suppressor function of Rb (Qin et al. 1992). The pocket is disrupted by most naturally occurring germ-line mutations in hereditary retinoblastoma patients (Harbour 1998) and by most tumor-derived mutations (Horowitz et al. 1990). Viral oncoproteins and a number of endogenous Rb-binding proteins contain an LXCXE motif that allows them to bind Rb (Whyte et al. 1988; Dyson et al. 1989; Ludlow et al. 1989; Lee et al. 1998). The crystal structure of the pocket in complex with an LXCXE peptide revealed that the binding site for LXCXE is in domain B (Lee et al. 1998). However, domain A is required

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for domain B to assume an active conformation, thus explaining the conservation of both domains (Kim and Cho 1997; Lee et al. 1998). A number of endogenous proteins that interact with Rb also contain an LXCXE-like sequence, including histone deacetylase (HDAC)-1 and HDAC2, and the ATPase, BRG1, from the SWI/SNF nucleosome remodeling complex (Dunaief et al. 1994; Brehm et al. 1998; Luo et al. 1998; Magnaghi et al. 1998).

The LXCXE binding site is the best characterized but not the only binding site in the pocket. E2Fs do not contain an LXCXE and thus bind Rb at a distinct site that appears to involve points of contact in both the pocket and in the carboxy-terminal region (Huang et al. 1992; Lee et al. 1998). This allows E2F to recruit complexes containing Rb and other proteins, such as those with the LXCXE motif, to a promoter. Still other binding sites appear to be in the pocket. Whereas HDAC1 and HDAC2 contain LXCXE-like motifs through which they can interact with Rb (Magnaghi et al. 1998), HDAC3 does not contain this sequence, and mutations in the LXCXE binding site of Rb do not affect its binding to Rb (Chen and Wang 2000; Dahiya et al. 2000). Although BRG1 contains an LXCXE, it does not require this sequence to bind Rb, which allows Rb to recruit BRG1 and HDAC1 into a single complex (Zhang et al. 2000). Three recent studies in which the LXCXE-binding site was mutated have provided additional, albeit somewhat conflicting, insights into its role. In each of these studies, the mutations inhibited binding to E1A. In two of the studies the mutations also inhibited binding to HDAC1 and HDAC2 but did not affect binding to HDAC3, BRG1, and E2F1 (Chen and Wang 2000; Dahiya et al. 2000). In the third study mutations did not affect binding to HDAC1 (N. Dyson, pers. comm.). Because mutation of the LXCXE-binding site had variable effects on Rb function in these studies, further work is needed to clarify its role and to characterize other binding sites on Rb.

Another functional domain of Rb is located within the carboxy-terminal region. This region contains binding sites for the c-abl tyrosine kinase and MDM2, which appear to be distinct from the E2F site in the carboxy-terminal region (Welch and Wang 1993; Xiao et al. 1995). The tyrosine kinase activity of c-abl is blocked when it is complexed with Rb (Welch and Wang 1993). This interaction appears to be important for Rb-mediated growth suppression (Whitaker et al. 1998). When Rb is hyperphosphorylated, active c-abl is released (Welch and Wang 1993). In addition to directly blocking c-abl, the carboxy-terminal region also appears to participate in the assembly of multimeric complexes containing Rb, E2F, c-abl, and potentially, other proteins. These complexes seem to be required for Rb function (Welch and Wang 1995). The importance of the Rb–MDM2 interaction is less clear. MDM2 interacts with the p53 tumor suppressor protein and opposes its proapoptotic activity by repressing p53 transcriptional activation and by mediating its degradation (Haupt et al. 1997; Kubbutat et al. 1997). Although initial results showed that MDM2 blocks Rb function, more recent studies have shown that Rb can form a trimeric complex with MDM2 and p53 and thereby block the antiapoptotic activity of MDM2 by preventing the degradation of p53 (Hsieh et al. 1999). Further work is needed to elucidate the role of these protein interactions involving the carboxy-terminal region of Rb in vivo.

The function of the amino-terminal region of Rb remains controversial. This region contains consensus cdk phosphorylation sites, which may regulate Rb activity when they are phosphorylated during the cell cycle. In addition, the amino-terminal region interacts with several proteins, including MCM7 (a replication licensing factor; Sterner et al. 1998), a novel G_2/M cycle-regulated kinase (Sterner et al. 1995), and other proteins (Durfee et al. 1994), but the function of these interactions is still unclear. In experiments with genetically engineered mice lacking Rb, introduction of an Rb transgene with an amino-terminal truncation mutation delayed the embryonic lethality caused by homozygous loss of Rb but did not prevent it, suggesting that this region may be important for complete Rb function during development (Riley et al. 1997). Similarly, this Rb mutant did not prevent the development of pituitary tumors in $Rb^{+/-}$ mice, although the onset of tumors was delayed, suggesting that this region may play some role in Rb-mediated tumor suppression (Riley et al. 1997). However, these experiments relied on expression of an Rb transgene that may not entirely recapitulate the expression pattern and protein level of endogenous Rb. In other experiments, tumor suppression by Rb was actually enhanced when the amino-terminal region was removed (Qin et al. 1992; Xu et al. 1994; Chow et al. 1996). Thus, a physiologic role of the amino-terminal region remains unclear and may only be settled once this region of the Rb gene is deleted in mice.

Rb-mediated inhibition of E2F

Rb can repress transcription by at least two different mechanisms. First, it can bind transcription factors such as E2F and block their ability to activate transcription (Flemington et al. 1993; Helin et al. 1993). Second, the Rb–E2F repressor complex that forms at promoters can actively repress transcription (Bremner et al. 1995; Sellers et al. 1995; Weintraub et al. 1995). The balance between these two activities in vivo is still in question. In this section, we address the potential mechanisms through which Rb might inactivate E2F.

Because Rb binds E2F within its transactivation domain (Flemington et al. 1993; Helin et al. 1993), it was assumed initially that it physically blocks E2F transactivation. This idea is supported by in vitro studies in which Rb inhibited transcriptional activation by E2F1 in the apparent absence of other corepressors (Ross et al. 1999). However, Rb may also inhibit E2F by recruiting chromatin remodeling enzymes, including the HDACs mentioned above. The HDACs are a family of at least seven different enzymes that remove acetyl groups from the tails of histone octamers, which appears to facilitate condensation of nucleosomes into chromatin. This, in turn, inhibits gene expression by blocking access of tran-

scription factors to the promoter (Kingston and Narlikar 1999; Kornberg and Lorch 1999; Wolffe and Hayes 1999). In contrast to experiments in vitro, transfection assays in cultured cells have suggested that interaction with HDAC is required for the inhibition of E2F1 by Rb (Brehm et al. 1998; Magnaghi et al. 1998). Other studies have only shown a partial requirement for HDAC activity in the Rb-mediated inhibition of E2F activity (Luo et al. 1998; Lai et al. 1999a). E2F1 has been shown to interact with the histone acetyl transferases p300/CBP and p/CAF (Trouche et al. 1996). Thus, it is possible that Rb-mediated recruitment of HDAC to E2F acts to offset this histone acetyltransferase (HAT) activity (Fig. 1). It has also been shown recently that E2F1 can be acetylated, which increases the binding of the E2F/DP complex to DNA (Martinez-Balbas et al. 2000). Therefore, recruitment of HDAC to E2F via Rb may inhibit E2F activity by deacetylation of the protein, thereby inhibiting its binding to DNA (Fig. 1).

