The Role of E2F•Pocket Protein Repressive Complexes in Cell Cycle Control and Differentiation.

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ABSTRACT

The pocket protein family is comprised of pRB (the protein product of the retinoblastoma susceptibility gene), p107, and p130. This family regulates the G₁/S transition by interacting with their major downstream target, the E2F transcription factor. E2F is a heterodimeric protein composed of one DP subunit and one E2F subunit. The E2F family can be subdivided into three categories based upon structural and functional homology: E2F6; the ‘activating E2Fs’ (1-3); and ‘the repressive E2Fs’ (4-5). The focus of this study is E2F4 and E2F5, the members of the repressive E2F subgroup. The repressive E2Fs function by occupying E2F responsive promoters during G₀ and recruiting in the pocket proteins. As cells begin cycling, E2F4 and E2F5 are replaced at promoters by members of the activating E2Fs subgroup. Loss of either E2F4 or E2F5 does not result in cell cycle defects but instead lead to the abnormal development of specific tissues. The lack of a cell cycle phenotype in single mutants could be due to compensation by the other repressive E2F.

In order to determine the role that E2F4•pocket protein repressive complexes play in regulating cell cycle control, differentiation, and development, mice lacking E2F4 and two members of the pocket protein family, p107 and p130, were generated. Analysis of mouse embryonic fibroblasts derived from mutant embryos revealed that while loss of E2F4 alone did not lead to defects in cell cycle control, it did significantly enhance the ability of cells to differentiate into adipocytes. This phenotype could be further enhanced by additional loss of p107 and p130. Analysis of mice lacking E2F4, p107, and p130 revealed a requirement for these proteins in regulating fetal hematopoiesis. Taken together, these data suggest that E2F•pocket protein repressive complexes are critical regulators of differentiation and development.

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CHAPTER ONE

INTRODUCTION
Part I. Cell Cycle Control and the G₁ Restriction Point

The process of cellular proliferation, or cell cycle, is tightly regulated and is composed of four basic stages. In response to growth cues such as mitogens, cells leave a period of rest known as G₀ (post-mitotic) or G₁ (gap-phase entered by cells that have recently divided prior to progressing through the cell cycle again) and synthesize a copy of their DNA (S phase). The cells then enter another rest phase, G₂ before dividing into two daughter cells in mitosis (M). A major decision point that controls the G₁ to S transition is the restriction point (R). “R” was originally defined by Pardee as a point reached during G₁ after which the need for mitogenic stimulation for cell cycle progression is eliminated (Pardee, 1974; Pardee, 1989). Once the requirements for passage through the restriction point are met, the cell is committed to S-phase progression.

This chapter will describe the pathway critical for the regulation of the G₁ to S transition. The first part of this chapter will focus on describing the proteins in this pathway and how each functions to control cell cycle progression. The second part of this chapter will focus on the contribution of this pathway to the regulation of differentiation.

A. Cyclins of the G₁/S transition

After stimulation, the cell cycle is driven by a cascade of cyclins and cyclin dependent kinases (cdks) (Sherr, 1994). Cyclins and cdks are regulated by several mechanisms including: the levels of cyclins present, complex formation, complex activity, and interaction with inhibitory molecules. Cdks are expressed constitutively
while cyclins are made only during defined phases (Sherr, 1994). A functional holoenzyme is formed only when the cyclin and cdk subunits are complexed together (Sherr, 1993). This temporal regulation is reflective of the fact that cyclin•cdk complexes are responsible for regulating distinct cell cycle transitions. During early G₁, three different D-type cyclins (D1, D2, D3) are synthesized in response to mitogenic stimulation and complex with either cdk4 or cdk6 (Sherr and Roberts, 1995). As cells progress through G₁, cyclin E (E1 and E2) becomes upregulated with expression peaking at the G₁/S transition. Cyclin E shows specificity for a different cyclin dependent kinase, cdk2. Both D- and E-type kinases require phosphorylation by cdk-activating kinase (CAK) to become catalytically active (Kato et al., 1994; Matsuoka et al., 1994). Both D and E type holoenzymes regulate passage through the restriction point as overexpression of either cyclin D or E shortens the G₁/S transition and reduces the requirement for mitogens (Ohtsubo et al., 1995; Quelle et al., 1993; Resnitzky et al., 1994). As cyclin D and E levels decrease during the G₁/S transition, the two forms of cyclin A (A1 and A2) become upregulated and complex with cdk2 (Sherr, 1994). Cyclin A is not required for passage through the restriction point, but is necessary for DNA synthesis and progression into mitosis (Sherr, 1994).

B. G₁ cyclin inhibitors

The cyclins of the G₁/S transition are regulated by two classes of inhibitors: the Ink4 family and the Cip/Kip family (as reviewed in Sherr and Roberts, 1995). The Cip/Kip family has three members, p21\(^{Cip1/Waf1}\), p27\(^{kip1}\), and p57\(^{kip2}\) (Sherr and Roberts, 1995). All three family members can bind to and inhibit the activity of cyclin D, E, and
A containing holoenzymes (Sherr and Roberts, 1995). \( p21^{\text{Cip1/Waf1}} \) is induced in response to mitogens and is present in cycling cells. The presence of \( p21^{\text{Cip1/Waf1}} \) is, in fact, required in low levels for the appropriate assembly of cyclin/cdk complexes. Under conditions of senescence or DNA damage, multiple \( p21^{\text{Cip1/Waf1}} \) molecules interact with cyclin/cdk complexes, inhibiting their activity (Harper et al., 1995; Sherr, 1993; Zhang et al., 1994). Consistent with this cell cycle inhibitory role, loss of \( p21^{\text{Cip1/Waf1}} \) in mice results in tumors including sarcomas and B-cell lymphomas (Martin-Caballero et al., 2001). \( p27^{\text{kip1}} \) is induced in response to transforming growth factor \( \beta \) (TGF\( \beta \)), cAMP, and confluence (Hunter and Pines, 1994). Its pattern of expression is therefore different than that of \( p21^{\text{Cip1/Waf1}} \), being present at high levels during G\(_0\)/G\(_1\) and decreasing over the G\(_1\)/S transition. Mice deficient for this cdk inhibitor exhibit organomegaly and a high incidence of pituitary tumors, confirming its importance as a cell cycle inhibitor (Fero et al., 1996; Kiyokawa et al., 1996). \( p57^{\text{kip2}} \) is the most recently identified member of this family. Studies using \( p57^{\text{kip2}} \) null mice reveal that loss of this cdk inhibitor leads to defects in a number of tissues, suggesting it is important in regulating differentiation and development (Yan et al., 1997; Zhang et al., 1997).

The Ink4 family (p15, p16, p18, and p19) consists of small proteins of about 32 amino acids that contain four ankyrin motifs (as reviewed in Sherr and Roberts, 1995). Members of this family specifically inhibit D-type cyclin containing kinases. Based on their broad expression patterns in embryonic and neonatal mice, \( p16^{\text{ink4c}} \) and \( p19^{\text{ink4d}} \) are thought to play a role in regulating differentiation and development (Sherr and McCormick, 2002). Mice deficient for \( p19^{\text{ink4d}} \) have testicular atrophy, but no other apparent defect suggesting that it is not specifically required for either tumor suppression
or development (Zindy et al., 2000). Mice lacking p18\textsuperscript{ink4c}, however, develop organomegaly and pituitary tumors confirming its importance in the regulation of both the cell cycle and development (Franklin et al., 1998; Latres et al., 2000). Both p16\textsuperscript{ink4a} and p15\textsuperscript{ink4b} are triggered in response to stress induced by plating primary cells into culture. p15\textsuperscript{ink4b} is also induced in response to TGF\textbeta treatment (Sherr and Roberts, 1995). 
p16\textsuperscript{ink4a} is upregulated as cells in culture are continually passaged, suggesting a role for p16\textsuperscript{ink4a} in mediating the process of senescence (Alcorta et al., 1996; Hara et al., 1996). Mice deficient for either of these cdk inhibitors have no developmental abnormalities but do develop tumors confirming their importance in inhibiting cell cycle progression in vivo (Krimpenfort et al., 2001; Sharpless et al., 2001).

The two cdk inhibitor families work in concert to regulate the cell cycle (as reviewed in (Sherr and Roberts, 1995). Under growth permissive conditions, levels of the Ink4 family remain low. During this time Cip/Kip family members are expressed and associate in low levels with cyclin/cdk complexes and facilitate their assembly. As cells arrest, levels of the Ink4 family increase and specifically associate with cdk4 and cdk6, preventing the activation of cyclin D containing holoenzyme and displacing the Cip/Kip family. The Cip/Kip family subsequently associates in high levels with cyclin E and cyclin A containing holoenzymes, inactivating them. Consistent with their proposed roles as key regulators of the G\textsubscript{i}/S transition, mutations resulting in the overexpression of cyclin D and cdk4 or loss of p16\textsuperscript{ink4a} have been found in a number of human cancers (Sherr and McCormick, 2002).
C. pRB regulates the G₁ restriction point

One key target of the cyclin D-ckd holoenzyme is the product of the retinoblastoma susceptibility gene (RB) (Ewen et al., 1993). RB was the first tumor suppressor gene to be identified as both copies of the RB gene were found to be mutated in retinoblastoma, a childhood cancer of the eye (Friend et al., 1986; Lee et al., 1987). Disabling the RB restriction point is a critical step for tumor progression as RB is deleted or mutated in one third of all human tumors (Weinberg, 1991; Weinberg, 1992). Tumors maintaining a functional RB usually carry mutations in genes encoding other members of the retinoblastoma pathway such as p16\textsuperscript{Ink4a} or cyclin D (as reviewed in Sherr, 1996).

Insight into the function of the retinoblastoma protein (pRB) came from studies investigating DNA tumor viruses such as adenovirus, simian virus 40 (SV40), and human papillomavirus (HPV). DNA viruses must gain control over the host’s cell cycle machinery in order to replicate their genome. Proteins produced by these viruses (adenovirus E1A, SV40 large T antigen (Lg TAg), and HPV E7) were found to specifically interact with a number of cellular proteins. One of these was a 105kD protein that was shown to be the hypo-phosphorylated form of pRB (DeCaprio et al., 1988; Dyson et al., 1989b; Munger et al., 1989; Whyte et al., 1988a). pRB interacts with a region of E1A that has been shown to be necessary for mediating transformation (Whyte et al., 1988b; Whyte et al., 1989). E1A and other DNA tumor viral proteins contain LXCXE domains through which they interact with pRB (Dyson et al., 1989b; Whyte et al., 1988a). The interaction with pRB occurs in a domain within pRB of about 400 amino acids referred to as the “pocket” (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990). The pocket domain can be further subdivided into an A and B pocket that are
separated by a small spacer of about 75 amino acids. Studies of the crystal structure of pRB bound to E7 and SV40 Lg TAg have revealed that the B pocket is critical for this interaction (Kim et al., 2001; Lee et al., 1998).

The phosphorylation status of pRB is regulated in a cell cycle dependent manner. Cells in G₀/G₁ contain hypo-phosphorylated pRB. After mitogenic stimuli, cyclin D•cdk4/6 and cyclin E•cdk2 kinases initiate a phosphorylation cascade resulting in a hyper-phosphorylated form of pRB (Buchkovich et al., 1989; DeCaprio et al., 1989; Ewen et al., 1993; Harbour et al., 1999; Lundberg and Weinberg, 1998). Oncoproteins produced by DNA tumor viruses specifically target the hypo-phosphorylated form of pRB. Injection of purified, hypo-phosphorylated pRB into cells in early G₁ inhibits S-phase progression, but injection of pRB had no effect on cells in late G₁ or at the G₁/S transition (Goodrich et al., 1991). These experiments serve as evidence that pRB plays a role in regulating the restriction point. It can significantly prevent cell cycle entry in early G₁ but as cells progress past the restriction point into late G₁ pRB has little effect.

D. p107 and p130

pRB is a member of a family of proteins collectively referred to as the pocket protein family. This family includes two related proteins, p107 and p130. p107 was originally identified as a cellular protein capable of interacting with the regions of E1A and SV40 Lg TAg necessary for transformation (Dyson et al., 1989a; Whyte et al., 1989). Cloning of the gene encoding p107 revealed that the protein product contains a pocket domain homologous to the one found in pRB (Ewen et al., 1991).
Two independent studies cloned and characterized p130. The first study identified p130 through its ability to interact with the same conserved region of E1A as p107 and pRB (Dyson et al., 1992). The second study cloned p130 and identified its protein product as capable of interacting with cdk2 through a yeast two-hybrid screen (Hannon et al., 1993). The cloned sequence showed that p130 encoded a protein containing a pocket domain homologous to pRB and p107. All of the pocket proteins are regulated by phosphorylation. Cyclins D and E phosphorylate pRB, p107, and p130, but only p107 and p130 are targets for phosphorylation by cyclin A (Ewen et al., 1992; Faha et al., 1992; Hannon et al., 1993; Li et al., 1993).

E. The E2F transcription factor

The pocket protein family regulates cell cycle progression primarily through association with the E2F transcription factor. E2F was originally identified through multiple studies as a cellular factor capable of association with and transactivation of a sequence in the E1A inducible adenoviral E2 promoter (Kovesdi et al., 1986; Yee et al., 1987). Another study identified an "E2 associated factor specifically downregulated in differentiated cells", Differentiation Regulated Transcription Factor 1 (DRTF1), that was later identified as also being the E2F transcription factor (La Thangue and Rigby, 1987). Further studies have shown that E1A functions to dissociate E2F from other cellular factors. Once free from cellular factors, E2F can drive transcription off of the E2 promoter (Bagchi et al., 1990).

