Emerging roles for E2F: Beyond the G1/S transition and DNA replication

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E2F activity is crucial for the G1/S transition and DNA replication in mammalian cells. The retinoblastoma (pRB) family of proteins is the primary negative regulator of E2F. Recent findings have begun to clarify distinct roles for E2F family members during cell cycle progression and have considerably broadened our understanding of E2F transcriptional control beyond S phase. In this review, we examine the relative contribution of two distinct subclasses of E2F to repression and activation and how this division of labor could explain the role of E2F in DNA damage and repair checkpoints as well as tumorigenesis.

In mammalian cells, the E2F family of transcription factors plays a pivotal role in regulating the expression of genes involved in the G1/S transition and DNA synthesis. In turn, E2F activity is controlled primarily by the retinoblastoma (pRB) family of proteins. Much is known about the regulation of E2F by pRB in the context of the cell cycle, and several reviews have provided excellent coverage of these topics (Dyson, 1998; Harbour and Dean, 2000; Trimarchi and Lees, 2002). This review will focus instead on recent findings suggesting diversification within the E2F family that allows the execution of specialized functions in response to various cellular cues that promote or inhibit proliferation. In particular, technical innovations have brought new insights regarding the roles for E2F family members beyond the G1-to-S transition and suggest that E2F contributes to the DNA damage and repair checkpoint response and tumorigenesis.

A role for E2F in repression versus activation

Current evidence suggests that there is a clear bifurcation of E2F family members with respect to their transcriptional roles in cell cycle control: E2F1–3 function mainly as activators of transcription while E2F4 and E2F5 act primarily as repressors. Hence, we refer to the activator and repressor classes of E2Fs (Figure 1A). The divergence of function between the two classes of E2F parallels their specificity of binding to the pRB family of proteins: E2F1–3 bind only to pRB, E2F5 binds to p130, and E2F4 can bind to all three pRB members (Figure 1B). E2F6 differs significantly from the other E2Fs, and since its role as a transcriptional repressor has not been as thoroughly investigated, it will be excluded from general discussion in this review. A more extensive summary of evidence in support of this current picture of the E2F family has been provided elsewhere (Trimarchi and Lees, 2002).

Promoter occupancy by E2F in vivo

In serum-deprived cells, E2F-responsive promoters are occupied exclusively by complexes containing E2F4, p130, and a histone deacetylase (HDAC)-associated corepressor complex (Rayman et al., 2002; Ren et al., 2002; Takahashi et al., 2000) (Figure 1C). As cells reenter the cell cycle and progress through mid-G1, E2F4 and p130 and the entire corepressor complex are displaced by the activators E2F1, 2, and 3, coincident with the induction of E2F-responsive genes (Rayman et al., 2002; Takahashi et al., 2000). These data are wholly consistent with the view that there are two distinct subgroups within the E2F family: E2F4 and E2F5 act as repressors of E2F transcription in quiescent and early G1 cells by associating with the pocket proteins, while E2F1, 2, and 3, devoid of associated pocket proteins, function as activators in late G1 and S phase. Importantly, these studies also suggested that occupancy of E2F-responsive promoters by repressive and activating E2Fs is sequential and mutually exclusive. Remarkably, a similar division of labor among distinct E2F family members has also been demonstrated recently in *Drosophila* (Stevaux et al., 2002).

Recent analysis of approximately 1400 proximal human promoters revealed E2F binding to a much larger set of cell cycle-regulated promoters than anticipated (Ren et al., 2002). Significantly, these target genes function not only during the G1/S transition and DNA replication but also during mitosis and in DNA damage and repair checkpoints. A number of these novel E2F targets were shown to be controlled by the E2F4/p107/p130 repressive complex since they were noticeably deregulated in $p107^{-/-}$; $p130^{-/-}$ mouse embryonic fibroblasts (MEFs) (Ren et al., 2002). Therefore, current data suggest a more prominent role for E2F in repression of the cell cycle machinery during G0 and early G1. Whether the activator E2Fs contribute equally to the activation of these novel E2F targets remains to be seen. However, it is likely that a number of them, such as those involved in DNA repair, are regulated by the activator E2Fs since their promoters were occupied by E2F1 during G1 and they were responsive to induction by E2F1 and E2F2 (Ishida et al., 2001; Ren et al., 2002).