Rb can also interact with BRG1 and BRM—the two ATPase components of the human SWI/SNF chromatin remodeling complex, which is discussed in more detail below (Dunaief et al. 1994; Singh et al. 1995). Some results have shown that overexpression of BRG1/BRM can facilitate Rb-mediated inhibition of E2F1transcriptional activity (Trouche et al. 1997); however, other studies have found that E2F activity is inhibited efficiently in cells that are BRG1/BRM deficient (Weintraub et al. 1992; Zhu et al. 1993; Zhang et al. 2000). Thus the relative importance in vivo of these two potential mechanisms for inhibiting E2F transactivation—direct binding and masking of the E2F transactivation domain versus Rb-mediated recruitment of chromatin remodeling enzymes to inhibit E2F—is still unclear.

Active transcriptional repression by Rb

Binding of Rb and other pocket proteins to E2F does not simply inhibit E2F activity. The resulting Rb–E2F complex binds to promoters and actively represses transcription by blocking the activity of surrounding enhancers on the promoter (Weintraub et al. 1992; Hsiao et al. 1994; Johnson et al. 1994; Adnane et al. 1995; Bremner et al. 1995; Neuman et al. 1995; Sellers et al. 1995; Weintraub et al. 1995; Chow et al. 1996; Ferreira et al. 1998; Meloni et al. 1999). Whereas Rb requires sequences in the pocket and in the carboxy-terminal region to bind and inhibit E2F, the pocket alone is sufficient for active repression when Rb is tethered directly to a promoter (e.g., through a Gal4 DNA binding domain; Bremner et al. 1995; Sellers et al. 1995; Weintraub et al. 1995). This is explained by the recent finding that active repression by Rb is attributable, at least in part, to the recruitment of pocket-binding corepressors. Perhaps the best studied of these corepressors are the chromatin remodeling enzymes.

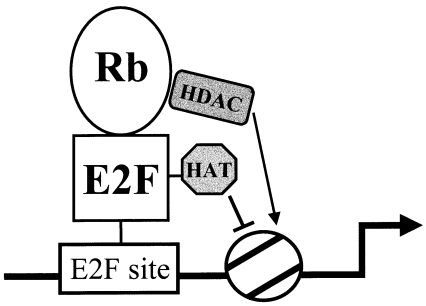

Figure 1. Potential roles of histone acetyltransferase (HAT) activity and histone deacetylase (HDAC) activity in regulating E2F activity. A cellular gene promoter with E2F-binding sites is depicted. HAT activity associated with E2F can promote binding of E2F to the promoter and it can inhibit nucleosome formation, thereby allowing further access of transcription factors to the promoter (see text). In contrast, HDAC recruited by Rb–E2F appears to promote nucleosome assembly on the promoter, blocking access to transcriptional machinery.

Modification of chromatin structure is an important mechanism for regulating gene transcription (Felsenfeld 1992; Kingston and Narlikar 1999). One manner in which chromatin structure can be altered is by acetylation of histones. HATs have been shown to act as transcriptional coactivators that alter chromatin structure, thereby allowing transcription factors access to the promoter. In contrast, HDACs have been associated with transcriptional inhibition and are found in corepressor complexes (Alland et al. 1997; Grunstein 1997; Hassig and Schreiber 1997; Hassig et al. 1997; Heinzel et al. 1997; Laherty et al. 1997). Three of the seven known HDACs (HDAC1–HDAC3) interact with Rb, and Rb can bind simultaneously to HDAC and E2F, allowing recruitment of an HDAC–Rb–E2F repressor complex at promoters of cell cycle genes (Brehm et al. 1998; Luo et al. 1998; Magnaghi et al. 1998; Lai et al. 1999a; Chen and Wang 2000; Dahiya et al. 2000). In two recent studies, Rb mutants that have amino acid substitutions in the LXCXE-binding site showed reduced binding to HDAC1 and HDAC2, but not HDAC3 (Chen and Wang 2000; Dahiya et al. 2000). The impaired interaction with HDAC1 and HDAC2 had no effect on the ability of the Rb mutants to inhibit transcriptional activation by E2F, but these mutants were unable to actively repress some genes and unable to maintain growth arrest. In addition, it has been shown that recruitment of an HDAC–Rb–E2F complex can actively repress transcription and regulate histone acetylation at the promoter (Luo et al. 1998). Furthermore, inhibition of HDAC with trichostatin A prevented repression of a set of cellular genes, including cyclin E, by Rb (Luo et al. 1998; Zhang et al. 2000). Together, these results suggest that HDAC activity has an important role in Rb function. In further support of this, results in *Caenorhabditis elegans* suggest that Rb, HDAC, and RbAp48 antagonize the Ras signaling pathway in vulval precursor cells by repressing transcription of genes that are required for determining the fates of vulval cells (Lu and Horvitz 1998). In addition, RbAp48 has recently been shown to be a component of the Rb–HDAC complex in mammalian cells (Nicolas et al. 2000).

Chromatin structure is also regulated through ATP-dependent nucleosome remodeling complexes (Tyler and

Kadonaga 1999). These complexes appear to influence the access of transcription factors to promoters by altering nucleosomal structure and the position of nucleosomes in a manner dependent on ATP hydrolysis (Schnitzler et al. 1998; Lorch et al. 1999). The first of these complexes to be identified was yeast SWI/SNF. Whereas all of the SWI/SNF-like complexes are made up of multiple subunits, each contains an ATPase that is central to its function. The ATPases in yeast SWI/SNF are SWI2/SNF2. A number of studies have suggested that SWI/SNF is important for transcriptional activation and that it is associated with recruitment of transcriptional activators and HAT activity (Cosma et al. 1999; Tyler and Kadonaga 1999). In a recent study, however, mutation of *SWI2/SNF2* activated more genes than it repressed, suggesting that the SWI/SNF complex may be involved in transcriptional repression as well as activation (Holstege et al. 1998).

The human homologs of SWI2/SNF2 are BRG1 and BRM, which are capable of remodeling mononucleosomes as purified proteins in vitro (Phelan et al. 1999). Both proteins can interact with Rb (Dunaief et al. 1994; Singh et al. 1995), which suggests that they may have a role in Rb function. It is interesting that Rb can bind simultaneously to BRM and E2F, suggesting that a SWI/SNF–Rb–E2F complex can form at promoters with E2F binding sites (Trouche et al. 1997). It has been shown that overexpression of BRG1 arrests cells that are deficient in BRG1 and BRM; this arrest is dependent on interaction with functional Rb (Dunaief et al. 1994). Expression of a dominant-negative form of BRG1 or BRM containing a mutant ATPase domain blocked growth suppression by Rb (Dunaief et al. 1994; Strobeck et al. 2000). Expression of BRG1 was also essential for Rb to growth arrest C33a cells, which are Rb^- and BRG1/BRM deficient (Strobeck et al. 2000; Zhang et al. 2000). Furthermore, in a genetic screen for modifiers of a dE2F1 overexpression phenotype in the *Drosophila* eye, enhancer mutations included alleles of *brahma*, *moira*, and *osa* (homologs of *SWI1*, *SWI2*, and *SWI3*, respectively), suggesting that SWI/SNF chromatin-remodeling activity is important for negatively regulating dE2F1 function in flies (Staehling-Hampton et al. 1999). Taken together, the above findings suggest a role for both HDAC and SWI/SNF nucleosome remodeling complexes in Rb/E2F function. Indeed, recent results indicate that Rb can recruit HDAC and SWI/SNF together into a single complex (Zhang et al. 2000).