One of the cellular factors that is endogenously associated with E2F and that can be dissociated by E1A is pRB (Bagchi et al., 1991; Bandara and La Thangue, 1991;
Chellappan et al., 1991; Chittenden et al., 1991). The other members of the pocket protein family have also been shown to interact with E2F (Cao et al., 1992; Cobrinik et al., 1993; Shirodkar et al., 1992). Association between E2F and the pocket protein family has been shown to be cell cycle regulated. The predominant form of E2F in G$_i$/G$_i$ is found in complexes with the pocket protein family whereas free E2F appears in S phase (Chittenden et al., 1993). The free form of E2F has been shown to be a potent initiator of DNA synthesis and S-phase entry (Johnson et al., 1993). Cell cycle regulated genes containing E2-like elements in their promoters such as the genes encoding dihydrofolate reductase (DHFR), thymidylate synthase (TS), ribonucleotide reductase, and DNA polymerase $\alpha$ (DNA pol $\alpha$) were among the first E2F responsive candidates identified (Hiebert et al., 1991; Mudryj et al., 1990; Wade et al., 1992). To date a number of genes responsible for driving cell cycle progression have been identified as E2F targets (DeGregori et al., 1995a; Dyson, 1998; Ren et al., 2002; Weinmann et al., 2001). The pRB family interacts with E2F through their pocket domains (Chow and Dean, 1996). This interaction masks and inhibits the transactivational activity of E2F (Flemington et al., 1993; Helin et al., 1993a; Hiebert et al., 1992; Lee et al., 2002; Weintraub et al., 1992; Zhu et al., 1993).

E2F can therefore function as both an activator and a repressor of cell cycle progression. Interaction with the pocket protein family can repress the transcriptional activity of E2F and thereby inhibits the expression of E2F responsive genes and cell cycle progression. Pocket proteins once bound to E2F can recruit enzymes that have histone-remodeling activities such as deacetylases and methylases (as described below). Phosphorylation of the pocket protein family releases free E2F that can then activate the
transcription of E2F responsive genes which, in turn stimulates cells to enter the cell cycle. Both the activation and repressive functions of E2F have been shown to be required for normal cell cycle regulation.

The next section will describe the mechanisms employed by the pocket protein family to mediate active transcriptional repression. The remainder of Part I will be devoted to the description of the E2F transcription factor, its activities, and the mechanisms governing its regulation.

F. Mechanisms of pocket protein mediated repression

Pocket proteins inhibit the function of E2F through two different mechanisms: by inhibition of activation and through direct repression. As described above, the pocket protein family can prevent activation by interacting with and inhibiting the function of the E2F transactivation domain (Flemington et al., 1993; Helin et al., 1993a). As described in the following sections, pRB directly inhibits cell cycle progression through the recruitment of chromatin remodeling factors. One mechanism through which chromatin structure is modified is histone modification. Histones are basic proteins with an amino terminus tail that can be modified by acetylation and methylation. The basic structure of chromatin, or nucleosome, consists of a complex of eight histone subunits (two of H2A, H2B, H3, and H4) in an octamer, around which 1 3/4 superhelical turns of DNA (146 base pairs) are wrapped (as reviewed in Lachner and Jenuwein, 2002; Turner, 2002). Modification to histone tails such as acetylation promotes gene expression while methylation represses. Described below are the chromatin remodeling factors that are recruited by pRB.
(i) Nucleosome remodeling complexes are recruited by pRB

pRB has been shown to interact with nucleosome remodeling complexes. Yeast SWI/SNF was the first such complex to be identified. The human homologs of SWI/SNF complexes are BRG1 and hBRM. Both of these proteins possess nucleosome remodeling activity. All nucleosome-remodeling complexes are comprised of multiple subunits and are dependent upon ATP-hydrolysis to alter the structure and position of nucleosomes by disrupting histone-DNA interactions (Kingston and Narlikar, 1999). Multiple groups have found that exit from the cell cycle is dependent upon an interaction between pRB and BRG1 and hBRM in complexes with or without the presence of HDAC (Strobeck et al., 2000; Zhang et al., 2000).

(ii) Pocket proteins interact with histone deacetylases (HDAC)

pRB and the other members of the pocket protein family have been shown to interact with histone deacetylases (HDACs) (Brehm et al., 1998; Dahiya et al., 2000; Ferreira et al., 1998; Luo et al., 1998). There are seven members of the HDAC family of enzymes whose function is to remove acetyl groups from the tails of histone octamers. Deacetylated histones condense and prevent the transcriptional machinery from interacting with promoters (as reviewed by Harbour and Dean, 2000). HDACs 1 and 2 contain a LXCXE motif that has been found to be necessary for recruitment by pRB (Dahiya et al., 2000).
(iii) pRB recruits histone methyltransferases to E2F responsive promoters

Histone methylation has been shown to be a mechanism through which transcription is silenced. Most recently, pRB has been shown to interact with and recruit SUV39H1 and HP1 to promoters (Nielsen et al., 2001; Vandel et al., 2001). SUV39H1 is a lysine histone methyltransferase with specificity for unmodified lysine 9 on the tail of histone 3 (H3) (Rea et al., 2000). HP1 can interact with methylated H3, forming a complex with SUV39H1 and propagating subsequent methylation of nearby histone tails (Bannister et al., 2001).

There is now a prevailing view of the mechanism through which pRB mediates active transcriptional repression. The recruitment of HDACs removes acetyl groups from histones, repressing transcription by E2F. Once lysine 9 of H3 becomes deacetylated it is a target for pRB recruited SUV39H1 and (once methylated) HP1. The combined action of SUV39H1 and HP1 propagates methylation on nearby histone tails and silence transcription.

In order for the pocket protein family to repress transcription and cell cycle progression, they need to be targeted to promoters. This is accomplished through interaction with the E2F transcription factor. E2F is a heterodimeric protein that contains one of seven possible E2F subunits. The remainder of Part I will be devoted to describing the proteins that comprise the E2F transcription factor, their activities, and the mechanisms governing their regulation.
G. The E2F family of proteins

The genes encoding the components of the E2F transcription factor can be grouped into two categories (the DP and E2F families) based upon structural and functional homologies. Maximal E2F activity requires that one member of the DP family and one member of the E2F family interact to form a heterodimeric protein (Bandara et al., 1993; Helin et al., 1993b; Huber et al., 1993; Krek et al., 1993). E2F has been cloned and identified in several species, including xenopus, caenorhabditis elegans, and drosophila melanogaster (Ceol and Horvitz, 2001; Ohtani and Nevins, 1994; Sawado et al., 1998; Suzuki and Hemmati-Brivanlou, 2000). The function of E2F identified in other species is highly homologous to mammalian E2F, the major focus of this study. There have been eight genes encoding components of mammalian E2F transcription factor identified to date. The E2F and DP families will be described in detail below.

(i) The DP family

There have been two members of the DP family cloned to date, *DP-1* and *DP-2* (Girling et al., 1993; Ormondroyd et al., 1995; Rogers et al., 1996; Wu et al., 1995; Zhang and Chellappan, 1995). Both family members contain conserved dimerization and transactivation domains. DP is required for potentiating the transactivational activity of the E2F subunit, but is not directly responsible for the DNA binding specificity of the complex (Bandara et al., 1993; Helin et al., 1993b; Huber et al., 1993; Krek et al., 1993).
(ii) The E2F family

The E2F family consists of six genes encoding seven different protein products (Trimarchi and Lees, 2002). All of the E2F family members contain conserved DNA binding and dimerization domains, and they can interact with the DP family (Dyson, 1998). The E2F subunit is responsible DNA binding specificity, showing the maximum affinity for nucleotide sequences containing TTTCCCCG (as reviewed in Trimarchi and Lees, 2002). The E2F family can be divided into three subgroups based upon structural and functional homologies: E2F1-3, E2F4-5, and E2F6. The sections below will describe the discovery of the members of each subgroup.

a) E2F1, E2F2, and E2F3

This E2F subgroup consists of E2F1, E2F2, and E2F3. E2F1 was originally identified as a protein capable of interacting with the pocket domain of pRB (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992). E2F1 has distinct domains involved in, cyclin A binding, DNA binding, dimerization, and transactivation (Trimarchi and Lees, 2002). Two additional genes encoding members of this subgroup have been identified, E2f2 and E2f3 (Ivey-Hoyle et al., 1993; Lees et al., 1993a). Both of the genes encode proteins highly homologous to E2F1 in all major domains.

Recent studies have shown that the E2F3 gene encodes two separate protein products, the original transcript E2f3a and E2f3b, which arises from a promoter embedded in the first intron and uses an alternate first coding exon (He et al., 2000a; Leone et al., 2000). The E2f3b transcript is regulated in a manner distinct from the activating E2F subgroup. E2F1-3a are expressed in a cell cycle dependent fashion with
peak expression occurring at the G_1/S boundary (Ikeda et al., 1996; Moberg et al., 1996). The E2F3b promoter lacks E2F responsive elements and is expressed constitutively throughout the cell cycle (Adams et al., 2000; He et al., 2000a). The E2F3b protein product lacks the N-terminal cyclin A binding domain and has six novel amino acids in its N-terminus. The remainder of E2F3b is identical to E2F3a. All four of these E2Fs are regulated solely by pRB in vivo (Dyson et al., 1993; Lees et al., 1993b).

b) **E2F4 and E2F5**

E2F4 and E2F5 were identified due to their ability to interact with p107 and p130 (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995). This E2F subgroup can interact with DP as well as the pocket protein family, as both E2F4 and E2F5 contain the DNA binding, dimerization, and transactivation domains found in E2F1-3 (Dyson, 1998). These E2Fs lack most of the sequence N-terminal to the DNA binding domain that is found in E2F1-3. E2F5 is regulated predominantly by p130, and E2F4, the predominant E2F in vivo, can associate with all of the members of the pocket protein family (Ginsberg et al., 1994; Hijmans et al., 1995; Moberg et al., 1996; Vairo et al., 1995).

c) **E2F6**

E2F6 was recently identified and differs both structurally and functionally from the rest of the E2F family. E2F6 contains the DNA binding and dimerization domains but lacks the domain responsible for pocket protein interaction and transactivation (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). This E2F
therefore, is not regulated by the pocket protein family. Cells overexpressing E2F6 show a delay in cell cycle entry and E2F target gene expression (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). Studies of the endogenous function of E2F6 revealed that it is present in the mammalian polycomb complex in association with Bmi-1, Ring1, Mel-18, Mph1, and the Ring1 and YY1 binding protein (RYBP) (Ogawa et al., 2002; Trimarchi et al., 2001). A second study also found that E2F6 associates with other polycomb proteins such as YAF2 and the histone methyltransferase, HP1γ (Ogawa et al., 2002). Polycomb complexes have been shown to be responsible for maintaining transcriptional silencing of the homeobox (Hox) gene family whose expression is important for regulating development. Mice deficient for members of the Bmi-1 polycomb complex display defects in anterior-posterior patterning (homeotic transformations) of the vertebrae (Akasaka et al., 1996; Bel et al., 1998; Core et al., 1997; del Mar Lorente et al., 2000; van der Lugt et al., 1994). Mice carrying mutations for E2f6 display homeotic transformations of the vertebrae consistent with E2F6’s proposed role as a member of the polycomb complex (Storre et al., 2002).

H. Functional specificity of the E2F subgroups

The three E2F subgroups discussed above have been shown to have different functions in vivo. As mentioned above, E2F6 plays a role in the mammalian polycomb complex. The focus of this section will be on the other two subgroups, E2F1-3, the ‘activating E2Fs’, and E2F4 and E2F5, the ‘repressive E2Fs’. Functional assignments were made based upon a combination of over expression studies, analysis of endogenous
complexes, and studies using cells carrying targeted deletions in one or more of the E2F family members. This section will discuss the evidence for the functional assignments.

(i) The ‘activating E2Fs’

E2F1, E2F2, and E2F3a are all members of ‘activating E2F’ subgroup. This assignment was made because overexpression of these E2Fs strongly induces quiescent cells to enter S-phase entry and activate the expression of E2F responsive genes (Johnson et al., 1993; Leone et al., 1999; Qin et al., 1994; Shan and Lee, 1994). Cells that overexpress the activating E2Fs also show an inability to appropriately arrest in response to cyclin dependent kinase inhibitors, transforming growth factor β, and irradiation (DeGregori et al., 1995b; Lukas et al., 1996; Mann and Jones, 1996; Schwarz et al., 1995).