Proliferation and cell cycle exit defects in E2F knockout cells

More recent evidence bolstering the view of functional divisions within the E2F family has been obtained from mouse knockout experiments. In E2F3-deficient MEFs, many known E2F-responsive genes are not upregulated in quiescent cells responding to mitogen stimulation, suggesting a requirement for E2F3 in transcriptional activation (Humbert et al., 2000b). Consequently, the rate of proliferation of both primary and transformed cells is reduced (Humbert et al., 2000b). Moreover, MEFs that are deficient for all three "activating" E2Fs display proliferative arrest, and E2F target genes critical for the G1/S transition and DNA replication are not appropriately induced (Wu et al., 2001). Furthermore, loss of E2F3, but not E2F1,

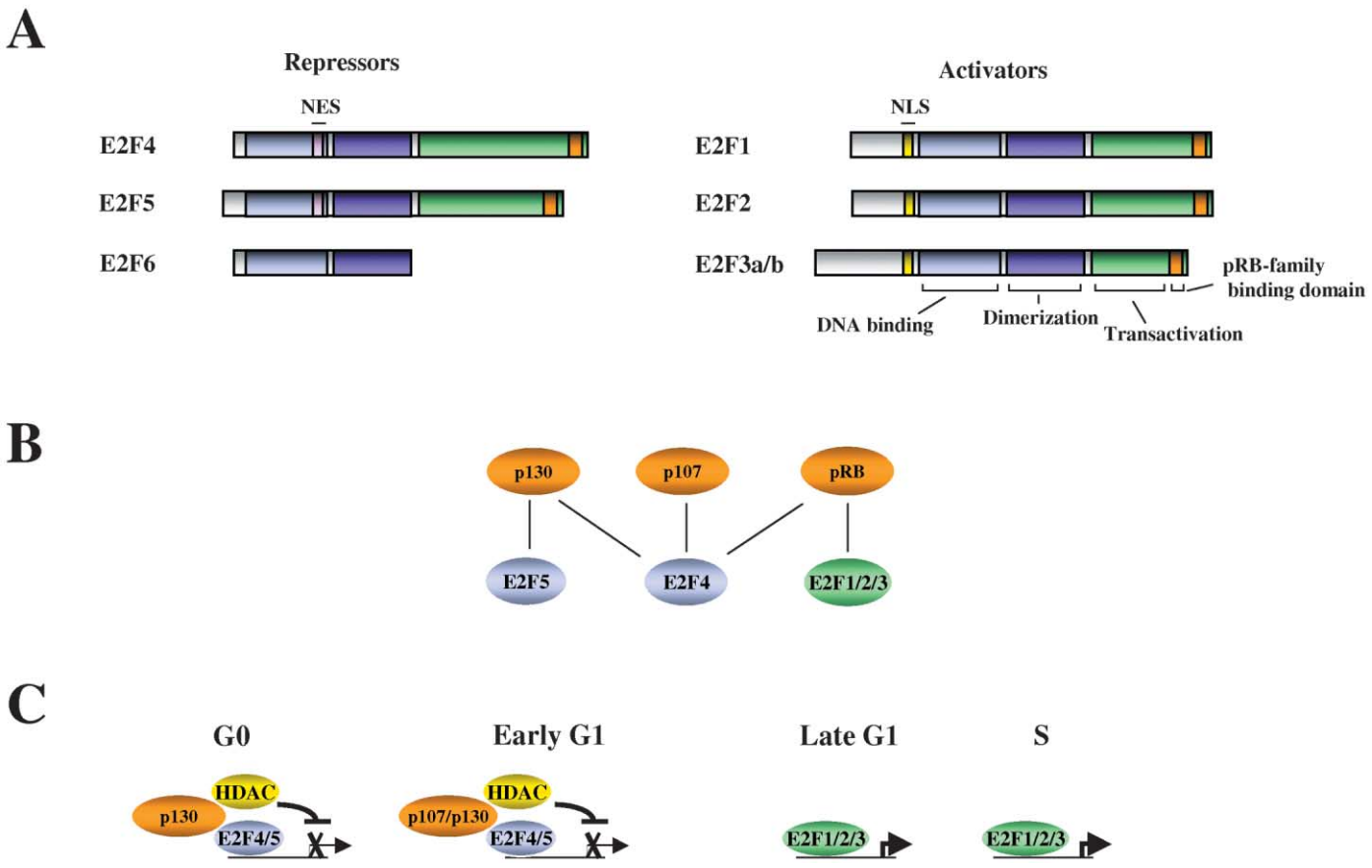

Figure 1. Structural divergence and preferential pRB binding explain cell cycle timing of promoter occupancy among E2F proteins