How might these two classes of chromatin remodeling enzymes cooperate to regulate transcription? With activation of the HO gene in yeast, recruitment of SWI/SNF precedes recruitment of HAT activity to the HO gene promoter (Cosma et al. 1999). It is unclear whether SWI/SNF recruitment is also an initial step in repression. However, it is possible that initial nucleosome remodeling by SWI/SNF is required for HAT to efficiently acetylate histones and to fix nucleosomes in an inactive state (Kingston and Narlikar 1999). Because virtually all transcription factors seem to interact with the p300/CBP coactivator, which has HAT activity, the default state on many promoters may be a balance in favor of HAT activity. Recruitment of SWI/SNF under such conditions would normally be associated with transcriptional activation. The Kingston group found that SWI/SNF can mediate a reversible reaction, causing continuous oscillation of nucleosomes between an active and inactive state (Fig. 2; Schnitzler et al. 1998). For repression to occur at the promoter in this model, SWI/SNF may need to bring along HDAC to alter the balance in the vicinity of the SWI/SNF complex in favor of HDAC activity (Fig. 2). Thus removal of acetyl groups by HDAC would unfix nucleosomes, forcing SWI/SNF-dependent reassembly of functional nucleosomes.

Other results have also suggested a role for Rb in transcriptional activation. In one study, Rb potentiated activation by the glucocorticoid receptor in a manner dependent on Rb interaction with BRM (Singh et al. 1995). Because the glucocorticoid receptor has been associated with HAT activity (Wallberg et al. 1999), one possibility is that transcriptional activation in this setting is a result of recruiting Rb–SWI/SNF to the glucocorticoid receptor where HAT activity is predominant. Transcriptional activation by Rb has also been implicated in differentiation. Rb is required for MyoD transactivation and thus its ability to induce myogenic differentiation (Gu et al. 1993). Some studies have suggested that this differentiation function of Rb involves direct transcriptional activation and does not require it to bind to E2F (Sellers et al. 1998). Might the roles for Rb in transcriptional activation all be mechanistically linked? This is still unclear,

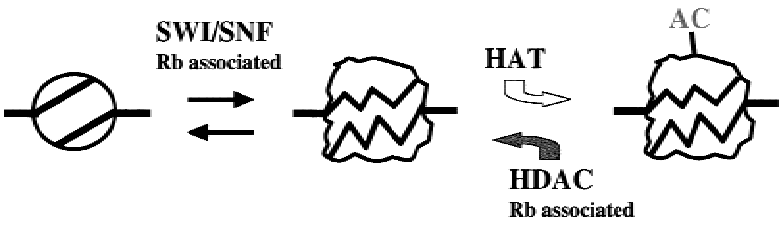

Figure 2. Potential interplay among SWI/SNF, HAT, and HDAC in transcriptional regulation. Kingston and coworkers have shown that SWI/SNF can remodel nucleosomes. It is important to note that this is a reversible reaction that is at least as efficient at assembling nucleosomes as it is at disrupting nucleosomes (for detailed review, see Kingston and Narlikar 1999). In this model, SWI/SNF ensures continuous oscillation of nucleosomes between a functional and disrupted structure. It is this disrupted nucleosome that may be targeted by HAT, which fixes the nucleosome in an inactive conformation, thereby driving the equation to the right. Conversely, recruitment of HDAC removes the inhibitory acetylation (AC), dumping unfixed disrupted nucleosomes back into the equation thereby forcing SWI/SNF to assemble them back into functional nucleosomes (moving the equation to the left toward function nucleosomes). Thus it is the balance between HDAC and HAT activity in the vicinity of SWI/SNF that determines whether SWI/SNF will facilitate transcriptional repression or activation.

and thus, the molecular role of Rb in transactivation must be further examined.

Several studies have suggested that Rb can also actively repress transcription by HDAC-independent mechanisms (Luo et al. 1998; Meloni et al. 1999). These mechanisms may involve other corepressors such as CtIP, RBP1, and HBP1 (Yee et al. 1998; Lai et al. 1999a; Meloni et al. 1999). CtIP interacts with CtBP, so named because it binds to the carboxy-terminal region of adenovirus E1A (Schaeper et al. 1998). The *Drosophila* homolog of CtBP is a transcriptional corepressor for Hairy, Knirps, and Snail (Nibu et al. 1998). CtIP binds the Rb pocket and has intrinsic repressor activity. This activity requires the PLDLS sequence, which mediates interaction with CtBP (Meloni et al. 1999). RBP1 is another pocket-binding protein that has transcriptional repressor activity, inhibits E2F transactivation, and suppresses cell growth when it is overexpressed (Lai et al. 1999b). RBP1 contains two repression domains, one of which binds HDAC, whereas the other domain appears to function independent of HDAC. Thus, it is possible that RBP1 may recruit HDAC to the Rb pocket for HDAC-dependent active repression (although an HDAC–RBP1–Rb complex has not yet been demonstrated), and it may also mediate HDAC-independent repression through the second repression domain (Lai et al. 1999a). HBP1 is a transcriptional repressor belonging to the high-mobility group family of proteins. It has two LXCXE sequences and it has been shown that interaction with either Rb or p130 is required for it to repress the *N-MYC* promoter (Yee et al. 1998; Lipinski and Jacks 1999). The function and relative contributions of these various corepressors that interact with Rb family members must still be defined in vivo.

Rb inhibition of E2F versus active repression by the Rb/E2F complex in cell cycle control

A number of studies have shown that transcriptional activation by E2F is important for the progression of cells through the cell cycle (Johnson et al. 1993), suggesting that Rb arrests cells in G_1 by inhibiting E2F transcriptional activity (Zhu et al. 1993; Qin et al. 1995). In this model, pocket proteins bind and inactivate E2F in G_0 and early G_1, but as the pocket proteins are phosphorylated by cdks in mid-to-late G_1, free E2F is released, and it drives the cell into S phase. Many of these studies have relied on overexpression of E2F, which could also have the effect of competitively replacing the Rb/E2F repressor complex on promoters with free E2F. Thus it is unclear in many of these studies whether the cells entered S phase as a result of transactivation by free E2F or displacement of the active Rb/E2F repressor complex (or both). Studies using a dominant-negative mutant of the E2F-binding partner, DP1, inhibited the progression of cells into S phase (Wu et al. 1996), supporting the idea that interaction of E2F/DP with promoters is important for cell cycle progression. However, DP has binding partners other than E2F and it may have E2F-independent functions (Sorensen et al. 1996; de la Luna et al. 1999). Most studies in which E2F genes have been deleted in mice have thus far failed to yield clear-cut evidence that transactivation is the primary function of E2F in cell cycle regulation, undoubtedly at least in part because of redundancy and functional compensation among the E2F family members. However, in one recent study, embryo fibroblasts derived from E2F3 gene knockout mice were delayed in entering S phase (Humbert et al. 2000). However, it is unclear whether this proliferative defect was present in the whole mouse or if this may be an E2F3-dependent stress response on the part of the cells to being placed in culture. Nevertheless, these studies show evidence of a major cellular defect in the $E2F3^{-/-}$ cultured cells, and thus provide a critical tool for further examination of the role of E2Fs in vivo.

In tumors triggered by expression of T antigen, which binds Rb and p53 and releases free E2F, backcrossing the mice into an $E2F1^{-/-}$ background-impaired tumor growth (Symonds et al. 1994). These results suggest that tumor growth depends on the free E2F1 that is released when Rb function is blocked by T antigen. Nevertheless, E2F1 does not seem to be required for essentially normal embryonic development. This suggests that free E2F1 may become essential for tumor cells because of their rapid growth relative to normal cells. However, this does not appear to be the case during development, when cells proliferate very rapidly, suggesting that E2F1 is not essential for rapid cell proliferation per se. Nevertheless, this may still explain the tumor-cell dependence on E2F1. In addition, crossing $Rb^{-/-}$ mice into an $E2F1^{-/-}$ background significantly reduced ectopic cell cycle entry in the CNS and lens compared to $Rb^{-/-}$ littermates (Tsai et al. 1998), providing evidence that release of free E2F1 can induce cells that would normally remain quiescent to enter the cell cycle.