Analysis of the endogenous proteins supports the assignment that E2F1, E2F2, and E2F3a belong to the activating E2F subgroup. First, the expression of E2Fs in this subgroup begins in mid G1 with peak levels of transcription occurring during the G1/S transition (Ikeda et al., 1996; Moberg et al., 1996). These E2Fs are autoregulated due to the presence of E2F responsive elements within their promoters (Johnson et al., 1994). Second, when expressed, the activating E2Fs are found in the nucleus due to a nuclear localization sequence present in their amino terminus (Magae et al., 1996; Muller et al., 1997; Verona et al., 1997). Finally, chromatin immunoprecipitation assays (ChIP) reveal that the activating E2Fs occupy the promoters of responsive genes during mid G1 through the S-phase transition, the time when these genes are expressed (Takahashi et al., 2000).
Mouse embryonic fibroblasts (MEFs) carrying targeted deletions for each of the activating E2Fs have been generated and analyzed. $E2f1^{-/-}$ MEFs display a slight increase in the time necessary for the cells to induce the expression of cyclin E and progress into S phase from a G₀ or G₁ arrest (Wang et al., 1998). In contrast, deletion of E2F3 (both a and b forms) leads to a severe impairment in the ability of MEFs to enter the cell cycle and induce E2F responsive gene expression from a serum withdrawal mediated G₁ arrest (Humbert et al., 2000b). Subsequent loss of the other activating E2Fs in an $E2f3^{-/-}$ background increase the severity of this phenotype (Wu et al., 2001). Taken together, the data summarized in this section demonstrates that E2F1, E2F2, and E2F3a are required for activation of E2F target gene expression and S-phase progression. It is important to note that E2F3b has not formally been assigned to an E2F subgroup to date. Though it has high homology to all of the activating E2Fs, it is expressed throughout the cell cycle (Adams et al., 2000; He et al., 2000b). The function of this E2F, therefore, is still under investigation.

a) E2F and apoptosis

Deregulation of the activating E2Fs has been shown to lead to a loss of cell cycle control (as discussed above). A second consequence of deregulated E2F activity is an increase in programmed cell death, or apoptosis (reviewed in Dyson, 1998). E2F-induced apoptosis has been found to be both dependent and independent of the pro-apoptotic tumor suppressor, p53. The following sections discuss the mechanisms through which the E2F family induces apoptosis by first focusing on its role in the p53-apoptotic pathway and then discussing alternate mechanisms for E2F-dependent apoptosis.
1) E2F and p53 dependent apoptosis

The p53 transcription factor is a downstream target of a number of signaling pathways that respond to conditions of DNA damage, hypoxia, and other cellular stresses (as reviewed in Sherr and McCormick, 2002). Under certain conditions, p53 responds to these signals by inducing cell cycle arrest through the transcription of targets such as the cdk inhibitor, \( p27^{kip1} \). Alternatively p53 can trigger apoptosis by upregulating the expression of genes encoding pro-apoptotic proteins such as bax. An additional p53 responsive gene is mouse \( MDM2 (HDM2 \text{ in humans}) \), whose protein product functions to bind to and target p53 for nuclear export and degradation (as reviewed in Woods and Vousden, 2001). The activity of Mdm2 is antagonized through interaction with mouse p19\(^{ARF}\) (p14\(^{ARF}\) in humans). Interaction between Mdm2 and p19\(^{ARF}\) increases the stability and activity of p53 (as reviewed in Sherr and Weber, 2000).

One mechanism through which E2F is thought to increase the activity of p53 is by directly regulating the transcription of the gene encoding p19\(^{ARF}\) (\( ARF \)) (Bates et al., 1998; DeGregori et al., 1997). Deregulation of E2F1 by overexpression of E1A can increase both the levels of \( p19^{ARF} \) expression and apoptosis (de Stanchina et al., 1998). Increased levels of p19\(^{ARF}\) can stabilize p53, predisposing the cell towards undergoing programmed cell death.

2) Mechanisms of E2F and p53 independent apoptosis

The activating E2Fs have been shown to induce apoptosis through pathways independent of p53. The other pathways that E2F has been implicated to be involved in are briefly described in this section. The p53 transcription factor is a member of a family
of proteins. One member of the p53 family, p73, has also been implicated in apoptosis induced by E2F. p73 has been shown to be a transcriptional target for E2F, and cells lacking functional p53 and p73 have defects in undergoing E2F induced apoptosis (Irwin et al., 2000; Lissy et al., 2000; Stiewe and Putzer, 2000).

E2F has also been shown to directly stimulate pro-apoptotic pathways. NF-κB is a transcription factor that is induced in response to survival signals. E2F has been implicated in impairing the activation of NF-κB through an as yet undetermined mechanism that is independent of its function as a transcription factor (Phillips et al., 1999).

E2F has been shown to be responsible for the transcription of genes encoding proteins required for the apoptotic machinery. E2F-responsive pro-apoptotic genes include the apoptosis protease-activating factor (Apaf-1), and caspase-3, caspase-7, caspase-8, and caspase-9 (Ma et al., 2002; Moroni et al., 2001; Muller et al., 2001; Polager et al., 2002; Stanelle et al., 2002).

b) Regulation of the activating E2Fs

As described above, deregulation of the activating E2Fs can lead to inappropriate cell cycle activation and apoptosis. The expression and activity of the activating E2Fs are tightly regulated at the level of transcription (as described above) and by post-translational modifications. This section will detail the mechanisms employed to regulate the activating E2Fs.
1) Acetylation

The activating E2Fs have been shown to associate with two transcriptional co-activators, P/CAF and p300/CBP (Martinez-Balbas et al., 2000; Marzio et al., 2000). Both co-activators are thought to stimulate the transcriptional activity of E2F through the transfer of acetyl groups (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). Acetylation of the activating E2Fs has been shown to enhance their activity by increasing their DNA binding affinity, stability, and transactivation potential (Martinez-Balbas et al., 2000; Marzio et al., 2000).

2) Phosphorylation

As stated above, phosphorylation by cyclin dependent kinases (cyclin D•cdk4, cyclin D•cdk6, and cyclin E•cdk2) regulates the activity of E2F indirectly through eliminating pocket protein repression. Many studies have shown that cyclin A directly interacts with the N-terminus of the activating E2Fs (Fagan et al., 1994; Krek et al., 1994; Krek et al., 1995). This interaction leads to phosphorylation of the E2F heterodimer regulating its activity in two ways. Phosphorylation of the E2F subunit leads to disruption of its ability to interact with the pocket protein family and stimulates its transcriptional activity (Fagan et al., 1994). Interaction with cyclin A also leads to phosphorylation of the DP subunit, inhibiting DNA binding and E2F activity (Dynlacht et al., 1994; Krek et al., 1994; Krek et al., 1995).
3) Ubiquitination

The activating E2Fs are regulated at the level of protein stability. The protein levels of this E2F subgroup peaks during the G1/S transition and declines as cells progress from S-phase to G2 (Flores et al., 1998; Moberg et al., 1996). Proteins are targeted for degradation by the 26 S proteasome through the addition of the small polypeptide, ubiquitin (Hershko and Ciechanover, 1998). Ubiquitin is added through the concerted action of three enzymes: E1, E2, and E3. The role of E1 is to activate the ubiquitin molecule so that it can be transferred to E2, the ubiquitin conjugating enzyme. E2 transfers ubiquitin to the ubiquitin-protein ligase (E3). E1 and E2 are general components of the ubiquitination machinery whereas the E3 confers substrate specificity to the complex (Hershko and Ciechanover, 1998). The E3 ligases fall into one of three categories: the anaphase promoting complex, HECT-domain proteins, and the Skp1-cullin-F-box (SCF) proteins. Skp2, a member of the SCF family has been found to regulate the stability of E2F1 (Harper and Elledge, 1999). This finding arose from the observations that SCF$^{Skp2}$ becomes active at the same time levels of E2F-1 declined. In addition, mutation of sites responsible for interaction with SCF$^{Skp2}$ increased E2F’s stability (Marti et al., 1999). Studies have shown that interaction with pRB can inhibit the ubiquitination and degradation of E2F (Hofmann et al., 1996).

(ii) The ‘repressive E2Fs’

The ‘repressive E2F’ subgroup consists of E2F4 and E2F5 and is thought to play a role in mediating active transcriptional repression through recruitment of the pocket protein family to E2F responsive promoters. When overexpressed in resting cells, both
E2F4 and E2F5 are inefficient at driving S-phase entry and E2F responsive gene expression (Muller et al., 1997).

Analysis of the endogenous transcripts and proteins revealed that the repressive E2Fs lack E2F responsive elements in their promoters, are expressed constitutively throughout the cell cycle, and are not subjected to auto-regulation (Ikeda et al., 1996; Moberg et al., 1996). The activity of this subgroup is instead regulated by localization. During G0/G1, E2F4 and E2F5 are found in the nucleus in complexes with the pocket protein family (Lindeman et al., 1997; Moberg et al., 1996; Muller et al., 1997; Takahashi et al., 2000; Verona et al., 1997). Unlike the activating E2Fs, the repressive E2Fs lack a nuclear localization signal and are brought into the nucleus through an undetermined mechanism. During the G1/S transition, the repressive E2Fs are shuttled into the cytoplasm and are predominantly cytoplasmic during S phase (Gaubatz et al., 2001; Lindeman et al., 1997; Muller et al., 1997; Verona et al., 1997). The members of this subgroup have been found to contain multiple nuclear export sequences and are exported from the nucleus in a manner dependent on the CRM1 nuclear export receptor (Gaubatz et al., 2001). The localization data has been supported by ChIP studies that have revealed that during G0 and G1, E2F4 and E2F5 are found at E2F responsive promoters in association with the pocket protein family (Takahashi et al., 2000; Wells et al., 2000). As the cell enters the cell cycle, E2F4 and E2F5 are removed from E2F responsive promoters and are replaced by the activating E2Fs (Takahashi et al., 2000; Wells et al., 2000).

Mouse embryonic fibroblasts (MEFs) deficient for either E2F4 or E2F5 have been generated. Neither E2f4−/− nor E2f5−/− MEFs appear to have any defects in cell cycle or E2F responsive gene regulation (Humbert et al., 2000a; Lindeman et al., 1998; Rempel et
al., 2000). Compound mutant MEFs were being generated at the beginning of this study. The generation and analysis of these cells will be discussed in detail in this study.

I. E2F and pocket protein mutant mouse models

Further insight into the function of both the pocket proteins and E2Fs in vivo has come from the generation of mice carrying targeted deletions for one or more members of the E2F or pocket protein families. This section will describe the phenotypes of these mice and their implications for the roles of these families in vivo.

(i) \( Rb^- \) mice

Mice deficient for pRB die in utero between 13 to 15 days post coitum (dpc) (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). \( Rb^- \) embryos also display defects in hematopoiesis and exhibit high levels of cells inappropriately cycling and undergoing programmed cell death (apoptosis) in the central and peripheral nervous systems, lens, and liver (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Consistent with its proposed role as a tumor suppressor, mice heterozygous for \( Rb \) develop both pituitary and thyroid tumors (Hu et al., 1994; Jacks et al., 1992; Williams et al., 1994).

(ii) \( p107^- \) and \( p130^- \) mice

Unlike \( Rb^- \), mice deficient for either p107 or p130 are completely viable in a mixed 129/Sv:C57BL/6J background and do not develop tumors (Cobrinik et al., 1996; Lee et al., 1996). Mice deficient for both p107 and p130 die shortly after birth indicating
that these two pocket proteins have overlapping roles in development. *p107<sup>−/−</sup>;p130<sup>−/−</sup>* mice have defects in endochondral ossification, the formation of long bones. The chondrocytes of the cartilage continue to proliferate instead of undergoing a program of cell cycle arrest, hypertrophy, and ossification (Cobrinik et al., 1996; Rossi et al., 2002). As a result of this defect, the long bones, including the ribs of *p107<sup>−/−</sup>;p130<sup>−/−</sup>* mice fail to form appropriately resulting in breathing difficulties and suffocation.

The *p107* and *p130* deficient mice were originally generated in a mixed 129/Sv:C57BL/6J background. Subsequent studies using *p107<sup>−/−</sup>* and *p130<sup>−/−</sup>* mice generated in a 129/Sv:Balb/cJ background revealed a phenotypic shift (LeCouter et al., 1998a; LeCouter et al., 1998b). *p107<sup>−/−</sup>* mice in the new genetic background are runted and developed myeloid hyperplasia (LeCouter et al., 1998a). *p130<sup>−/−</sup>* mice generated in this background died *in utero* between 11-13 dpc. These embryos suffer from defects in heart development and exhibit inappropriate cell cycle entry and apoptosis in the brain and neural tube (LeCouter et al., 1998b). These novel phenotypes of both *p107<sup>−/−</sup>* and *p130<sup>−/−</sup>* mice are due to strain specific modifiers and can be rescued by subsequent crosses into a C57BL/6J background.

(iii) MEFs deficient for the pocket protein family

MEFs deficient for members of the pocket protein family have defects in cell cycle control. MEFs that lack either *p107* or *p130* do not have a cell cycle phenotype (Cobrinik et al., 1996). Both *Rb<sup>−/−</sup>* and *p107<sup>−/−</sup>;p130<sup>−/−</sup>* MEFs exhibit a decrease in the time required to transverse from G<sub>1</sub> to S phase (Herrera et al., 1996; Hurford et al., 1997; Mulligan et al., 1998). *Rb<sup>−/−</sup>* MEFs exhibit deregulation of only two E2F responsive
genes: *p107* and *cyclin E* (Herrera et al., 1996; Hurford et al., 1997). Cells deficient for both *p107* and *p130* display deregulation of a wide spectrum of E2F responsive genes including *B-myb, cdc2, E2F-1, thymidylate synthase, ribosomal reductase subunit M2, cyclin A2,* and *dihydrofolate reductase* (Hurford et al., 1997; Mulligan et al., 1998). The cell cycle and gene expression phenotype was more severe in *p107<sup>-/-</sup>;*p130<sup>-/-</sup> MEFs than in the *Rb<sup>−/−</sup>* MEFs, suggesting an overlap between all three of these proteins in regulating cellular proliferation. Consistent with this, *Rb<sup>−/−</sup>;*p107<sup>−/−</sup>;*p130<sup>−/−</sup> MEFs never undergo senescence and fail to respond to any form of *G<sub>1</sub>* arrest (Dannenberg et al., 2000; Sage et al., 2000).