A: The E2F family contains six members that can be divided into at least two subgroups: E2F1–3 function primarily as activators of transcription, while E2F4 and E2F5 as repressors of transcription, and E2F6, which lacks a transactivation domain altogether, is likely to be involved in gene silencing. NES (nuclear export signal); NLS (nuclear localization signal).
B: Selective E2F binding to pRB family proteins.
C: Sequence and timing of target gene promoter occupancy by repressor and activator E2Fs is illustrated.

E2F2, E2F4, or E2F5, results in deregulated cyclin E-dependent kinase activity, defects in nucleophosmin B association with centrosomes, and premature centriole separation and duplication (Saavedra et al., 2003).

In contrast, cells lacking E2F4, E2F5, or both do not display cell cycle perturbations or aberrant gene expression (Humbert et al., 2000a; Rempel et al., 2000). However, these cells do not properly respond to growth-inhibitory signals provoked by p16 overexpression, and the fact that similar results were obtained with *p107*$^{-/-}$;*p130*$^{-/-}$ MEFs reinforces conclusions from recent ChIP studies indicating that E2F4 and E2F5, in association with p107 and/or p130, are the primary repressors of E2F target genes (Bruce et al., 2000; Hurford et al., 1997; E. Balciunaite and B.D.D., unpublished data). These results may also explain why mice lacking *E2f4*, *E2f5*, *p107*, or *p130* individually or in combination tend to suffer developmental abnormalities presumably related to defects in cell cycle exit prior to terminal differentiation.

E2F and DNA damage

Data from E2F knockout mice suggested that activator E2Fs are not only essential for cell proliferation but that they also play a role in apoptosis (Field et al., 1996; Yamasaki et al., 1996). How E2F triggers apoptosis is not clear. Several studies have focused on E2F-mediated apoptosis via p53/p14ARF pathways (Ginsberg, 2002). One recent study suggested that direct interaction of the cyclin A binding domain of E2F1 with p53 was able to induce apoptosis in response to DNA damage (Hsieh et al., 2002), while another demonstrated that p53 phosphorylation was critical for E2F1-mediated apoptosis (Rogoff et al., 2002). Intriguingly, E2F1 might play a more direct role in apoptosis: overexpression of E2F1, like loss of pRB function through E1A or mutation, induced caspase expression, provoking apoptosis and suggesting that activator E2Fs can be effectors of cell death signals (Nahle et al., 2002).

Several recent studies suggest an additional role for E2F1 in a DNA damage checkpoint and apoptosis (Huang et al., 1997; Lin et al., 2001; Maser et al., 2001). In one study, ionizing radiation led to the upregulation of E2F1 protein, induction of S phase, and subsequent cell death (Huang et al., 1997), and a second report showed that cells treated with a DNA-damaging agent stabilized E2F1 levels (Lin et al., 2001). Stabilization of E2F is thought to be due to phosphorylation of E2F1 by the ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) kinases (Lin et al., 2001). The