It is interesting to consider the possibility that free E2F1 provides the tumor cells (and cells in the lens and CNS) with a growth advantage beyond simply shortening G_1. Clearly, overexpression of E2F1 can drive serum-starved cells in G_0 into G_1. In this situation, the growth-promoting function of E2F1 negates the requirement for mitogens and drives quiescent growth-factor-starved cells into the cycle. Therefore, it is conceivable that the excess free E2F1 that is released by T antigen binding to Rb might similarly serve to reduce dependence on growth factors and provide tumor cells with a proliferative advantage under adverse conditions in which growth factors may be limiting (a situation that is common early in tumor progression). This could also provide lens and CNS cells with a growth factor–like boost from G_0 into G_1 under conditions in which the cells would normally remain quiescent.

Taken together, there is abundant evidence from overexpression assays in cultured cells and in several mouse models to support a role for transcriptional activation by E2F in regulation of the transition from G_1 to S phase. However, this role needs to be further examined by dominant-negative, gene knockout, and other genetic approaches that do not rely on overexpression of E2F or forcing serum-deprived cells in G_0 into the cell cycle.

There is also growing evidence from recent studies that E2F forms an active repressor complex with Rb (and other pocket proteins) and that this complex is important for regulation of the cell cycle by Rb (Sellers et al. 1995; Zhang et al. 1999; He et al. 2000). In one study, plasmids containing multiple E2F-binding sites were used to titrate Rb–E2F repressor complexes and to prevent their interaction with promoters (He et al. 2000). These competitor plasmids were able to prevent arrest in G_1 following accumulation of endogenous hypophosphorylated Rb. In a second study, a dominant-negative mutant of E2F1, which contained the DNA-binding domain but lacked the Rb-binding site and transactivation domain, was used to displace Rb–E2F complexes from E2F-responsive genes. Expression of this E2F mutant prevented Rb-dependent arrest of G_1 by either p16 or TGF-β (Zhang et al. 1999). However, overexpression of Rb (in contrast to activation of endogenous Rb) still caused arrest of G_1 in the presence of this dominant-negative E2F, suggesting that artificially high levels of Rb can arrest cells in an E2F-independent (and potentially nonphysiologic) fashion. Taken together, such studies show that active repression by Rb–E2F has an important role in growth suppression by Rb. However, these studies do not show that E2F does not have a role in transcriptional activation in the cell cycle—it is unclear from either of these studies whether or not binding of free E2F to endogenous promoters was eliminated. Therefore, it is still unclear how this active repression by Rb/E2F contributes to control of the cell cycle in vivo relative to Rb-mediated inhibition of transcriptional activation by free E2F. Perhaps a current consensus view may be that the switch from active transcriptional repression by Rb/E2F family complexes to activation by free E2F is important and it provides a mechanism for efficient regulation of cell cycle genes.

A role for E2F may be forthcoming from work in *Drosophila*. Two E2Fs have been identified in *Drosophila*—dE2F1 contains a transactivation domain, whereas dE2F2 does not (Dynlacht et al. 1994; Ohtani and Nevins 1994; Duronio et al. 1995; N. Dyson, pers. comm.). Mutation of *dE2F1* led to a defect affecting the entry of cells into S phase, suggesting that its transactivation function may be important for driving cells into S phase. However, it is also conceivable that with mutation of *dE2F1*, a higher percentage of dE2F2 becomes complexed with *Drosophila* Rb proteins (RBF1 and RBF2), leading to an accumulation of repressor complexes and arrest of cells in G_1. Mutation of *dE2F1* or *dE2F2* led to embryonic defects, and crossing the mutants should provide genetic insight into the general role of dE2F.

Regulation of Rb function by phosphorylation

Cell cycle progression normally occurs when Rb is inactivated by phosphorylation catalyzed by cdks in complex with their cyclin partners (Chen et al. 1989; Hinds et al. 1992; Lundberg and Weinberg 1998). Rb contains 16 potential sites for cdk phosphorylation, and it oscillates between hypophosphorylated and hyperphosphorylated forms during the cell cycle. At least three different cyclin–cdk complexes have been suggested to phosphorylate Rb during the cell cycle. It is thought that cyclin D–cdk4/6 phosphorylates Rb early in G_1, cyclin E–cdk2 phosphorylates the protein near the end of G_1, and cyclin A–cdk2 may maintain phosphorylation of Rb during S phase (Sherr 1996). Phosphorylation of specific sites appears to regulate distinct Rb functions, suggesting complex regulation of Rb by these phosphorylation events. For example, binding of E2F, LXCXE proteins, and c-abl are regulated by distinct sets of phosphorylation sites (Knudsen and Wang 1996, 1997). Adding to the complexity, recent studies have suggested that Rb is unphosphorylated and inactive in G_0, and that initial phosphorylation by cdk4/6 leads to a hypophosphorylated, active protein (Ezhevsky et al. 1997). Subsequent hyperphosphorylations by cdk4/6 later in G_1 are then thought to inhibit Rb function. These results highlight the complexity of Rb phosphorylation events and our lack of a detailed understanding of this process.

Most of the studies of Rb phosphorylation have relied on overexpression of cyclins and/or cdks, so it is still unclear which cyclin–cdks normally phosphorylate Rb in vivo. Nevertheless, there appears to be a consensus that Rb can be phosphorylated sequentially by different cdks during the cell cycle. In one study, successive phosphorylation by cyclin D–cdk4/6 and cyclin E–cdk2 was necessary to completely hyperphosphorylate Rb (Lundberg and Weinberg 1998). It is interesting, however, that a "knockin" of the cyclin E gene into the cyclin D1 gene locus in mice prevented at least some of the phenotypic manifestations of the cyclin D1 gene deletion, suggesting that cyclin E is a downstream target of cyclin D1 function (Geng et al. 1999). If this is indeed the case, then why would Rb need to be phosphorylated by both cyclin D–cdk4/6 and cyclin E–cdk2? Recently, a mechanistic explanation was suggested for how cyclin D–cdk4/6 and cyclin E–cdk2 may regulate distinct Rb functions (Harbour et al. 1999). In this study, cyclin D–cdk4/6 appears to phosphorylate specific phosphoacceptor sites in the carboxy-terminal region of Rb, and this triggers an intramolecular interaction between the phosphorylated carboxy-terminal region and a positively charged "lysine patch" encircling the LXCXE-binding site in domain B of the pocket. This interaction displaces HDAC from the pocket, and it was proposed that this inhibits the ability of Rb to repress the cyclin E gene (Harbour et al. 1999; Zhang et al. 1999). Overexpression of cyclin E is sufficient to overcome growth arrest induced by a phosphorylation-resistant Rb mutant, RbΔcdk (Leng et al. 1997; Lukas et al. 1997), indicating that cyclin E–cdk2 has a critical target or targets in the Rb pathway other than Rb itself. Taken together, the above studies provide support for a model wherein Rb regulates the sequential expression of cyclins as the cell cycle progresses. Cyclin D–cdk4/6 allows expression of cyclin E by disrupting the Rb–HDAC complex (Harbour et al. 1999; Zhang et al. 2000), and then cyclin E expression is sufficient to overcome Rb-imposed arrest of G_1 (Zhang et al. 2000). Such a model could explain why cyclin D–cdk4/6 might not

be necessary for cell cycle progression if cyclin E is expressed constitutively or prematurely during the cell cycle.