(iv) Mice deficient for the activating E2Fs

Mice carrying targeted mutations in the activating E2Fs have been generated. *E2f1<sup>−/−</sup>* mice are viable at birth, but develop testicular atrophy and a spectrum of late onset tumors revealing a role for E2F1 as a tumor suppressor (Yamasaki et al., 1996). *E2f2<sup>−/−</sup>* mice are predisposed to developing T-cell lymphomas and myeloid leukemias (Zhu et al., 2001). *E2f3<sup>−/−</sup>* mice exhibit embryonic and neonatal lethality in a strain specific manner. More specifically, in a pure 129/Sv background *E2f3<sup>−/−</sup>* mice die *in utero* in mid to late gestation (between 13.5 dpc and birth). *E2f3<sup>−/−</sup>* mice in a pure 129/Sv background are not viable at birth, but mutants in a mixed 129/Sv:C57BL/6J background are born alive at 25% of the expected frequency (Cloud et al., 2002). This suggests that like *p130* and *p107* (LeCouter et al., 1998a; LeCouter et al., 1998b), there are strain specific modifiers of the activity or expression levels of E2F3. The surviving adults show no tumor phenotype but die prematurely from congestive heart failure (Cloud et al., 2002).
Generation of compound mutants (E2f1<sup>−/−</sup>;E2f3<sup>−/−</sup>, E2f1<sup>−/−</sup>;E2f2<sup>−/−</sup>, or E2f2<sup>−/−</sup>;E2f3<sup>−/−</sup>) reveal overlapping roles for these proteins in regulating cell cycle control and development. E2f1<sup>−/−</sup>;E2f3<sup>−/−</sup> and E2f2<sup>−/−</sup>;E2f3<sup>−/−</sup> mice have an earlier window of embryonic lethality, dying *in utero* between 9.5-10.5 dpc (Wu et al., 2001). Heterozygous adults also display an increase in onset and severity of both E2f1<sup>−/−</sup> and E2f3<sup>−/−</sup> associated phenotypes with varying degrees of penetrance (Cloud et al., 2002). E2f1<sup>−/−</sup>;E2f2<sup>−/−</sup> mice are viable at birth but develop autoimmunity and show an increase in severity of the hematopoietic cancers found in E2f2<sup>−/−</sup> mice and the tumor spectrum seen in E2f1<sup>−/−</sup> mice (Zhu et al., 2001).

(v) *Rb<sup>−/−</sup>* compound mutants

*Rb<sup>−/−</sup>;p53<sup>−/−</sup>* embryos exhibit a reduction in apoptosis (but not proliferation) in the CNS and lens but still display apoptosis in the PNS, which indicates that apoptosis in this tissue occurs independently of p53 activation (Macleod et al., 1995; Morgenbesser et al., 1994). Elimination of either E2F1 or E2F3 in an *Rb<sup>−/−</sup>* background rescues the inappropriate proliferation observed in the lens, CNS, and (in the case of E2F3) PNS indicating that the activity of these E2Fs contributes to the cell cycle arrest defect that is observed upon loss of pRB (Tsai et al., 1998; Ziebold et al., 2001).

*Rb<sup>−/−</sup>;E2f1<sup>−/−</sup>* mice live about three months longer than *Rb<sup>−/−</sup>* animals. This is thought to be due to a reduction in the severity and incidence of both the pituitary and thyroid tumors observed in these animals (Yamasaki et al., 1998). This supports the idea that tumors arising from the loss of *Rb* are in part due to the deregulation of the activating E2Fs. *Rb<sup>−/−</sup>;E2f3<sup>−/−</sup>* mice have also been generated. These animals exhibit a reduction in
the appearance of pituitary tumors but display an increase in severity of tumors derived from the thyroid (Ziebold et al., submitted). These animals ultimately die in the same time frame as Rb<sup>+/−</sup> mice due to metastasis from the thyroid tumors (Ziebold et al., submitted). The role of E2F3 in mediating this phenotype is still under investigation.

(vi) E2f4<sup>−/−</sup> and E2f5<sup>−/−</sup> mice

At the time this study began, mouse models deficient for the members of the repressive E2F subgroup had not been generated. This study and others have shown that mice deficient for members of this subgroup do not display defects in cell cycle regulation, but instead exhibit tissue specific abnormalities (Appendix A and Lindeman et al., 1998; Rempel et al., 2000). E2f4<sup>−/−</sup> mice display defects in end stage hematopoiesis, leading to fetal anemia and craniofacial abnormalities (Appendix A and Rempel et al., 2000). Loss of E2F5 results in neonatal lethality due to hydrocephaly that results from the accumulation of cerebral spinal fluid (Lindeman et al., 1998).

The mutant mice studies demonstrate that E2F-pocket protein repressive complexes regulate cell cycle progression and are also required for appropriate differentiation and development. Part II will describe the evidence to date for pocket proteins (in particular pRB) and E2Fs in regulating differentiation and development.

**Part II. Regulation of Differentiation By the Retinoblastoma pathway**

As discussed in Part I, the retinoblastoma tumor suppressor protein (pRB) and its family members p107 and p130 are key regulators of the G1/S transition. The retinoblastoma regulatory pathway has also been shown to be critical for normal
differentiation and development of a number of tissues. The pRB pathway regulates these processes through two distinct mechanisms. The first is to induce terminal cell cycle withdrawal that is mediated through the cell cycle repressive effect of the pocket protein family. Repression mediated by the pocket protein family is critical for differentiation and development, as cells deficient for all three pRB family members are severely impaired in their ability to undergo terminal differentiation (Dannenberg et al., 2000; Sage et al., 2000). The second mechanism is direct participation of pRB in the differentiation process by functioning as a coactivator for tissue specific transcription factors. This section will first examine the evidence from analysis of mutant mouse models and in vitro tissue culture studies for the role of pRB in the differentiation process. Next, I will discuss the involvement of p107 and p130 in regulating differentiation. Third, I will discuss the evidence for the involvement of E2F in regulating differentiation and development. Finally, I will close with a discussion of the mechanisms governing the formation of white adipose tissue, a major focus of this study. The discussion will concentrate on the interplay between proteins essential to adipogenesis and cell cycle regulatory proteins.

A. Regulation of differentiation by pRB

As stated above, pRB has been shown to play a role in regulating a number of differentiation processes including: erythropoiesis, neurogenesis, osteogenesis, myogenesis, and adipogenesis. This section will focus on describing the data supporting the role for pRB in each of these processes.
(i) Mouse models show a role for pRB in erythropoiesis and neurogenesis

As previously discussed in Part I, Rb⁻/⁻ embryos display anemia due to defects in fetal liver hematopoiesis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). This defect is characterized by the presence of an abnormal number of immature nucleated erythrocytes. The severe reduction in the number of mature enucleated erythrocytes suggests a role for pRB in the regulation of erythropoiesis. Compound mutant mice lacking either E2f1 or E2f3 in an Rb⁻/⁻ background survive longer than Rb⁻/⁻ embryos due to a reduction in abnormal cell cycle entry and apoptosis (Tsai et al., 1998; Ziebold et al., 2001). Despite the extension in life span, both Rb⁻/⁻;E2f1⁻/⁻ and Rb⁻/⁻;E2f3⁻/⁻ embryos exhibit the Rb⁻/⁻ hematopoietic phenotype (Tsai et al., 1998; Ziebold et al., 2001). This serves as evidence for a direct role for pRB in regulating hematopoiesis in a manner distinct from its role in regulating cell cycle progression. Subsequent studies examined hematopoiesis in chimeras generated using Rb⁻/⁻ embryonic stem cells. These investigations found that in chimeric animals erythrocytes deficient for pRB developed normally (Maandag et al., 1994; Williams et al., 1994). It therefore is thought that this erythocyte phenotype arises due to an as yet undefined non-cell autonomous role for pRB.

Rb⁻/⁻ embryos exhibit high levels of apoptosis in both the central and peripheral nervous systems that can be rescued by subsequent loss of E2f1 or E2f3 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Tsai et al., 1998; Ziebold et al., 2001). This suggests that cell cycle repression mediated by pRB is required for the appropriate differentiation and development of neurons. The Rb mutation was crossed to mice carrying a β-galactosidase (β-gal) transgene driven off of the Tα1 α-tubulin promoter, a
gene whose expression is initiated as neurons terminally exit the cell cycle. These experiments demonstrate that Rb\(^{\text{\textsuperscript{\text{-}}}f}\) embryos initiate expression of \(\beta\)-gal at the appropriate stage in gestation, but the levels of \(\beta\)-gal positive neurons decrease as development progresses (Slack et al., 1998). The conclusion from this experiment was that pRB is dispensable for cell cycle withdrawal but is required for maintaining cell cycle arrest in the differentiated state. The requirement for pRB in regulating neurogenesis has also been investigated in Rb\(^{\text{\textsuperscript{\text{-}}}f}\) chimeras. Rb\(^{\text{\textsuperscript{\text{-}}}f}\) cells can differentiate into mature neurons in chimeric animals (Lipinski et al., 2001). Neurons deficient for pRB arrest after the accumulation of a 4n DNA content but do not undergo programmed cell death. This shows that pRB is required for maintaining cell cycle arrest in a cell-autonomous manner and inhibiting apoptosis in a non-cell autonomous fashion (Lipinski et al., 2001). The exact mechanism underlying the role of pRB in both hematopoiesis and neurogenesis is currently under investigation.

(ii) pRB regulates myogenesis through multiple mechanisms

Myogenesis is mediated by the coordinated action of a family of myogenic regulatory factors (MRFs). The four MRFs (MyoD, Myf-5, myogenin, and MRF4) are basic helix-loop-helix (bHLH) transcription factors that activate transcription through dimerization with E-proteins (as reviewed in Sabourin and Rudnicki, 2000). In response to the appropriate stimuli (such as the reduction of mitogens in culture) uncommitted cells induce the expression of Myf-5 and MyoD and become proliferating myoblasts. The combined action of Myf-5 and MyoD induce the expression of myogenin, MRF4, and an additional muscle specific transcription factor, MEF2 (as reviewed in Yun and Wold,
1996). The action of muscle-specific transcription factors induces the expression of muscle-specific genes such as *myosin heavy chain* (*MHC*) and *muscle creatine kinase* (*MCK*). The myoblasts then exit the cell cycle and fuse into multinucleate myotubes (Walsh and Perlman, 1997).

Analysis of mice deficient for pRB reveal a requirement for this pocket protein family member in myogenesis. As described in Part I and above, both *Rb<sup>-/-</sup>*;*E2f1<sup>-/-</sup>* and *Rb<sup>-/-</sup>*;*E2f3<sup>-/-</sup>* embryos survive to a later point in gestation than *Rb<sup>-/-</sup>* embryos. Analysis of *Rb*;*E2f* compound mutant embryos reveal defects in the development of skeletal muscle (Tsai et al., 1998; Ziebold et al., 2001). The *Rb* mutation was crossed with mice expressing low levels of *RB* from a transgene. *Rb<sup>-/-</sup>* in this background exhibit defective skeletal muscle with a large number of apoptotic cells and polyploid nuclei, suggesting a role for pRB in achieving and maintaining the differentiated state (Zacksenhaus et al., 1996). The role of pRB in myogenesis has been analyzed in *in vitro* tissue culture assays. These studies, described below, reveal both direct and indirect roles for pRB in regulating muscle differentiation.

**a) pRB is required to mediate terminal cell cycle withdrawal in myogenesis**

There are three mechanisms through which the pRB is thought to promote terminal cell cycle withdrawal during myogenesis. The first is through repression of E2F target gene expression and S-phase progression. *Rb<sup>-/-</sup>* mouse embryonic fibroblasts (MEFs) show defects in differentiating into mature myocytes but can undergo myogenesis in response to overexpression of MyoD (Novitch et al., 1996; Schneider et al., 1994). The ability of *Rb<sup>-/-</sup>* to successfully differentiate in response to MyoD
expression is attributed to compensation by p107. When Rb<sup>−</sup> myotubes are placed into high serum conditions, however, they inappropriately enter the cell cycle indicating a requirement for pRB in maintaining terminal cell cycle withdrawal and the differentiated state (Novitch et al., 1996; Schneider et al., 1994).

The second mechanism through which pRB mediates cell cycle withdrawal during myogenesis, is by interacting with the transcriptional repressor, HBPI. pRB can interact with the LXCXE domains of this member of the HMG family of transcription factors (Lavender et al., 1997; Tevosian et al., 1997). The expression of HBPI increases during myogenesis and the interaction between HBPI and pRB is thought to block the expression of E2F responsive genes (Tevosian et al., 1997). HBPI, when associated with pRB, can also inhibit the activity of Myf5 and prevent the induction of MyoD and myogenin (Shih et al., 1998). The interaction between HBPI and pRB is thought to regulate the transition between primary and differentiated cells. When levels of HBPI•pRB complexes are low, cells withdraw from the cell cycle but do not differentiate. High levels of HBPI•pRB complexes, however, promote differentiation (Lipinski and Jacks, 1999; Shih et al., 1998).