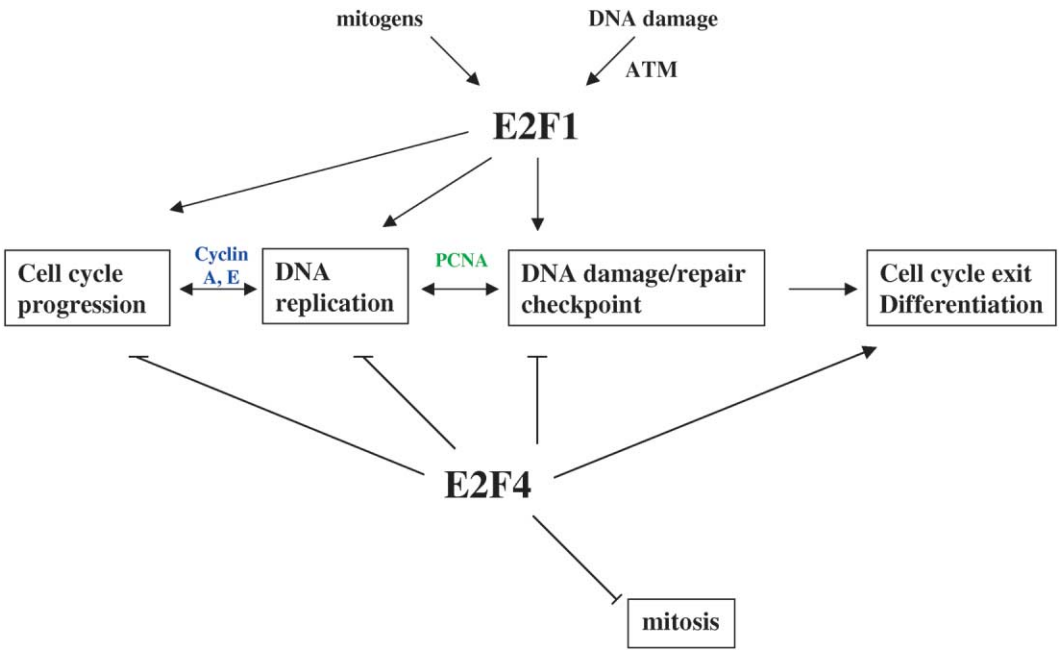

Figure 2. Cellular processes that involve E2F regulation

Recent gene profiling and location analysis experiments suggest more extensive control by E2F family members than originally anticipated: E2F plays a role in regulating genes involved not only in S phase entry, but DNA repair and mitosis as well.

ATM/ATR phosphorylation site is unique to E2F1 and lies in the amino-terminal region of E2F1 that overlaps with the $p45^{SKP2}$ binding site, suggesting that E2F1 phosphorylation by ATM/ATR kinases may confer resistance to $p45^{SKP2}$-mediated degradation of E2F1 (Lin et al., 2001; Marti et al., 1999). That E2F1 may play a role in an S phase checkpoint was suggested previously by experiments in which expression of a stabilized form of E2F1 provoked an S phase delay and apoptosis (Krek et al., 1995). Stabilization of E2F1 during S phase may trigger a sensor for abnormally elevated E2F activity that could lead to an apoptotic response similar to that observed in cells overexpressing activator E2Fs (DeGregori et al., 1997). Interestingly, E2F1 has also been implicated recently in the recruitment of NBS1 and the MRE11 recombination/repair complex to origins of DNA replication where it may implement activation of a checkpoint by suppressing origin firing when cells entering S phase are irradiated (Maser et al., 2001).

These findings are intriguing in light of other recent observations. First, pRB has been shown to regulate progression through S phase, at which point it is necessary for cell cycle arrest in response to DNA damage (Knudsen et al., 1998; Knudsen et al., 2000). In addition, recent ChIP studies suggest that pRB is not present at E2F-responsive promoters in cycling cells (Rayman et al., 2002; Takahashi et al., 2000), inviting speculation that this protein may act as a tumor suppressor at least in part by virtue of its localization to other regulatory regions, such as origins. Certain cues, such as cell cycle withdrawal induced by p16 overexpression or terminal differentiation, might cause pRB to relocalize to promoters (Dahiya et al., 2001; Thomas et al., 2001). Other work, however, directly implicates a role for pRB during S phase (Bosco et al., 2001; Kennedy et al., 2000). In particular, Bosco et al. recently demonstrated a requirement for RBF (a pRB homolog) binding to dE2F to enforce normal origin function in *Drosophila* (Bosco et al., 2001). Although much work will be needed to fully understand the mechanisms through which E2F1 (and pRB) could enforce a potential checkpoint, it is tempting to speculate that enhanced E2F1 levels may be one sensor that prevents further cell cycle progression by recruitment of proteins that suppress origin firing or by inappropriately elevating target gene expression and thereby engaging an apoptotic pathway, or both. Regardless of the mechanism, these studies illustrate how E2F1 and their regulators play a central role in control of both cell cycle progression and genome integrity and how the loss of appropriate responses promotes tumorigenesis.