The cyclin D–cdk4/6-dependent intramolecular interaction between the carboxy-terminal region of Rb and the pocket not only inhibits HDAC-mediated repression by Rb, it also appears to recruit cyclin E–cdk2 to the pocket through RXL docking sites located in the carboxy-terminal region (Adams et al. 1999; Harbour et al. 1999). This recruitment of cyclin E–cdk2 then facilitates phosphorylation of Ser 567, an otherwise inaccessible phosphoacceptor site buried within the domain A–domain B interface (Harbour et al. 1999). Ser 567 makes critical contacts between domain A and B (Lee et al. 1998), and this phosphorylation disrupts the A–B interface and blocks Rb binding to E2F. The sensitive location of Ser 567 is further illustrated by the fact that it is the only potential cdk phosphoacceptor site in Rb that is a target of naturally occurring missense mutations in tumors (Templeton et al. 1991). However, this phosphorylation of Ser 567 has been shown only in vitro, and it is not yet clear whether it actually occurs in vivo or if it is the only target of cyclin E–cdk2 on Rb in the absence of cyclin/cdk overexpression. It is also important to point out that these studies have relied on cyclin overexpression to activate endogenous cdks, which could lead to elevated levels of cdk activity and altered specificity. In addition, these studies were performed in tumor cells, which may not display a normal cell cycle. Nevertheless, such studies have provided evidence that phosphorylation of Rb by successive cyclin–cdk complexes can progressively block the interaction of Rb with regulatory proteins, which is consistent with the notion that different phosphorylated forms of Rb have distinct roles during the cell cycle (see below).

Roles for Rb beyond G_1 phase

Although it is widely accepted that Rb arrests cells in G_1, there is growing evidence that it also has regulatory effects on the cell cycle beyond G_1. Microinjection of a phosphorylation-resistant Rb mutant blocked DNA synthesis in cells that had passed the G_1 restriction point, suggesting that Rb can inhibit progression of the cell cycle beyond G_1 (Knudsen et al. 1998). In other experiments, the arrest of G_1 imposed by ectopic expression of either p16 or a phosphorylation-resistant Rb could be overcome by coexpression of cyclin E, resulting in completion of S phase and the remainder of the cell cycle (Lukas et al. 1997). In addition, this cyclin E-induced S phase did not require E2F transactivation, suggesting that cyclin E can act downstream from Rb/E2F. What then could be the downstream target of cyclin E–cdk2 in the Rb pathway? One possible target is the BRG1 component of SWI/SNF. Cyclin E has been found to associate with BRG1 and to inhibit growth arrest induced by BRG1 (Shanahan et al. 1999). Also, BRG1 is phosphorylated in a cell cycle–dependent fashion (Sif et al. 1998), and this phosphorylation inhibits SWI/SNF function (Shanahan et al. 1999). Because SWI/SNF function appears to be required for growth suppression by Rb, BRG1 could serve as an important downstream target of cyclin E–cdk2 in the Rb/E2F pathway. Thus cyclin E in S phase may play a major role in the inhibition of SWI/SNF.

A recent study supports this idea and provides evidence that Rb regulates progression through S phase via its interaction with SWI/SNF (Zhang et al. 2000). In this study, evidence is presented that an HDAC–Rb–SWI/SNF complex is required to inhibit cyclin E expression and to prevent entry into S phase (Fig. 3). Phosphorylation of Rb by cyclin D–cdk4/6 inhibited Rb binding to HDAC, but not to BRG1. The persisting Rb–SWI/SNF complex allowed accumulation of cyclin E—cyclin E–cdk2 is required for assembly of origins of DNA replication and thus for S phase (Krude et al. 1997), but cyclin A and cdc2 genes remained inhibited. It is unclear whether an HDAC-independent corepressor is required for this Rb–SWI/SNF-mediated inhibition of the cyclin A gene. It is then proposed that the resulting increase in cyclin E levels leads to inactivation of the Rb–SWI/SNF complex by cyclin E–cdk2, through its phosphorylation of Rb and/or BRG1. However, this was not directly shown in the studies. This inactivation of Rb–SWI/SNF is suggested to allow the accumulation of cyclin A and cdc2. But, it is unclear whether this is a direct effect on the cyclin A promoter, and the link between Rb–SWI/SNF and cyclin A expression is still somewhat circumstantial. It is interesting that the Rb–SWI/SNF complex could not arrest cells in G_1 when Rb–HDAC activity was diminished, but the complex could still inhibit cells from exiting S phase. These cells also underwent endoreduplication with >4N DNA content. It has been suggested that this abnormality was attributable to Rb–SWI/SNF-mediated inhibition of cdc2 and cyclin A, which are required for activation of cyclin B–cdc2 and M phase. Although cyclin A and cdc2 levels did decrease in these experiments, it has not yet been shown that these are the only targets of Rb–SWI/SNF and that they are responsible for the effect. However, in this regard it is interesting that a link between Rb/E2F and cyclin B–cdc2 was recently established (Lukas et al. 1999). Results have shown that the anaphase promoting complex (APC), a

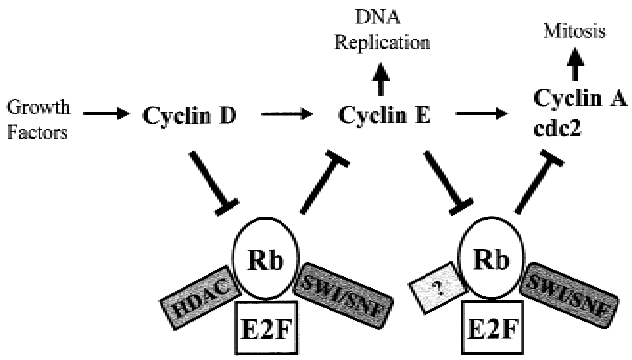

Figure 3. Potential roles for different Rb-chromatin remodeling complexes during the cell cycle. (?) Potential unknown corepressor. (See text for details.)

ubiquitin ligase that causes the degradation of cyclin B, remains active in the cell into S phase (Brandeis and Hunt 1996). APC depresses the level of cyclin B until cyclin A–cdk2 phosphorylates the Cdh1 subunit of the complex, blocking its activity and thereby allowing accumulation of cyclin B near the end of S phase—formation of cyclin B–cdc2 is then required for entry into mitosis. This process was shown to be controlled by Rb/E2F inhibition of the cyclin A gene (Fig. 4) (Lukas et al. 1999).

The HDAC and SWI/SNF studies described above have relied heavily on the C33A cell line, which is Rb^- and deficient in BRG1/BRM. Although HDAC readily forms complexes with Rb and Mad when proteins are overexpressed in these cells, these complexes are not capable of efficiently repressing transcription (Zhang et al. 2000). This was critical for allowing examination of various Rb-repressor complexes in some degree of isolation. However, it is important to emphasize that these studies have thus far been restricted to tumor cell lines and that they need to be reproduced in more normal cells and in genetic models. As pointed out to us by a reviewer, we had originally listed C33A among the different cell lines that we had used to examine Rb–HDAC activity (Luo et al. 1998). This was an oversight on our part (the Saos-2 cell line should have been listed in these studies in place of C33A)—in fact the C33A cells show only very limited evidence of Rb– or Mad–HDAC activity (Zhang et al. 2000), and they have been very useful in analyzing Rb–SWI/SNF activity for this reason. Despite diminished levels of BRM/BRG1 and the inability to form an efficient Rb–HDAC repressor complex, Rb still represses some promoters (e.g., the SV40 promoter/enhancer) in C33A cells, suggesting that Rb may interact functionally with yet another corepressor such as CtIP/CtBP, RBP1, HBP1, or an unidentified factor.