The third mechanism through which pRB regulates myogenesis by promoting cell cycle withdrawal is through interactions with histone deacetylases (HDACs). The transcriptional activity of MyoD has been shown to be inhibited through interaction with Class I HDACs (Mal et al., 2001). The interaction between hypo-phosphorylated pRB and the HDACs correlates with the timing of the dissociation of MyoD-HDAC complexes (Puri et al., 2001). Arrested cells have high levels of hypo-phosphorylated pRB that can interact with the MyoD-HDAC complexes and titrate away the HDAC.
pRB-HDAC repressive complexes form and release MyoD that activates muscle-specific gene expression. HBP and HDAC have not been placed into the same complex with pRB to date. A model reconciling how these two mechanisms are coordinated has yet to be determined.

b) pRB plays a direct role in myogenesis

There is substantial evidence that pRB interacts directly with muscle-specific transcription factors and promotes differentiation in a manner independent of its ability to interact with E2F (Sellers et al., 1998). MyoD has been shown to interact with the pocket domain of pRB (Gu et al., 1993). This interaction is required for stimulation of the transactivational activity of another differentiation specific transcription factor, MEF2 (Novitch et al., 1999). Consistent with this finding, Rb\(^{-}\) fibroblasts induced to differentiate into muscle have normal expression of early myogenic genes but fail to induce the expression of later myogenic markers such as MHC and MCK (Novitch et al., 1996). The activity of pRB, therefore, is required for maintaining terminal exit from the cell cycle, as well as interacting with MyoD to enhance the activity of MEF2.

(iii) pRB promotes osteogenesis and adipogenesis through interacting with differentiation specific transcription factors

*In vitro* differentiation assays using MEFs deficient for pRB has defined a role for this pocket protein family member by directly regulating osteogenesis and adipogenesis. When treated with bone morphogenic protein Rb\(^{-}\) MEFs fail to form osteoblasts. This has been shown to be due to a requirement for pRB to function as a coactivator for
CBFA1, a transcription factor necessary for the induction of late osteogenic genes such as *osteopontin* and *osteocalcin* (Thomas et al., 2001).

*Rb<sup>−/−</sup>* MEFs also fail to differentiate into adipocytes in response to treatment with hormones. This failure to form adipocytes is caused by a requirement for pRB to serve as a coactivator for a C/EBPβ, a member of the CCAAT/enhancer binding protein family (Charles et al., 2001; Chen et al., 1996a; Chen et al., 1996b). The requirement for pRB in adipogenesis will be discussed in greater detail in later sections.

**B. The role of p107 and p130 in osteogenesis**

Mice deficient for p107 and p130 display defects in long bone development (Cobrinik et al., 1996). The bones of the skeleton develop through two distinct mechanisms: intramembranous ossification and endochondral ossification. Intramembranous ossification (the process through which the flat bones of the skeleton form) occurs when the mesenchymal stem cells differentiate directly into osteoblasts without the need for a cartilage precursor (as reviewed in Erlebacher et al., 1995). The long bones of the skeleton arise through endochondral ossification, the multi-step process in which the chondrocytes that compose the cartilage template cease to proliferate and undergo hypertrophic differentiation. The steps of this differentiation process include hypertrophy, calcification, and vascularization. The end result is the recruitment of osteoblasts that replace the cartilage template with bone matrix and osteoclasts that modify bone structure (Erlebacher et al., 1995).

In the long bones of mice, cartilage first arises from the mesenchyme between 10.5 and 12.5 days post coitum (dpc). The chondrocytes form a growth plate where they
begin to arrest and undergo hypertrophic differentiation between 14.5 and 15.5 dpc (as reviewed in Karsenty, 1999). A key regulator of endochondral ossification is the transcription factor CBFA1, which is induced as cartilage first differentiates from the mesenchyme and is required for hypertrophic differentiation (as reviewed in Karsenty, 1999).

Chondrocytes from p107<sup>-/-</sup>;p130<sup>-/-</sup> embryos cannot arrest appropriately and display a reduction in expression of Cbfa1 and its responsive target genes (Rossi et al., 2002). This finding suggests that p107 and p130 mediated repression is required for induction of Cbfa1 and the onset of hypertrophic differentiation.

p107 and p130 are also required to regulate the differentiation of osteoblasts. Studies using MEFs deficient for members of the pocket protein family have further defined their roles in osteogenic differentiation. MEFs deficient for p107 and p130 display an enhanced ability to differentiate into osteoblasts in response to treatment with bone morphogenic protein (BMP2), suggesting that these two pocket protein family members function to inhibit osteogenesis (Thomas et al., 2001). The exact mechanism for p107 and p130 in this process, and whether or not it is independent of their roles in cell cycle regulation has yet to be determined.

C. Regulation of differentiation by E2F

To date, there is little published evidence that E2F regulates differentiation in a manner distinct from its role in regulating the cell cycle. Much of the evidence for E2F in directly regulating differentiation and development has come from the study of mutant mice. Mice deficient for E2F1 and E2F2 have no distinct developmental defects
(Yamasaki et al., 1996; Zhu et al., 2001). Both E2f1−/− and E2f2−/− mice are predisposed to tumors, but this is thought to be attributed to their roles as cell cycle regulators. A study investigating the role of E2F2 in neuronal differentiation has implicated this E2F family member in maintaining terminally differentiated neurons in G0 (Persengiev et al., 2001). The mechanism for E2F2’s role in this process is still unknown. E2f3−/− mice develop dilated cardiac myopathy, but this primary heart defect appears to arise from a reduction in the proliferation of fetal cardiac myocytes as opposed to a direct defect in differentiation (J.E. Cloud, personal communication and Cloud et al., 2002). The loss of E2F5 leads to hydrocephaly due to an accumulation of excess cerebral spinal fluid (Lindeman et al., 1998). The role of E2F5 in preventing hydrocephaly has yet to be determined.

E2F4 is the major E2F species present in terminally differentiated cells (Moberg et al., 1996; Puri et al., 1997; Richon et al., 1997; Smith et al., 1996; Vairo et al., 1995). Differentiation and development of most tissues is unaffected in E2F4 deficient mice. E2f4−/− mice have a cell autonomous defect in fetal hematopoiesis leading to macrocytic anemia (Appendix A and Rempel et al., 2000). The exact role for E2F4 in regulating hematopoiesis is still under investigation. Though E2f4−/− mice have not been shown to have defects in neurogenesis, a study investigating the role of E2F4 in neuronal differentiation has found that overexpression of this E2F family member can promote and accelerate neuronal differentiation of cells in culture (Persengiev et al., 1999). The authors of this study speculate that E2F4 could directly interact with factors responsible for regulating neuronal differentiation. A subsequent study identified an interaction between E2F4 and a retina specific transcription factor, PATF (Crisanti et al., 2001).
This interaction is thought to promote cell cycle withdrawal and differentiation in the retina through the repression of E2F responsive promoters. These studies have suggested that E2F4 containing repressive complexes could be critical in regulating cell cycle withdrawal and differentiation. Future studies are required to determine the mechanisms through which E2F4 regulates differentiation.

D. The role of the pRB pathway in regulating adipogenesis

Adipocytes, like chondrocytes, osteoblasts, and myocytes, originate from mesenchymal precursor cells during gestation (as reviewed in Gregoire et al., 1998). There are two major types of adipose tissue, brown adipose tissue (BAT) and white adipose tissue (WAT). BAT regulates body temperature and protects against obesity (as reviewed in Rangwala and Lazar, 2000; Rosen and Spiegelman, 2000). WAT is involved in storing energy in the form of lipid filled vacuoles. While BAT forms during gestation, WAT does not visibly accumulate until after birth (Rangwala and Lazar, 2000). Studies of adipose tissue development have come in part from the culturing of cell lines that can be induced hormonally to differentiate into adipocytes.

Confluent cells can be induced with the hormone insulin, the glucocorticoid receptor agonist dexamethasone, and the cAMP phosphodiesterase inhibitor methylisobutylxanthine (Rosen and Spiegelman, 2000). Upon induction two members of the CCAAT/enhancer binding protein family C/EBPβ and C/EBPδ, become expressed. The C/EBPs are a family of basic-leucine zipper transcription factors that have been demonstrated by mutant mouse models to be essential in mediating adipogenesis (as reviewed in Rosen and Spiegelman, 2000). Through mechanisms that have yet to be
determined, the cells undergo one to two rounds of mitotic clonal expansion. At the end
of the clonal expansion phase, three more transcription factors, C/EBPα, ADD1/SREBP1, and peroxisome proliferator-activated receptor γ (PPARγ) are induced. Cells terminally arrest and begin to express adipose specific genes and accumulate lipids (Rosen and Spiegelman, 2000). The expression of C/EBPα and PPARγ is induced initially by C/EBPβ and C/EBPδ (Darlington et al., 1998; Rosen and Spiegelman, 2000). The mechanisms responsible for inducing ADD/SREBP1 have yet to be determined, but once induced, this transcription factor binds to and activates transcription from the PPARγ promoter (Rosen and Spiegelman, 2000). It has been shown that C/EBPα is required for both PPARγ induction and for enhanced insulin sensitivity. PPARγ can induce the expression of C/EBPα, but recently it has been shown that its predominant role is to control the expression of adipose tissue specific genes (Rosen et al., 2002).

The mechanisms governing the transition between the stages of adipogenesis are still poorly understood. There has been evidence to suggest that cell cycle regulation by the pRB pathway is critical to adipogenesis. Studies using viral oncoproteins such as SV40 large TAg have shown that sequestration of the pocket proteins decreases the ability of cells to differentiate into WAT (Higgins et al., 1996). The E2F and pocket protein family members at E2F responsive promoters change as cells progress through adipogenesis. Growth arrested preadipocytes have high levels of E2F4•p130 complexes at the promoters of E2F responsive genes. As cells progress into the clonal expansion phase, the pocket protein family becomes phosphorylated and is replaced at E2F responsive promoters by the activating E2Fs (Richon et al., 1997).
The activating E2Fs are necessary for progression through clonal expansion, but the activity of this subgroup must be repressed for terminal differentiation and lipid accumulation to occur. A number of strategies are employed to induce adipocytes to withdraw from the cell cycle by inhibiting E2F. Induction of PPARγ at the end of the clonal expansion phase results in cell cycle repression through up-regulation of cyclin dependent kinase inhibitors p21, p27, and p18 (Morrison and Farmer, 1999). PPARγ has also been shown to inhibit E2F DNA binding activity by downregulating the serine-threonine phosphatase, PP2A (Altior et al., 1997). PP2A removes phosphates from Dp and its reduction inhibits the ability of E2F to bind to DNA. C/EBPα induction has been shown to repress cell cycle entry indirectly through upregulation of p21 (Timchenko et al., 1996). C/EBPα has also been show to directly interact and inhibit the activity of both E2F and cyclin dependent kinases (Porse et al., 2001; Slomiany et al., 2000; Wang et al., 2001).

Studies using cells derived from Rb−/− embryos have demonstrated a requirement for this pocket protein family member in adipogenesis. Rb−/− embryonic lung-bud fibroblasts (ELFs) failed to significantly form WAT when subjected to hormone induction (Chen et al., 1996b). The reduced ability to differentiate into WAT observed in Rb−/− ELFs is due to a requirement for pRB to function as a coactivator for C/EBPβ (Chen et al., 1996a; Chen et al., 1996b). Both C/EBPα and C/EBPβ have also been found to require interaction with pRB to achieve maximal transcriptional activity (Charles et al., 2001). The C/EBP family interacts with pRB in the pocket domain independent of its interaction with the E2F transcription factor (Charles et al., 2001; Chen et al., 1996a; Chen et al., 1996b).
The preponderance of the studies investigating pocket proteins in regulating differentiation and development suggest that pRB is the only member of this family that can directly participate in differentiation. Only a few studies (as discussed above) have shown that members of the E2F family can directly regulate differentiation in a manner independent of their ability to control cell cycle progression. The focus of this study is to investigate the role of E2F•pocket protein repressive complexes in regulating cell cycle control, differentiation, and development. In particular I was interested if members of the E2F family, p107, or p130 could regulate differentiation in a manner that is distinct from their ability to control the cell cycle. In this study I used MEFs deficient for E2Fs and pocket proteins in white adipose tissue in vitro differentiation assays. I demonstrated that E2F4 functions to inhibit adipogenesis in a manner that is distinct from its ability to regulate the cell cycle and interact with the pocket protein family (Chapter 2). I also examined the effects in vivo when E2F4•pocket protein repressive complexes are eliminated. I have found that loss of E2F4, p107, and p130 has a profound effect on fetal hematopoiesis (Chapter 3). Taken together these results show that E2F4•pocket protein repressive complexes are critical for appropriate differentiation and development.
REFERENCES


Hu, Q. J., Dyson, N., and Harlow, E. (1990). The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. Embo J 9, 1147-1155.


E2F4 is essential for normal erythrocyte maturation and neonatal viability. Mol Cell 6, 281-291.


Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S.,
growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell
85, 721-732.

factor involved in E1A trans-activation. Cell 45, 219-228.

Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G., Jr., and Livingston, D.
M. (1994). Negative regulation of the growth-promoting transcription factor E2F-1 by a

retinoblastoma gene product promoted by complex formation of different E2F family
members. Science 262, 1557-1560.

DNA binding function underlies suppression of an S phase checkpoint. Cell 83, 1149-
1158.

p16Ink4a confers susceptibility to metastatic melanoma in mice. Nature 413, 83-86.

is regulated during the differentiation of murine embryonal carcinoma stem cells. Cell 49,
507-513.


CHAPTER TWO

The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity.