Consistent with these observations, mouse embryos lacking *Rb* display numerous apoptosis-induced developmental defects, which were dramatically alleviated in the absence of either E2F1 or E2F3 (Yamasaki et al., 1998; Ziebold et al., 2001). However, it is not clear whether or how the role of E2F1 in S phase-induced apoptosis is related to suppression of origin firing by E2F1, and possibly other E2Fs. Intriguingly, recent expression profiling and location analyses have identified more than a dozen E2F targets, including *p53*, *chk1*, *pcna*, *fen1* that are known to be involved in the DNA damage response, hinting at a pervasive involvement of E2F in a DNA damage response pathway (Ishida et al., 2001; Muller et al., 2001; Polager et al., 2002; Ren et al., 2002). In addition, a cohort of genes involved in DNA repair was also identified as targets of E2F4 (Ren et al., 2002), inviting speculation that a tight coupling of DNA replication and repair in mammalian cells requires the E2F family. Furthermore, a more general requirement for repressive E2F

activity in growth arrest associated with TGFβ signaling, contact inhibition, p16, and p19ARF/p53-dependent senescence has been demonstrated (Rowland et al., 2002; Zhang et al., 1999). Although the complex circuitry through which these E2F targets are regulated by the two subclasses of E2F needs further study, it is nevertheless clear that E2F plays a more pervasive role than anticipated in processes other than S phase entry (Figure 2).

E2F and tumorigenesis

Recent studies have also shed new light on the connections between E2F, apoptosis, and tumorigenesis. Overexpression of activator E2Fs is not only able to drive cells out of quiescence, but in some settings, it also confers transforming potential to primary cells (Pierce et al., 1998; Trimarchi and Lees, 2002). Several lines of evidence support the view that pRB-E2F interactions play a major role in restraining E2F oncogenic activity. First, inactivation of pRB function by viral oncoproteins or cancerous mutations also tends to prevent pRB binding to E2F (Sellers and Kaelin, 1996). Second, overexpression of E2F mutants that cannot bind pRB promotes anchorage-independent growth more efficiently than wild-type E2F1 (Xu et al., 1995). Finally, loss of E2F1 in $Rb^{+/-}$ mice partially suppresses the incidence of pituitary and thyroid tumors (Yamasaki et al., 1998). Whether loss of the other activator E2Fs such as E2F2 and E2F3 also contributes to tumor suppression in $Rb^{+/-}$ mice has yet to be determined. However, Ziebold et al. recently reported that E2F3 loss in $Rb^{-/-}$ mice rescues most of the abnormal proliferation and apoptosis associated with $Rb^{-/-}$ embryos (Ziebold et al., 2001). This phenotype is reminiscent of $Rb^{-/-}$;$E2f1^{-/-}$ mouse embryos in which loss of E2F1 suppressed almost all of the p53-dependent apoptosis associated with $Rb^{-/-}$ embryos (Tsai et al., 1998). Therefore, it is tempting to speculate that E2F3 loss might similarly contribute to a lower incidence of pituitary and thyroid tumors in mice simultaneously deficient for pRB. These results are especially interesting in light of recent data indicating that apoptosis observed in *Rb* null embryos stems from placental defects (Wu et al., 2003). However, these non-cell-autonomous roles for pRB in neurogenesis and erythropoiesis are likely to be different from its known function as a tumor suppressor since all pituitary tumors in chimeric mice originate strictly from $Rb^{-/-}$ cells (Maandag et al., 1994; Williams et al., 1994).