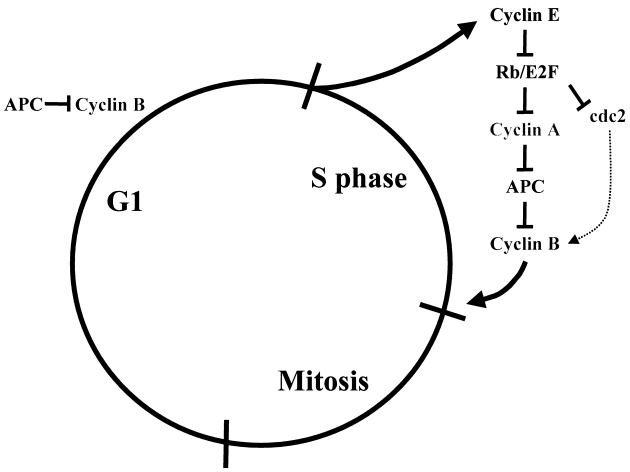

Figure 4. A novel link between cyclin A–cdk2, the anaphase promoting complex (APC), and assembly of cyclin B–cdc2. Recent studies from Bartek and coworkers suggest that cyclin A–cdk2 phosphorylates a component of the anaphase promoting complex, APC, blocking activity of the ubiquitin ligase which normally inhibits cyclin B expression (Lukas et al. 1999). These studies suggest that Rb/E2F and cyclin A–cdk2 have roles in regulation of APC and formation of cyclin B–cdc2, which is required for entry into mitosis.

The results described above suggest an intricate relationship between Rb/E2F and cyclin/cdks, which may serve at least in part to maintain the sequential order of cyclin/cdk activity during the cell cycle and regulate the exit of cells from G_1 and S phase. However, it is important to point out that the relative significance of these G_1 and S phase checkpoints in Rb function in vivo has yet to be established. And, although it has been shown that BRG1/BRM is required in Rb growth suppression using dominant-negative BRG1 and BRM, which does not rely on overexpression assays, there are still questions that need to be addressed before such a model for Rb function with HDAC and SWI/SNF can be fully accepted. For example, association of HDAC, SWI/SNF, and Rb family members must be examined during the cell cycle without protein overexpression, and association of these complexes with endogenous promoters must be evaluated during the cell cycle to determine if the effects are actually direct. In addition, the components of this potential pathway need to be evaluated in a genetic system in which their interplay can be examined in vivo. In this regard, further analysis of the Rb–E2F pathway in *Drosophila* and *C. elegans*, where initial genetic screens have implied roles for both SWI/SNF- and HDAC-like components in the pathway, must be pursued.

In *Drosophila*, dE2F1 appears to play a role in regulation of the localization of origin of replication complex (ORC) and in DNA replication (Royzman et al. 1999). Ovarian follicle cells in *Drosophila* undergo four endoreduplication cycles, after which there is additional amplification of the chorion genes to produce sufficient protein for the eggshell. A dE2F1 mutant that is truncated after the DNA-binding domain and thus lacks an Rb binding domain or transactivation domain leads to both premature and excessive amplifications in the follicle cells, suggesting that this process may be negatively regulated by an Rb/E2F complex (which cannot form with the mutant). Such a complex could regulate ORC activity by at least two mechanisms. First, it may recruit chromatin-remodeling enzymes to the origins, promoting nucleosome assembly and thereby physically blocking replication. However, dE2F has not yet been localized to the amplification origins. Alternatively, dE2F/RBF may actively repress expression of a component of the ORC complex. Indeed, ORC1 has been shown to be transcriptionally regulated by E2F in both *Drosophila* and in human cells (Ohtani et al. 1996; Asano and Wharton 1999). It is then possible that the regulation of exit from S phase in human cells by Rb/E2F complexed with chromatin-remodeling enzymes that has been observed in cultured cells (Zhang et al. 2000) may be occurring via direct effects on DNA replication or through control of expression of an ORC component.

Roles for Rb in apoptosis

Rb is inactivated either by mutation or by hyperphosphorylation in many tumors. Such a loss of Rb function

can trigger a p53-dependent apoptotic pathway, which may serve as an intrinsic protective mechanism to eliminate cells in which the Rb pathway is deregulated (Morgenbesser et al. 1994). Accordingly, loss of Rb function may also create a survival pressure for the cell to acquire mutations in this apoptotic pathway, thus explaining the high apoptotic index that is often found in $p53^+$ tumors and the frequent mutation of p53 in cancer (Symonds et al. 1994). A link between Rb and p53 appears to be the free E2F1, which is released when Rb function is lost. In support of this idea, transgenic mice in which Rb was inactivated developed slowly growing tumors of the choroid plexus with high apoptotic rates, whereas the additional inactivation of p53 led to rapidly growing tumors at least in part because of an 85% reduction in apoptosis (Symonds et al. 1994). Concomitant deletion of the E2F1 gene (instead of p53 inactivation) led to an 80% reduction in p53-dependent apoptosis in mice in which Rb was inactivated (Pan et al. 1998). In addition, Rb-deficient mice die in midgestation with widespread apoptosis (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992), whereas embryos that are mutants for both Rb and E2F1 show a significant reduction in apoptosis and down-regulation of the p53 pathway (Tsai et al. 1998). Taken together, these results suggest that release of free E2F1 resulting from loss of Rb function is responsible for triggering much of this p53-dependent apoptosis. How does this free E2F1 trigger p53-dependent apoptosis? A potential target of free E2F1 is the ARF gene (the alternate reading frame encoded by the p16INK4a locus; Bates et al. 1998). One function of ARF appears to be inhibition of MDM2-mediated turnover of p53, which in turn leads to an increase in p53 and apoptosis (Fig. 5) (Pomerantz et al. 1998; Zhang et al. 1998). Although it appears that E2F1 can activate ARF directly in overexpression assays (DeGregori et al. 1997) and that E2F1-dependent negative selection of thymocytes is associated with ARF expression (Zhu et al. 1999) it is still unclear whether or not this is a primary mechanism through which free E2F triggers apoptosis in vivo. Thus further studies are needed to define the mechanism of E2F1-induced apoptosis. Previous studies have suggested that E2F1 is somewhat unique among the E2F family members in its ability to trigger apoptosis (DeGregori et al. 1997). However, more recent studies now suggest that other E2F family members can also trigger apoptosis, and that this activity is regulated by their subcellular localization (Loughran and La Thangue 2000).

If Rb is hyperphosphorylated by cyclin D–cdk4/6 and cyclin E–cdk2 and free E2F is released as cells progress through G_1 into S phase, then why does this not free E2F trigger apoptosis? One possibility is that release of free E2F during the cell cycle is not sufficient to trigger an apoptotic response because another required pathway is not activated under these conditions (e.g., accumulation of free E2F is not sufficient for the apoptosis). This does not seem likely given that overexpression of E2F is sufficient to trigger the apoptotic response in cells and that a significant portion of the apoptosis resulting from mutation of the Rb gene is alleviated when the mice are crossed into an $E2F1^{-/-}$ background. Alternatively, free E2F may not be completely released during the cell cycle and its concentration may remain at a level that is insufficient to trigger an apoptotic response. Results from Farnham and coworkers may provide some support for this possibility (Wells et al. 2000). Using chromatin immunoprecipitation assays to assess which proteins are bound to endogenous genes containing E2F sites at different points during the cell cycle, this group found evidence of E2F–pocket protein complexes still associated with many cell cycle gene promoters during S phase. At first glance, this observation seems contrary to expectations. However, it appears that chromatin remodeling corepressors may be removed progressively from Rb–E2F as cells move through G_1 into S phase (Zhang et al. 2000), and this loss of corepressor activity seems to prevent active repression by Rb family members and to allow expression of S-phase genes. If the above model is correct, then free E2F may only accumulate sufficiently to trigger apoptosis when Rb function is lost, or perhaps when abnormal proliferative conditions in the cell lead to high cyclin E–cdk2 activity sufficient to phosphorylate Rb on Ser 567, at the A–B pocket interface, leading to a complete release of free E2F.