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Author’s contribution: Figures 1A, 1B, 1C, 2, 3A, 3C, 3D, and 3E


ABSTRACT

The E2F and pocket protein families are known to play an important role in the regulation of both cellular proliferation and terminal differentiation. In this study, we have used compound E2F and pocket protein mutant mouse embryonic fibroblasts (MEFs) to dissect the role of these proteins in adipogenesis. This analysis shows that loss of E2F4 allows cells to undergo spontaneous differentiation. The ability of E2F4 to prevent adipogenesis appears to be quite distinct from the known properties of E2F. First, it can be separated from any change in either E2F-responsive gene expression or cell cycle regulation. Second, it is a specific property of E2F4, and not other E2Fs, and it occurs independently of E2F4’s ability to interact with pocket proteins. In addition, E2F4-loss does not over-ride the differentiation defect resulting from pRB-loss even though it completely suppresses the proliferation defect of $Rb^{-/-}$ MEFs. This finding definitively separates the known, positive role of pRB in adipogenesis from its cell cycle function and shows that this pocket protein is required to act downstream of E2F4 in the differentiation process.
INTRODUCTION

The E2F transcription factors play a key role in regulating the cell cycle by controlling the transcription of genes encoding key components of the cell cycle and DNA replication machinery (reviewed by Dyson, 1998; Trimarchi and Lees, 2002). The activity of E2F is controlled by the pocket protein family that includes the retinoblastoma protein (pRB), a known tumor suppressor, and its relatives p107 and p130. The pocket proteins bind to E2F and inhibit the activation of E2F-responsive genes through two mechanisms (reviewed by Dyson, 1998; Trimarchi and Lees, 2002). First, their binding interferes with the function of the E2F transactivation domain. Second, the resultant E2F•pocket protein complexes can actively repress transcription through recruitment of histone deacetylase (HDAC) and methylase activities. Consequently, the pocket proteins are key regulators of the G1 to S transition.

The E2F proteins can be divided into three subgroups based upon structural and functional homology (reviewed by Dyson, 1998; Trimarchi and Lees, 2002). E2F6 is the sole member of one subgroup. It does not interact with the pocket proteins and has been shown to play a role in vertebrate patterning through interaction with the mammalian polycomb complex (Storre et al., 2002; Trimarchi et al., 2001). The second E2F subgroup includes E2F1, E2F2, and E2F3a. These E2Fs are specifically regulated by pRB, and not p107 and p130, in vivo. Combined analysis of both over-expressing and deficient cells indicates that they play a key role in activating E2F-responsive genes and thereby promoting S-phase progression (Helin, 1998; Humbert et al., 2000b; Wu et al., 2001). E2F3b, a recently identified second product of the E2F3 gene, is most closely related to E2F1, 2 and 3a but its biological properties remain to be established (Adams et al., 2000; He et al., 2000; Leone et al., 2000).
The final E2F subgroup, comprising E2F4 and E2F5, is the focus of this study. E2F5 binds specifically to p130 in vivo (reviewed by Dyson, 1998; Trimarchi and Lees, 2002). In contrast, E2F4 associates with pRB, p107 and p130 and comprises the majority of the E2F•pocket protein complexes in vivo (Moberg et al., 1996). These E2Fs are thought to play a key role in the repression of responsive genes through the recruitment of pocket proteins and their associated HDACs. Chromatin immunoprecipitation (ChIP) assays show that the E2F4/5•p107/p130 complexes specifically associate with E2F-responsive promoters during G0/G1 when these genes are repressed and are then replaced by the activating E2Fs, E2F1, 2 and 3, as cells re-enter the cell cycle (Takahashi et al., 2000). Importantly, cells that lack either E2F4 and E2F5 or p107 and p130 have a defect in their ability to exit the cell cycle in response to a variety of growth arrest signals (Bruce et al., 2000; Gaubatz et al., 2000; Hurford et al., 1997). In the case of p107−/−;p130−/− cells, this correlates with a failure to mediate the normal repression of certain E2F-responsive genes (Hurford et al., 1997; Mulligan et al., 1998). The repressive E2F complexes are also required for normal development. Mice lacking E2F4 or E2F5 display distinct developmental defects in one or a few tissues that in each case result in neonatal lethality (Humbert et al., 2000a; Lindeman et al., 1998; Rempel et al., 2000). It is currently unclear whether the developmental defects reflect a requirement for E2F4 and/or E2F5 in cell cycle control or whether these proteins play a more direct role in the differentiation process. In contrast, there is considerable evidence that the essential requirement for p107 and p130 in long bone development is due to their role in mediating cell cycle exit (Cobrinik et al., 1996; Rossi et al., 2002).

pRB is also an important developmental regulator (as reviewed by Lipinski and Jacks, 1999; Trimarchi and Lees, 2002). pRB-deficient embryos die in mid-gestation because of a combination of inappropriate proliferation and apoptosis in a wide variety of tissues. These
defects are suppressed by the loss of either E2F1 or E2F3 indicating that they result from the inappropriate release of the activating E2Fs. However, the analysis of Rb;E2f1 and Rb;E2f3 double mutant embryos reveals a key role for pRB in development that is unrelated to the regulation of E2F. In vitro differentiation assays support a role for pRB in myogenesis, osteogenesis and adipogenesis and pRB can function as a coactivator for transcription factors that promote these differentiation processes (as reviewed by Lipinski and Jacks, 1999).

In this study, we investigated the role of the repressive E2F complexes in terminal differentiation. We have focused on adipogenesis because this pathway is well defined and it involves both a proliferation and a differentiation phase. Specifically, confluence arrested MEFs can be triggered to undergo differentiation by treatment with the hormone insulin, the glucocorticoid receptor agonist dexamethasone and, the cAMP phosphodiesterase inhibitor methyl-isobutylxanthine (Rosen and Spiegelman, 2000). Following treatment the cells express C/EBPβ and C/EBPδ and then undergo one to two rounds of mitotic clonal expansion before arresting again and inducing expression of the C/EBPα and PPARγ transcription factors that are required for lipid accumulation and adipocyte induction. The E2F and pocket protein complexes have already been linked to adipogenesis at several different levels. First, hormonal induction correlates with the dissociation of the repressive E2F4•p130 complex and the expression of the activating E2Fs (Fajas et al., 2002b; Richon et al., 1997). This induces clonal expansion and the simultaneous transcriptional activation of PPARγ, an E2F-responsive gene (Fajas et al., 2002b). Consistent with this scheme, p107\textsuperscript{ΔC}•p130\textsuperscript{ΔC} MEFs have an increased propensity to undergo hormone-induction adipogenesis (Classon et al., 2000). Second, pRB functions as a coactivator for C/EBPβ and thereby plays a positive role in adipogenesis (Charles et al., 2001; Chen et al., 1996; Richon et al., 1997). Finally, C/EBPα and PPARγ can repress E2F activity and may
thereby contribute to the switch from the clonal expansion to the differentiation phase (Altijk et al., 1997; Porse et al., 2001; Slomiany et al., 2000).
MATERIALS AND METHODS

Cell Culture and Analysis. Mouse embryonic fibroblasts (MEFs) were generated as previously described (Humbert et al., 2000b; Lee et al., 2002). For each genotype, two to four independently derived, passage 4 MEF lines were analyzed. Confluent monolayers were fed every other day with MEF media (DMEM containing 10% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine) in either the absence (cell cycle and spontaneous differentiation assays) or presence (hormone-induced adipogenesis) of 1μM dexamethasone (Sigma), 0.5mM 3-isobutyl-1-methylxanthine (Sigma), and 5μg/mL insulin (Sigma). Lipid accumulation was assessed by quantification of Oil Red O staining (Ramirez-Zacarias et al., 1992). Cell cycle progression was determined by northern blotting (Humbert et al., 2000b) or 24hr incubation in media containing 3mg/ml BrdU and 0.3 mg/ml FdU to assess DNA synthesis.

E2F4 Expression and Immunoblotting. pBabe-E2F4ΔC was generated by digesting pCMV-E2F444 (Verona et al., 1997) with BamHI and HindIII and subcloning into the pBABE vector. 293 T cells were infected at 60% confluence with 1μg pBabe vector and 1μg of pCL-Eco packaging construct (Naviaux et al., 1996) using FuGENE 6 (Roche Diagnostics). The media was replaced 24 hours later, and supernatants were harvested at 48 and 72 hrs, filtered and used to infect MEFs with 8μg polybrene. Infected cells were selected by culturing in 2μg/mL puromycin for 48 hrs. Western blotting assays were performed using 50μg whole cell lysates and the LLF4-1 monoclonal antibody (Moberg et al., 1996).
RESULTS

It has previously been shown that the loss of E2F4 or p107/p130 promotes the ability of primary fibroblasts to undergo adipogenesis in response to hormone induction (Classon et al., 2000; Fajas et al., 2002b). We wished to determine whether this reflects the shared role of these proteins in the formation of repressive E2F complexes and whether it correlates with, or can be separated from regulation of cell cycle exit. To address these questions we intercrossed the E2f4 (Humbert et al., 2000a) and p107; p130 (Cobrinik et al., 1996) mutant mouse strains to allow the generation of compound E2f4⁺/⁻; p107⁺/⁻; p130⁺/⁻ (TKO) mutant MEFs. Figure 1a depicts how these mutations would affect the various E2F and pocket protein complexes that exist in normal cells. The properties of the TKO cells were directly compared to those of the parental p107⁺/⁻; p130⁺/⁻ (DKO) and E2f4⁺/⁻ mutant MEFs.

E2F4-deficient MEFs undergo spontaneous adipogenesis.

We first examined the ability of the TKO, DKO and E2f4⁺/⁻ MEFs to differentiate into adipocytes using the standard hormone induction conditions. After two weeks of induction, the degree of differentiation was determined by staining with Oil Red O, a lipophillic dye that can be extracted and quantified to assess for induction efficiency. As previously reported (Classon et al., 2000; Fajas et al., 2002b), the absence of either E2F4 or p107 and p130 led to a 2 to 5-fold increase in adipogenesis (Figure 1b). Strikingly, the combined loss of all three proteins yielded a significant increase in lipid accumulation (10-fold). The relative effects of the E2f4⁺/⁻, DKO and TKO genotypes were observed with numerous preparations of MEFs, indicating that it is highly reproducible. Thus, E2F4 and the pocket proteins appear to have additive, negative effects on the differentiation process. Clearly, this effect could result from their contribution to either the same or to distinct regulatory processes.
During these studies we discovered that $E2f4^{-/-}$ MEFs were predisposed to undergoing adipogenesis when they were maintained at confluence without hormone induction (Figure 1c,d). Importantly, this spontaneous differentiation was observed with every preparation of $E2f4^{-/-}$ MEFs but never with the wild-type controls MEFs (Figure 1d and data not shown). Given this finding, we also tested the ability of DKO and TKO MEFs to accumulate lipids without hormone induction. In this assay, DKO cells behaved differently from $E2f4^{-/-}$ MEFs in that they consistently failed to undergo spontaneous adipogenesis. This suggests that E2F4 and p107/p130 repress adipocyte induction through distinct mechanisms. This hypothesis is supported by the phenotype of the TKO MEFs. These cells were highly predisposed to undergo spontaneous differentiation, consistently yielding many more Oil Red O positive cells than the $E2f4^{-/-}$ MEFs (Figure 1c,d). Taken together, these data indicate that E2F4 plays a gate-keeping role to block spontaneous adipocyte differentiation while p107/p130 inhibit induced-differentiation in a distinct manner.

**E2F4’s role in adipogenesis can be separated from its role in cell cycle regulation.**

The induction of adipogenesis *in vitro* involves transitions in cell cycle regulation from confluence arrest, to mitotic clonal expansion and then terminal cell cycle exit. The $p107^{-/-};p130^{-/-}$ MEFs have a well documented defect in their ability to arrest in response to certain growth conditions (Bruce et al., 2000; Hurford et al., 1997). It has previously been suggested that this could account for, or at least contribute to, the altered differentiation properties of these cells (Classon et al., 2000). Given this finding, we wished to establish whether there is any correlation between the differentiation and cell cycle phenotypes of the various mutant genotypes.

Since the *in vitro* adipogenesis is thought to require confluence arrest (reviewed by Rosen and Spiegelman, 2000), we wished to establish the cell cycle properties of the $E2f4^{-/-}$, DKO, and
TKO MEFs under these conditions (Figure 2). First, we determined how the mutation of the E2F and pocket proteins affected the regulation of E2F-responsive genes (Figure 2a). As confluence arrest proceeded, the levels of known E2F-responsive mRNAs declined with identical kinetics in the wild-type and E2f4−/− MEFs (Fig 2a). This is entirely consistent with previous conclusions that there are no defects in the regulation of either E2F target genes or cell cycle exit and re-entry in E2f4−/− MEFs in serum-starvation/re-stimulation assays (Humbert et al., 2000a; Rempel et al., 2000). In contrast, we found that specific E2F-responsive mRNAs were maintained at high levels in the DKO MEFs even eight days after confluence was established (Figure 2a). This correlates with the known defect in the down-regulation of a subset of E2F-responsive genes in serum-starved DKO MEFs (Hurford et al., 1997). Significantly, the pattern of target gene expression in the TKO MEFs was identical to that observed in the DKO MEFs (Figure 2a). Thus, mutation of E2f4 does not alter the regulation of E2F-responsive genes in wild-type cells or modulate (either qualitatively or quantitatively) the gene expression defect that results from the loss of p107/p130.