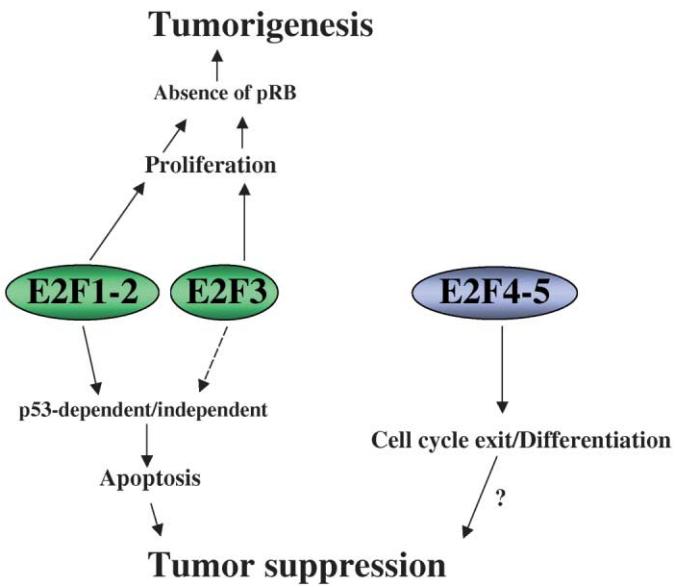

Figure 3. Current model for how E2F contributes to tumorigenesis and tumor suppression

Elevated levels of activator E2Fs can trigger apoptosis via p53-dependent or p53-independent pathways. Unrestrained activity of activator E2Fs, which are essential for proliferation of mammalian cells, results from the absence of pRB and leads to tumorigenesis. While *E2f3* loss suppresses apoptosis associated with simultaneous *Rb* loss, unlike the *E2f1* knockout, it does not exhibit tumor suppressor activity (dotted line). Although abundant evidence exists for the importance of E2F4 and E2F5 in cell cycle exit associated with enforced expression of p16 or cellular differentiation, whether the combined activities of E2F4 and E2F5 can function in tumor suppression is not known.

The role of E2F as an oncogenic factor in tumorigenesis is firmly established. However, a report that adult $E2f1^{-/-}$ mice developed a broad spectrum of tumors was unanticipated, suggesting that E2F1 has a dual role in tumorigenesis both as an oncogene and as a tumor suppressor (Field et al., 1996; Yamasaki et al., 1996). Interestingly, the types of tumors present in $E2f1^{-/-}$ mice are distinct from $Rb^{+/-}$ mice: whereas $Rb^{+/-}$ mice consistently develop pituitary adenomas and thyroid tumors, $E2f1^{-/-}$ mice develop reproductive tract sarcomas, lymphoma, and lung tumors. Therefore, E2F1 loss may not be equivalent to pRB loss as an underlying cause of tumor appearance in $E2f1^{-/-}$ mice. Since E2F1 has been implicated in p53-dependent apoptosis, it is now thought that E2F1 tumor suppressor function could at least partly stem from its ability to relay apoptotic signals to p53 (Pan et al., 1998). E2F2 may also act as a tumor suppressor since E2F2 deficiency further predisposes $E2f1^{-/-}$ mice to additional tumor development (Zhu et al., 2001).

While several recent reports have begun to clarify the roles of activator E2Fs in tumorigenesis, little was known about the tumorigenic roles of repressor E2Fs. Mice deficient for various combinations of *E2f4* and *E2f5* have been generated. These studies suggest that besides developmental defects in certain lineages, E2F4-deficient mice are tumor free (Gaubatz et al., 2000; Humbert et al., 2000a; Rempel et al., 2000). Although $E2F5^{-/-}$ mice are born with hydrocephalic syndrome, they are viable, but most died after 6 weeks, preventing the study of the role of E2F5 in tumor suppression (Lindeman et al., 1998). Predictably, $E2f4^{-/-}$;$E2f5^{-/-}$ mice died as neonates making it impossible to assess the compound loss of both E2F4 and E2F5 on tumorigenesis (Gaubatz et al., 2000).

pRB binds both to activator E2Fs and E2F4, the principal repressor E2F. How or if the pRB-E2F4 interaction contributes to tumor suppression is not clear. However, recent work has begun to address this question (Lee et al., 2002). Given the role of E2F4 as a major transcriptional repressor, the expectation would be that compound loss of this gene and *Rb* would exacerbate the tumor phenotype. Surprisingly, $Rb^{+/-}$;$E2f4^{-/-}$ mice were virtually tumor free up to 14 months of age. This finding is remarkable given that most $Rb^{+/-}$ mice died at or before 11 months of age due to pituitary and thyroid tumors (Yamasaki et al., 1998). Several older $Rb^{+/-}$;$E2f4^{-/-}$ mice eventually developed pituitary tumors, but the tumor severity varied widely among them (Lee et al., 2002).