However, a recently published report from the Dynlacht group has reached different conclusions using similar chromatin immunoprecipitation assays to assess promoter occupancy of some of the same genes by Rb/E2F family members during the cell cycle (Takahashi et al. 2000). In this study the investigators found an E2F4/p130 complex at promoters during G_0, which was associated with deacetylation of histones H3 and H4 on the pro-

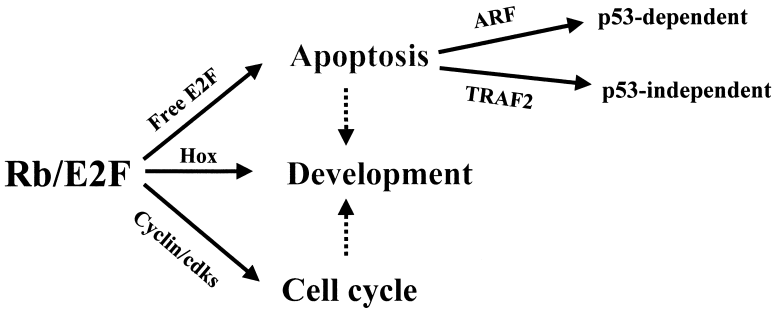

Figure 5. Previous studies have implicated the Rb/E2F pathway in regulation of the cell cycle and in control of apoptosis. Recent studies in *Xenopus* now raise the possibility that Rb/E2F plays a direct role in the regulation of Hox genes and development (Suzuki and Hemmati-Brivanlou 2000). Although this will need to be further confirmed, these results raise the possibility that roles for the Rb/E2F pathway may be about to expand.

moters. By late G_1, there was a switch to E2F1–3 on most of the promoters, which was associated with an increase in acetylation of histones. E2F binding to the promoters diminished significantly or was absent during S phase. Little or no Rb was detected in association with any of the genes at any stage of the cell cycle. This result is surprising, particularly given that the investigators detected abundant Rb–E2F complexes forming in G_1 and persisting into S phase in their cells by gel-shift assay. Although such a negative result is sometimes difficult to interpret (particularly in such indirect assays), these studies suggest that, although Rb–E2F complexes form in the cells, they do not make their way to the promoters. These results from the Dynlacht group raise the possibility that Rb may not have a general role in normal cell cycling—for example, it may only regulate the cell cycle in response to G_1-checkpoint activation. At first glance, this possibility is somewhat difficult to accept, given the substantial developmental and apoptotic defects seen in mice that lack Rb. However, it is important to point out that the developmental roles of Rb might not depend on its recruitment to a promoter via E2F, or the Rb–E2F complex may target genes other than those that control the cell cycle that were assayed in these studies (see below). Alternatively, these results in culture, where cells are exposed to very high concentrations of serum growth factors (and thus growth is maximally stimulated), may not entirely replicate cell cycle control in vivo, and under such conditions Rb (and perhaps p107) function could be limited to a checkpoint-like role. Additional experiments examining other genes, differing conditions (e.g., positive controls activating G_1 checkpoints and leading to accumulation of hypophosphorylated Rb), and more normal cells may be required to further assess this occupancy issue, which is critical for our understanding of the role of Rb/E2F family proteins.

Loss of Rb can also trigger p53-independent apoptosis (Pan and Griep 1995), but unlike p53-dependent apoptosis, this apoptosis does not require E2F transactivation and can be triggered by expression of the E2F DNA-binding domain alone (Hsieh et al. 1997; Phillips et al. 1997). This suggests that, in this distinct apoptotic pathway, free E2F may serve to displace the Rb–E2F repressor complex from genes. However, it is important to point out that this apoptosis occurs readily in functionally Rb^- cells, so the role of the E2F DNA-binding domain may instead be to displace free E2F from promoters. Although this would seem counterintuitive to the well-accepted idea that free E2F triggers apoptosis, it points out that free E2F is likely to be triggering apoptosis through two distinct pathways. This form of apoptosis is also associated with down-regulation of antiapoptotic proteins such as TRAF2 (Fig. 5; Phillips et al. 1999), but the mechanism of this down-regulation is still unclear. Both of these forms of apoptosis may be operative in vivo, as apoptosis in the central nervous system of $Rb^{-/-}$ mice is p53-dependent, whereas the apoptosis that occurs in the peripheral nervous system is p53-independent (Macleod et al. 1996). Thus an important direction for future research is to determine the relative contributions and the molecular targets of these p53-dependent and p53-independent apoptotic pathways in vivo.

Expanding roles for E2F and Rb in development?

E2F sites are found in the promoters of a number of genes that control the cell cycle and genes, such as ARF, that regulated p53-dependent apoptosis, leading to a widely held belief that the Rb/E2F pathway is restricted to cell cycle control and apoptosis. However, recent results now challenge this paradigm. In a screen for genes that are involved in anterior–posterior axis formation in *Xenopus*, an xE2F gene was identified that is most closely related to *E2F3* (Suzuki and Hemmati-Brivanlou 2000). Overexpression of xE2F led to ectopic expression of ventral and posterior markers and to suppression of the development of dorsoanterior structures. Conversely, when the investigators expressed a dominant-negative construct where the xE2F DNA-binding domain was fused to the engrailed repressor domain, ventral and posterior structures were inhibited. Further, the author showed that ectopic xE2F directly regulated Hox genes to control anterior–posterior axis formation, and it did not appear to function in cell cycle control. In these assays, however, the xE2F DNA-binding domain alone (which should displace endogenous xE2F from Hox genes) had no detectable effect. Thus although xE2F or xE2F–engrailed can regulate Hox genes when overexpressed, these results raise the question as to whether endogenous xE2F is actually regulating Hox genes during the course of these assays. If this is the case, it would highlight a hitherto unanticipated role for Rb/E2F. Accordingly, these results in *Xenopus* now raise the question of whether the defects observed in mice in which Rb/E2F-pathway genes are mutated are the result solely of aberrant cell cycle control and apoptosis, or if these defects are caused at least in part by deregulation of developmental controls genes (Fig. 5). Furthermore, these results suggest that E2F may directly regulate developmental genes through a mechanism that is independent of the cell cycle. Taken together with recent results from the Dynlacht group (Takahashi et al. 2000), which suggest that Rb is not associated with genes that control the cell cycle in cultured cells, this may eventually force us to readdress an old question in the field as to whether the Rb–E2F pathway is actually critical for control of the normal cell cycle, or if it serves primarily as a checkpoint that becomes activated in response to cellular deregulation.

The Rb-related proteins p107 and p130 and Rb function

Two other pocket proteins, p107 and p130, are homologous with Rb within the pocket, and they also bind viral oncoproteins and E2F (Ewen et al. 1991; Hannon et al. 1993). All three pocket proteins can inhibit E2F-responsive promoters (Zamanian and La 1993), recruit HDAC to the pocket (Ferreira et al. 1998), actively repress transcription (Bremner et al. 1995; Starostik et al. 1996), and

arrest the growth of cells when they are overexpressed (Zhu et al. 1993; Claudio et al. 1994; Starostik et al. 1996). Although this review has focused on Rb, it is important to convey that this protein does not function in isolation, and that there is mounting evidence that the pocket protein family members collaborate functionally. Therefore, we have included a brief discussion of p107 and p130 below, focusing primarily on recent results relating their function to that of Rb.