In parallel with the gene expression studies, we also monitored the confluent monolayers for evidence of inappropriate cell cycle entry by culturing them in the presence of BrdU for 24 hours (Figure 2b). As expected, there was no evidence of proliferating cells in the wild-type controls. In contrast, we observed sporadic cells, or pairs of cells, within the E2f4−/− monolayer that were BrdU-positive. Importantly, this cell division specifically occurred in E2f4−/− cells that were subjected to confluence and not serum starvation arrest (Figure 2b, (Humbert et al., 2000a; Rempel et al., 2000). Given this specificity, and the lack of any detectable gene-expression defect, we believe that the presence of proliferating cells within the confluent E2f4−/− monolayer is indicative of cells that are undergoing the clonal expansion phase of the adipocyte differentiation process as opposed to evidence of an intrinsic cell cycle defect. This hypothesis is
supported by our finding that E2f4 mutation had no detectable effect on the magnitude of the cell cycle defect in the DKO MEFs. Even after 8 days of confluence arrest, a similar high proportion of the DKO (60%) and TKO (64%) monolayers underwent inappropriate DNA synthesis during the 24 hour labeling window (Figure 2b). The arrest defects of the DKO and TKO MEFs were also similar to one another at shorter BrdU labeling times (data not shown). We therefore conclude that the DKO and TKO MEFs display indistinguishable defects in their ability to down-regulate E2F-responsive genes and arrest in G₀/G₁ in response to confluence arrest.

Importantly, these data strongly suggest the role of E2f4 in differentiation can be separated from its role in cell cycle regulation. E2f4⁻⁄ cells have an increased propensity to undergo spontaneous adipogenesis without intrinsic defects in the regulation of E2F-responsive genes and therefore cell cycle entry/exit. Moreover, E2f4-loss greatly increases the differentiation of DKO MEFs without modulating their cell cycle phenotype.

**p130 is the predominant mediator of the p107/p130 differentiation effect.**

We wished to established whether the enhanced differentiation of TKO versus E2f4⁻⁄ MEFs is dependent upon the combined mutation of p107 and p130 or whether it can be attributed to the mutation of one of these genes. To address this question, we compared the differentiation phenotypes of E2f4⁻⁄;p107⁻⁄ and E2f4⁻⁄;p130⁻⁄ MEFs with those of E2f4⁻⁄ and TKO controls (Figure 3a and data not shown). p107-loss had no effect on the differentiation of E2f4⁻⁄ MEFs. In contrast, the E2f4⁻⁄;p130⁻⁄ MEFs displayed a wide range of phenotypes in this assay: some were equivalent to E2f4⁻⁄ MEFs while others phenocopied the TKO MEFs (Figure 3a and data not shown). This degree of variation was specifically observed with E2f4⁻⁄;p130⁻⁄ MEFs and not those derived from any other mutant genotype. Moreover, it only arose between E2f4⁻⁄;p130⁻⁄ MEF lines isolated from different embryos and never the same embryo (data not shown). It has
recently been reported that the p130 mutant allele used in this study has some residual activity (Rossi et al., 2002) and strain-specific modifiers of p130 have been described (LeCouter et al., 1998). It therefore seems likely that this phenotypic range arises because the mixed (C57Bl/6 x 129S/v) genetic background of the MEFs modulates the residual p130 activity such that it is either above or below a critical threshold level.

Importantly, at least in certain settings, our data show that the p130 mutation was sufficient to enhance the E2f4+/- differentiation phenotype as well as the combined mutation of p107 and p130. Since, p107-loss has no detectable effect in these assays, this strongly suggests that p130 must be the predominant mediator of the p107/p130 differentiation role. We also examined the cell cycle properties of E2f4+/-;p107+/- and E2f4+/-;p130+/- confluent monolayers, as judged by both BrdU incorporation and E2F-responsive gene expression (data not shown).

Regardless of their differentiation phenotype, all preparations of E2f4+/-;p107+/- and E2f4+/-;p130+/- MEFs had cell cycle phenotypes that were indistinguishable from that of the E2f4+/- MEFs. This suggests that the ability of p130 mutation to enhance the differentiation phenotype of the E2F4-deficient cells occurs independently of p130's role in cell cycle control.

**Pocket protein binding is not required for the repression of adipogenesis by E2F4.**

E2F4 and 5 have many overlapping properties in vivo including regulation by p130 (reviewed by (Trimarchi and Lees, 2002). We therefore tested whether E2F5 inhibits adipogenesis in a manner analogous to E2F4 (Figure 3b). Significantly, E2F5-loss had little or no effect on the differentiation phenotype of either wild-type or E2f4+/- MEFs. Thus, the inhibition of adipogenesis appears to be a unique property of E2F4 and not E2F5. It has yet to be tested if overexpression of E2F5 can suppress the phenotype resulting from loss of E2F4.
It was important to establish whether the increased differentiation of \( E2f4^{+/−} \) MEFs results for the loss of E2F4 and not a subsequent change in the resulting cell lines. To address this issue, we introduced full length E2F4 into wild type and \( E2f4^{+/−} \) MEFs using retroviral infection (Figure 3c). We then compared the ability of control (pBABE) and E2F4-expressing cells to undergo adipogenesis (Figure 3d). Significantly, wild-type E2F4 reduced the differentiation potential of \( E2f4^{+/−} \) MEFs to wild type levels. Thus, the differentiation defect of the \( E2f4^{+/−} \) MEFs is due to the loss of E2F4.

Our data indicates that E2F4 and p130 act synergistically to inhibit adipocyte differentiation. There is also extensive evidence to indicate that pRB is essential for adipogenesis (Charles et al., 2001; Chen et al., 1996). Given these findings, we wanted to determine whether E2F4's ability to repress differentiation is dependent upon its ability to bind to pocket proteins. To address this question, we generated a mutant form of E2F4 that lacks the C-terminal pocket protein binding domain (E2F4AC) and introduced this into wild type or \( E2f4^{+/−} \) MEFs by retroviral infection (Figure 3c). Although this truncated protein is unable to bind pRB-family members (data not shown), it suppressed the differentiation defect of the \( E2f4^{+/−} \) MEFs almost as efficiently as the wild-type E2F4 protein. This suggests that E2F4 inhibits differentiation independently of pocket protein binding.

**pRB is required for adipogenesis in the absence of E2F4.**

Previous studies have shown that \( Rb^{−/−} \) cells fail to significantly undergo adipogenesis (Charles et al., 2001; Chen et al., 1996). pRB has been shown to serve as a coactivator for C/EBPβ, suggesting that pRB-loss impairs differentiation by reducing the activity of C/EBPβ. However, it is also possible that the differentiation defect is a consequence of the requirement for pRB in mediating cell cycle arrest. We have recently shown that E2F4-loss completely
suppresses the inappropriate proliferation of pRB-deficient cells under growth arrest conditions (Lee et al., 2002). Given this finding, we wished to establish whether E2F4-loss would modulate the differentiation phenotype of \( Rb^+ \) MEFs. Consistent with our previous studies, the \( E2f4^- \) MEFs differentiated much better than the wild-type controls in response to hormone induction (Figure 3e). In contrast, the \( E2f4^-;Rb^- \) MEFs failed to undergo hormone-induced differentiation (Figure 3e). Moreover, confluence arrested \( E2f4^-;Rb^- \) MEFs never spontaneously differentiated (data not shown). We therefore conclude that E2F-loss is unable to over-ride the differentiation defect of the \( Rb^- \) MEFs even though it suppresses the cell cycle defect. This supports the prevailing view that pRB promotes differentiation independently of its role in cell cycle regulation. Furthermore, our data indicate that in adipogenesis pRB is required to act downstream of E2F4.
DISCUSSION

In this study, we have continued to investigate the roles of the E2F and pocket proteins in the regulation of adipocyte differentiation. It was previously shown that hormone-induced adipogenesis is promoted by the loss of either E2F4 or p107 and p130 (Classon et al., 2000; Fajas et al., 2002b). It seemed highly likely that the shared activity of E2F4 and p107/p130 simply reflects their participation in transcriptionally repressive complexes. However, our current analyses of compound mutant MEFs do not support this hypothesis. Instead, they suggest that the E2F and pocket proteins contribute to the regulation of adipocyte differentiation through three distinct mechanisms. Moreover, each one of these can be separated from effects on cell cycle control.

The first mechanism involves the E2F4 transcription factor. We have found that E2F4-loss predisposes MEFs to undergo adipogenesis. This includes increasing the proportion of cells that differentiate in response to the standard hormone treatment as well as enabling confluent monolayers to undergo spontaneous differentiation. We do not understand the precise mechanism by which E2F4 mediates this apparent gatekeeper function but our analysis suggests that it is quite distinct from the known properties of E2F. First, the increased differentiation of the E2F4-deficient cells can be separated from any gross changes in either E2F-responsive gene expression or cell cycle regulation. Second, this differentiation phenotype is neither shared nor augmented by the loss of E2F5, with which it is known to cooperate in the control of cell cycle exit, or any other E2F family member (Figure 3; our unpublished findings; Fajas et al., 2002b) indicating that it is a specific activity of E2F4. Finally, E2F4’s ability to suppress differentiation can occur in the absence of its C-terminus sequences. These include the region of the protein that mediates both transcriptional activation and pocket protein binding functions. Thus, E2F4’s
ability to inhibit differentiation must occur independently of its ability to either directly activate
target genes, or to mediate their repression through recruitment of the pocket proteins and their
associated histone deactylates and/or methylases.

Our observations do not rule out the possibility that E2F4 contributes to the regulation of adipogenesis by influencing, either positively or negatively, the activity of other transcription factors. Indeed, there is some precedence for this mode of action. Chen et al. recently showed that the E2F4/p107 complex binds to the Smad proteins in a TGFβ-responsive manner and the resulting complex represses the c-myc gene (Chen et al., 2002). Since E2F4’s role in adipogenesis does not require pocket protein binding, it cannot function through this precise mechanism but one can envisage variations on this theme. Importantly, the finding that E2F4 participates in both TGFβ signaling and adipogenesis indicates that its role extends well beyond the regulation of classical E2F-responsive genes. It also raises the possibility that E2F4 might play a direct role in the differentiation of other cell lineages. In this regard, it is interesting to note that the erythroid defect in the E2F4-deficient mice seems to result from a failure in terminal differentiation without any obvious affect on cell cycle regulation (Humbert et al., 2000a; Rempel et al., 2000).

The second mechanism by which the E2F and pocket proteins contribute to the regulation of adipocyte differentiation involves p107 and p130. Consistent with previous reports (Classon et al., 2000), we find that DKO MEFs undergo hormone induced differentiation with increased efficiency. Our analysis of compound mutant MEFs provides additional insight into their action. First, the loss of these proteins cannot trigger the differentiation process in an analogous manner to the absence of E2F4. However, their loss significantly enhances adipogenesis that has been induced by either classic hormone induction or E2F4-loss. Although our data show that E2F4
can function in a pocket protein independent manner, it is entirely possible that p107 and p130 repress adipocyte induction in association with one or more E2F proteins, including E2F4. Since E2F4 and p107/p130 both regulate adipogenesis negatively, we cannot order the action of these proteins. However, we were able exploit the cooperative effects of E2F4- and p107/p130-deficiency to determine the relative contribution of p107 and p130. While p107-loss had no detectable effect on the differentiation phenotype of the E2f4<sup>−/−</sup> MEFs, p130 mutation could increase it as well as the combined mutation of p107 and p130. This strongly suggests that p130 is the predominant mediator of the p107/p130 differentiation function.

These observations provide important insight into the relative roles of p107 and p130. To date, the analysis of both development and cell cycle defects in the p107; p130 compound mutant mice have largely highlighted the overlapping properties of these proteins (Bruce et al., 2000; Cobrinik et al., 1996; Hurford et al., 1997; Mulligan et al., 1998; Rossi et al., 2002). For example, the p107<sup>−/−</sup> and p130<sup>−/−</sup> mice have no obvious developmental defects but the p107<sup>−/−</sup>; p130<sup>−/−</sup> compound mutants are neonatal lethal (Cobrinik et al., 1996; Lee et al., 1996). In contrast, our differentiation studies provide clear evidence for differential properties of p130 and p107 that is commensurate with the specificity of their expression in arrested (p130) versus cycling (p107) cells. Given these findings, it will be interesting to compare the phenotypes of the E2f4<sup>−/−</sup>; p130<sup>−/−</sup> and E2f4<sup>−/−</sup>; p107<sup>−/−</sup> mutant mice to determine whether the differential roles of p130 and p107 can be observed <i>in vivo</i> and whether they are specific for adipocyte differentiation or extend to other tissues.

The analysis of the E2f4<sup>−/−</sup>; p130<sup>−/−</sup> MEFs also raises some question of the role of cell cycle effects. The DKO MEFs have a major defect in their ability to exit the cell cycle and this was thought to account for, or at least contribute to, the propensity of these cells to undergo
adipogenesis (Bruce et al., 2000; Classon et al., 2000; Hurford et al., 1997). However, the *E2f4^−/−;p130^−/−* MEFs do not have an obvious cell cycle defect even though they display a differentiation defect as profound as TKO MEFs. Thus, at least in the absence of E2F4, the effect of *p130* mutation appears largely cell cycle independent.