Examination of the pocket protein-E2F complexes in various organs and MEFs of *Rb*$^{+/-}$;*E2f4*$^{-/-}$ mice revealed that loss of E2F4 promotes formation of novel complexes between p107 and p130 and the activator E2Fs that could suppress improper E2F activity in the absence of *Rb*. These novel complexes probably have a repressive effect on E2F-responsive promoters since appropriate transcription of the *cyclin E* gene, which is deregulated in *Rb*$^{-/-}$ MEFs, was restored in *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs (Lee et al., 2002). p130 was also detected in complexes with E2F5 in *Rb*$^{-/-}$;*E2f4*$^{-/-}$ MEFs. However, it is unlikely that the p130/E2F5 complex is responsible for repressing *cyclin E* transcription because in the absence of *Rb* alone, there is an abundance of the p130/E2F4 complex, which functionally resembles the p130/E2F5 complex. Interestingly, compound loss of E2F4 and E2F5 enhanced p107 and p130 association with the activator E2Fs (Lee et al., 2002; H.C., J. Rayman, and B.D.D., unpublished data), which could explain why *E2f4*$^{-/-}$;*E2f5*$^{-/-}$ MEFs do not display deregulation of E2F activity and therefore no cell cycle defects in cycling and serum-starved cells (Gaubatz et al., 2000). Apparently, in the absence of the E2F4 and E2F5 repressors, p107 and p130 are still capable of forming repressor complexes by binding to the activator E2Fs, enabling their recruitment to, and inhibition of, E2F-responsive promoters (H.C., J. Rayman, and B.D.D., unpublished data). Taken together, these findings also suggest that pRB may be the only pocket protein with tumor suppressor activity by virtue of the fact that it is uniquely able to sequester and inhibit activator E2Fs.

Conclusions and perspectives

The initial discovery of E2F and work that followed suggested that this transcription factor promoted proliferation by activating critical target genes required for DNA replication. Another key discovery, the association of E2F with the pRB family, suggested a simple way in which E2F activity could be restrained until it was required for gene activation. Recent studies have clearly demonstrated that E2F family members are not simply activators held in check. Rather, a subset of the E2F family members function as active repressors that are subsequently replaced by a new set of polypeptides that function as activators. Moreover, several recent studies have considerably expanded our understanding of the roles of E2F in transcriptional regulation of the cell cycle and how its mechanisms of transcriptional activation and repression contribute to tumor formation. Surprisingly, given the known roles of the two subfamilies of E2F in transcription, current data suggest that while activator E2Fs can behave both as oncogenes and tumor suppressors, the repressors E2F4 and E2F5 have no direct effect in tumorigenesis (Figure 3). This is hard to reconcile with known properties of E2F4 and E2F5 as the primary repressors of E2F activity. However, given that pRB is the only tumor suppressor of the pocket protein family and the only one that normally binds to all three activator E2Fs, it is possible that repressor E2Fs are essential only in cell cycle exit that occurs during terminal differentiation (Figure 3). Indeed, mouse knockout experiments suggest that the ability of pRB, but not p107/p130, to exclusively inhibit activator E2Fs under normal conditions underlies its unique role as tumor suppressor. However, additional knockout experiments will be required to determine whether E2F4 and E2F5 play a role as activators as well as repressors of transcription in other settings since both of these proteins possess transactivation domains (Figure 1A).

Advances in DNA microarray technology and the combined use of location analysis and gene expression profiling will enable the identification of the complete set of physiological targets of all E2F and pRB family members and will illuminate the role of E2Fs not only in the control of replication and tumorigenesis but also in other processes not discussed in this review, including development and differentiation and how these processes interconnect (Figures 2 and 3). Significantly, the recent implication of E2F in the DNA damage response pathway and the identification of a number of E2F targets involved in DNA damage and repair by expression and location analysis strongly suggest a more extensive involvement of E2F in this pathway. Future studies should be able to dissect the relative contribution of E2F activation and repression in DNA damage and repair pathways and explain how its involvement in these pathways is related to other processes such as apoptosis. Such an analysis will enable us to link cell cycle events with apoptosis, and ultimately, with the failure of surveillance and apoptotic mechanisms that are known to give rise to tumors.

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