There are significant differences between the pocket proteins. For example, the spacer region between domains A and B in Rb is not conserved across species or in p107/p130 and has no known function, whereas this region is conserved between p107 and p130 and contains a p21-like sequence through which these proteins bind and inhibit cyclin E– and cyclin A–cdk2 complexes (Ewen et al. 1992; Zhu et al. 1995b; Adams et al. 1996; Lacy and Whyte 1997). This binding has been shown to mediate growth suppression by p107 (Zhu et al. 1995a). Studies examining interactions of the pocket proteins with E2F during cell growth and terminal differentiation have pointed out additional differences between the pocket proteins. In general, Rb can bind E2F1–4, whereas p107 and p130 bind to E2F4 and E2F5 (Hijmans et al. 1995; Sardet et al. 1995; Nevins 1998). E2F4–p130 is the most abundant complex in quiescent cells, and during differentiation of muscle cells. For example, Rb–E2F complexes are replaced by p130–E2F complexes, which are required to maintain inhibition of DNA synthesis in myotubes (Corbeil et al. 1995; Kiess et al. 1995; Shin et al. 1995). It is unclear why such a switch to p130–E2F is functionally important in quiescent cells, but it is common during development as cells differentiate.

Most experiments in genetically altered animals suggest that the pocket proteins partially overlap in function. Chimeric mice containing Rb-deficient cells do not develop retinal tumors but they are frequent in mice that are also p107-deficient (Robanus-Maandag et al. 1998). Similarly, mice that are heterozygous for Rb develop normally (Jacks et al. 1992), but the additional homozygous loss of p107 leads to growth retardation and early mortality (Lee et al. 1996). Homozygous Rb deletion leads to embryonic lethality in midgestation (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992), whereas the additional homozygous loss of p107 results in lethality two days earlier (Lee et al. 1996). All of these results suggest that p107 and Rb have overlapping but distinct roles in tumor suppression and development. Not only are the phenotypes of mice deficient for Rb, p107, or p130 affected by the presence of the other family members, the phenotypes are also modified dramatically by genetic background (LeCouter et al. 1998), suggesting the presence of strain-specific modifiers of pocket protein activity. Identification and characterization of such modifiers may prove important to ultimately assessing the relative contributions of pocket proteins in vivo and facilitate further understanding of their molecular action.

Primary cells from $p107^{-/-}$ or $p130^{-/-}$ mice showed no deregulation of E2F-responsive genes, whereas cells from $p107^{-/-}/p130^{-/-}$ mice showed deregulation of different genes than were found in analogous $Rb^{-/-}$ cells (Hurford et al. 1997). Similarly, in growth-limiting conditions, $Rb^{-/-}$ mouse embryo fibroblasts (MEFs) entered S phase and E2F target genes were deregulated, suggesting that expression of p107 and p130 are not sufficient substitutes for Rb in the arrest of G_1 and the repression of some E2F genes (Almasan et al. 1995). Arrest of cell growth by p16 depends on Rb, suggesting that the other pocket proteins cannot substitute for Rb in p16-mediated growth arrest. In contrast, p16 did not arrest cell growth in $Rb^{+/+}$ MEFs that lacked p107 and p130, suggesting that transcriptional repression by p107 and p130 may be required in addition to that of Rb for p16-mediated growth arrest (N. Dyson and L. Zhu, pers. comm.). Alternatively, p107 and p130 bind to cyclin E–cdk2 and cyclin A–cdk2 (Hannon et al. 1993; Zhu et al. 1995b) and they may be required to titrate cdk2 activity down to a level at which transcriptional repression by Rb can effectively arrest cells. Mutations in p107/p130 that block repression but leave the p21-like spacer intact, or those that delete the space, leaving the repression domain intact (Starostik et al. 1996) may ultimately prove useful in defining the role of p107/p130 in this situation. In any event, new results point to an intimate relationship between Rb and the other pocket proteins and indicate that Rb function depends on p107/p130 (Fig. 6). Additional studies are now warranted to examine the molecular basis of this relationship.

Conclusions

Although Rb was the first of the tumor-suppressing genes to be identified, and the Rb/E2F pathway has been studied widely, new findings in the past several years challenge our notions regarding the molecular mechanisms of this pathway and its role in vivo. Although there has been some success in characterizing the molecular basis of Rb function, the elaborate mechanism of Rb regulation by cdks, and the linkage of these pathways to extracellular signals, many of the basic issues regarding the role of the Rb/E2F pathway remain unanswered. Some of the fundamental issues are:

1. What is the relative contribution in vivo of inhibition of E2F transcriptional activation by Rb versus active repression by the Rb/E2F complex mediated by chromatin-remodeling enzymes?
2. How does the cell distinguish between the free E2F that is thought to accumulate as a result of Rb hyperphosphorylation in a normal cell cycle, and the free E2F that triggers apoptosis as a result of loss of Rb function?
3. What are the mechanisms and relative roles for p53-dependent and p53-independent forms of apoptosis, which appear to be triggered through distinct pathways in response to release of free E2F?
4. Do the developmental defects that are observed when Rb/E2F pathway members are mutated result from altered cell cycle control or do they result from direct effects on developmental control genes?
5. What is the role for Rb in transcriptional activation

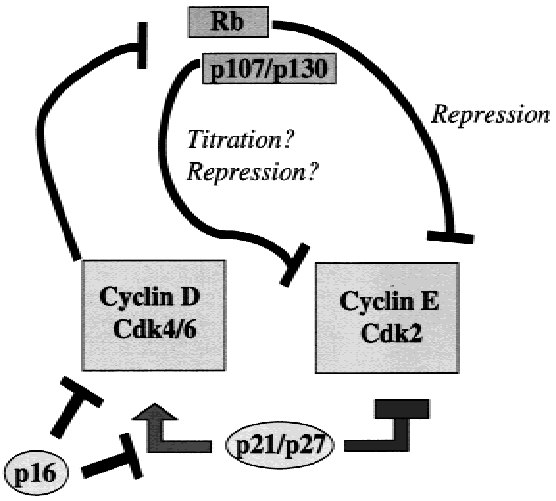

Figure 6. Potential interactions among cdks, cdk inhibitors, and Rb pocket protein family members. Several different studies have shown that the cdk2 inhibitors p21 and p27 not only inhibit cdk2 but also associate with cyclin D–cdk4/6. However, in contrast to cdk2, this association cdk promotes assembly of an active kinase and titrates the inhibitors away from cdk2, thereby increasing cdk2 activity in the cell. p16 binds to cdk4/6, inhibiting its activity, and displaces p21 and p27, freeing them to inhibit cdk2. Both the accumulation of hypophosphorylated Rb, which results from the p16-mediated inhibition of cdk4/6, and the inhibition of cdk2 activity by p21/p27 (displaced from cyclin D–cdk4/6 by p16) appear to be required for complete inhibition of cdk2 and growth suppression. (For detailed review of this pathway, see Sherr and Roberts 1999). New studies from both the Dyson and Zhu groups indicate that p107/p130 is also required for this growth suppression. p107 and p130 can bind to cdk2 (via a p21-like sequence in the spacer region of the pocket), blocking its activity. Thus, these proteins may serve a function similar to p21/p27—titration of active cdk2. However, like Rb, they are capable of repressing transcription. Thus it is also possible that p107/p130 contributes to the transcriptional repression displayed by Rb. Nevertheless, such recent results link the three pocket proteins together functionally.

(e.g., in transcriptional activation by MyoD during muscle differentiation or in response to glucocorticoids)?

6. Rb is frequently mutated in tumors, whereas p107 and p130 do not yet seem to be, highlighting a unique function for Rb. These results emphasize the fact that we still know little about the functions of p107 and p130 or about the intricate relationship between the three pocket proteins that is only now becoming apparent.

Resolving these and other issues regarding the Rb/E2F pathway in the upcoming years should eventually allow us to assess its complex and expanding roles in vivo.

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