The final mechanism by which the E2F and pocket proteins contribute to the regulation of adipocyte differentiation involves pRB. Numerous studies have shown that pRB is required for the differentiation of MEFS *in vitro*. This is believed to be due to pRB’s role as a coactivator for C/EBPβ but it could also result from the profound cell cycle defect of the *Rb^−/−* cells (Charles et al., 2001; Chen et al., 1996; Richon et al., 1997). Indeed, Fajas and coworkers recently reported that pRB also negatively regulates adipogenesis via inhibition of PPARγ and suggested that pRB’s dominant positive role might be primarily dependent upon its key role in cell cycle exit (Fajas et al., 2002a). Since E2F4-loss suppresses the inappropriate proliferation of pRB-deficient cells (Lee et al., 2002), we have also examined the differentiation phenotype of the *E2f4^−/−;Rb^−/−* MEFs. Significantly, these cells are completely impaired in their ability to undergo either spontaneous or hormone-induced differentiation. This represents the first evidence that the requirement for pRB in adipogenesis is independent of any cell cycle effect. Moreover, it shows that pRB acts downstream of E2F4 in the regulation of adipogenesis. Since C/EBP and E2F bind to the same region of pRB, we initially suspected that E2F4 would inhibit adipogenesis by preventing the pRB-C/EBPβ interaction. However, our data show that E2F4’s inhibitory activity does not require the C-terminal, pocket protein binding domain. Thus, while this competitive mechanism may operate in some situations, it cannot fully account for the adipogenic properties of the E2F4-deficient cells.
Our data clearly show that the three distinct mechanisms by which the E2F and pocket proteins regulate adipogenesis can be separated from one another and, in certain situations, from cell cycle control. However, \textit{in vivo}, it seems highly likely that there will be extensive cross-talk between these pathways to ensure ordered progression through sequential stages of the differentiation process and link each one to the appropriate cell cycle condition. Thus, the future challenge will be to establish the underlying basis of each mechanism and determine how they are coordinated \textit{in vivo}.

\section*{ACKNOWLEDGEMENTS}

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Fig. 1. E2F4/pocket protein repression is required for repression of adipogenesis. (A)
Cartoon indicating the spectrum of E2F complexes that exist in normal cells and the components (denoted by the X’s) that are affected by the combined mutation of E2f4, p107, and p130. (B) Wild-type (+/+), DKO and TKO MEFs were induced to differentiate for 2 weeks, stained with Oil Red O and quantitated. (C) MEFs of all four genotypes maintained at confluence for 3 weeks, stained with Oil Red O and quantitated. (D) Wild-type (+/+), E2f4+/−, and TKO MEFs stained with Oil Red O after 6 wks at confluence.
Fig. 2. Loss of repression leads to defects in cell cycle control and deregulation of E2F
target gene expression. (A) Wild-type (+/+), $E2f4^{-/-}$, DKO, and TKO MEFs were grown and
analyzed at 0, 4, 6, and 8 days of confluence for expression of E2F responsive genes. Gene
expression was quantitated using the loading control ($ARP\ PO$). (B) (i) Wild-type (+/+), (ii)
DKO, (iii) TKO, and (iv) $E2f4^{-/-}$ MEFs were grown and maintained at confluence for eight days
and then pulsed with BrdU for 24 hours. The percentage of cycling cells was based upon
counting 300 dapi positive nuclei for each genotype.
Fig. 3. Analysis of E2F4 mediated adipocyte repression. (A) E2f4−/−;p107−/− and E2f4−/−;p130−/− MEFs were induced to differentiate in response to hormones for 2 weeks, stained with Oil Red O, quantitated, and compared with wild-type (+/+), E2f4−/−, and TKO MEFs. (B) Wild-type, E2f5−/−, E2f4−/−, and E2f4−/−;E2f5−/− MEFs were induced to differentiate in response to hormones for 2 weeks and analyzed as in (A). (C) Full length or a C-terminal truncated (E2F4AC) E2F4 were expressed in control and E2f4−/− MEFs by retroviral mediated infection. (D) Infected cells were induced by hormones to differentiate for 2 wks and analyzed as above. (E) Wild-type (+/+), E2f4−/−, Rb−/−, and E2f4−/−; Rb−/− MEFs were differentiated in response to hormones for two weeks and analyzed as above.
REFERENCES


CHAPTER THREE

E2F4•pocket protein repressive complexes are essential for development

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Author’s contribution Figures 1, 2, 3, 4, 5, 6 and Tables I, II, III

ABSTRACT

E2F•pocket protein repressive complexes have been shown to be critical for
regulating cell cycle control and differentiation. $E2f4^{−/}$ and $p107^{−/};p130^{−/}$ mice have been
shown to display developmental defects. To further understand how E2F•pocket protein
repressive complexes contribute to development, mice lacking E2F4, p107, and p130
were generated. Analysis of compound mutant animals revealed a requirement for
E2F•pocket protein repressive complexes in the development of a number of tissues.
Most striking, $E2f4^{−/};p107^{−/};p130^{−/}$ mice display a cell autonomous defect in the
development of the hematopoietic compartment. This phenotype suggests that
E2F4•pocket protein repressive complexes are critical regulators of fetal hematopoiesis.
INTRODUCTION

The pRB (pocket protein) family are critical regulators of the G₁ to S transition of the cell cycle. This family also includes two related proteins, p107 and p130. The cell cycle inhibitory effects of these proteins are largely due to their interactions with their major downstream target, the E2F transcription factor. Pocket proteins complex with E2F, inhibit its transactivational activity, and prevent the expression of E2F target genes that are required for DNA replication and S-phase entry (as reviewed in Dyson, 1998; Trimarchi and Lees, 2002). E2F·pocket protein complexes can actively repress E2F responsive gene expression through recruitment of methylase and histone deacetylase (HDAC) activity (Bannister et al., 2001; Brehm et al., 1998; Lachner et al., 2001; Luo et al., 1998; Nielsen et al., 2001).

There have been eight genes encoding components of the E2F transcription factor identified to date. E2F is a heterodimeric factor composed of one Dp subunit, of which there are two family members (DP-1, DP-2) and one of the seven E2F family members (as reviewed in Trimarchi and Lees, 2002). The E2F family can be divided into three subgroups based upon structural and functional homology. E2F6 does not interact with any of the pocket protein family members and has been shown to play a role in vertebrate patterning through interaction with the mammalian polycomb complex (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Ogawa et al., 2002; Storre et al., 2002; Trimarchi et al., 1998; Trimarchi et al., 2001). The second subgroup consists of E2F1, E2F2, E2F3a, and E2F3b which are repressed only by pRB. E2F1, E2F2, and E2F3a play a key role in activating E2F target gene transcription and thereby promoting S-phase progression (Helin et al., 1992; Kaelin et al., 1992; Lees et al., 1993). E2F3b was
recently identified as a second product of the E2F3 gene (Adams et al., 2000; He et al., 2000; Leone et al., 2000). E2F3b most closely resembles the activating E2Fs but its function is still under investigation. The last subgroup consists of E2F4 and E2F5, the repressive E2Fs. E2F5 interacts only with p130, but E2F4 interacts with all three of the pocket protein family members and comprises the majority of the E2F•pocket protein repressive complexes in vivo (Moberg et al., 1996). Consistent with their proposed roles, chromatin immunoprecipitation (ChIP) assays have shown that the repressive E2Fs occupy E2F responsive promoters during G0/G1 and are replaced by the activating E2Fs during the G1/S transition (Takahashi et al., 2000).

Mutant mouse models have been generated for E2Fs and pocket proteins. Embryos deficient for pRB have defects in end stage erythropoiesis characterized by a decrease in mature, enucleated erythrocytes (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). These embryos die in utero exhibiting a high number of apoptotic cells in the central and peripheral nervous systems, liver, and lens due to inappropriate proliferation. The inappropriate proliferation and apoptosis are due to deregulated E2F activity and can be suppressed by loss of either E2f1 or E2f3 (Tsai et al., 1998; Ziebold et al., 2001). Mice deficient for pRB and either of these E2Fs die later in gestation, revealing a requirement for pRB in the development of skeletal and cardiac muscle. pRB regulates differentiation and development by serving as a coactivator for differentiation specific transcription factors in a manner independent from its ability to interact with E2F and regulate the cell cycle. In vitro differentiation assay systems using cells derived from Rb- mice reveal a requirement for pRB in myogenesis, osteogenesis, and adipogenesis.
(Charles et al., 2001; Chen et al., 1996; Gu et al., 1993; Novitch et al., 1996; Thomas et al., 2001).

Loss of p107 and p130 also leads to developmental abnormalities. p107<sup>−/−</sup>;p130<sup>−/−</sup> mice die shortly after birth and display defects in long bone development (Cobrinik et al., 1996). Analysis of these defects suggest that they arise from a loss of cell cycle control as opposed to a direct impairment of differentiation (Cobrinik et al., 1996; Rossi et al., 2002). Consistent with this, mouse embryonic fibroblasts (MEFs) derived from p107<sup>−/−</sup>;p130<sup>−/−</sup> embryos exhibit G<sub>1</sub> arrest defects and deregulation of a large number of E2F responsive genes (Bruce et al., 2000; Hurford et al., 1997; Landsberg et al., Submitted). Based on this data, p107 and p130 appear to regulate differentiation and development by maintaining cell cycle arrest through interaction with and repression of the E2F transcription factor.

Mice deficient for the repressive E2Fs have been generated. E2f5<sup>−/−</sup> mice die neonatally from hydrocephaly due to an accumulation of cerebral spinal fluid (Lindeman et al., 1998). E2f4<sup>−/−</sup> mice exhibit macrocytic anemia during gestation. A subset of the E2f4<sup>−/−</sup> mice die neonatally from chronic sinal infections due to craniofacial abnormalities (Humbert et al., 2000; Rempel et al., 2000). The phenotypes observed in these animals appear not to be due to loss of cell cycle control as MEFs derived E2f4<sup>−/−</sup> and E2f5<sup>−/−</sup> embryos show no defects in proliferation or E2F target gene regulation (Humbert et al., 2000; Lindeman et al., 1998; Rempel et al., 2000). Previously we established an in vitro white adipose tissue differentiation assay to study the role of E2F•pocket protein repressive complexes in regulating adipogenesis. We found that E2F4 represses adipogenesis in vitro in a manner distinct from its ability to interact with the pocket
proteins or regulate the cell cycle (Landsberg et al., Submitted). This phenotype can be enhanced through subsequent loss of p130. Significantly, $E2F4^{-/-};p130^{-/-}$ MEFs do not display defects in withdrawing from the cell cycle, indicating a role for p130 in adipogenesis that is independent from its ability to regulate cell cycle progression. To investigate how E2F•pocket protein complexes contribute to differentiation and development $in vivo$, we have generated mice lacking E2F4, p107, and p130. Analysis of these mice have revealed a role for these proteins in mediating the development of a number of tissues.
MATERIALS AND METHODS

Production of E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (TKO), E2f4<sup>−/−</sup>;p107<sup>−/+</sup>;p130<sup>−/−</sup> (KHK) and E2f4<sup>−/−</sup>;p130<sup>−/−</sup> mice. E2f4<sup>−/−</sup>;p130<sup>−/−</sup> mice were bred with p107<sup>−/−</sup> animals; any resulting triple heterozygotes had to have undergone the cross-over event, linking the E2F4 mutation with the p130 mutation. Further crosses to p107<sup>−/−</sup> yielded E2f4<sup>−/+</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> mice. These males were bred with triple heterozygous females to yield E2f4<sup>−/−</sup>;p107<sup>−/+</sup>;p130<sup>−/−</sup> (KHK) and E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (TKO) mice at an expected frequency of one in eight. E2f4<sup>−/−</sup>;p130<sup>−/−</sup> mice were generated by breeding triple heterozygotes to wild type animals and interbreeding E2f4<sup>−/−</sup>;p130<sup>−/−</sup> animals.

Histological Analysis. Embryos and tissues were fixed in either Boiuns solution or 10% formalin, processed, and stained as previously described (Humbert et al., 2000). Alizarin red and alcian blue staining was performed on 16.0 dpc and 18.5 dpc embryos. Soft tissue was cleared by treatment with alkali and staining was performed as previously described (Kessel and Gruss, 1991).

Adoptive Transfer Assays. Adoptive transfer experiments were performed as described (Humbert et al., 2000).
RESULTS

In order to determine the contribution of E2F-pocket protein repressive complexes to differentiation and development, we generated mutant mice lacking E2F4, p107, and p130. In mice, E2F4 and p130 are approximately 10cM apart on the long arm of chromosome 8. In order to generate a triple mutant individual, the two mutant alleles needed to be linked on the same chromosome. The breeding scheme outlined in Figure 1 and described in the materials and methods section illustrates the strategy used to generate the meiotic cross-over event and the subsequent crosses that yielded $E2f4^{−/−};p107^{−/−};p130^{−/−}$ (TKO) and $E2f4^{−/−};p107^{−/−};p130^{−/−}$ ("knock out; heterozygote; knock out" or KHK) animals. Due to the nature of the crosses pure wild type littermate controls are not generated. For all experiments $p107^{−/−}$ or $p107^{−/−}$ were used as controls as they have been shown to have no obvious developmental or cell cycle phenotype (Cobrinik et al., 1996; Hurford et al., 1997; Mulligan et al., 1998).

**E2F-pocket protein repressive complexes are required for neonatal viability.**

Using the breeding scheme shown in Figure 1, $E2f4^{−/−};p107^{−/−};p130^{−/−}$ (KHK) animals were generated, and the viability of these mice was investigated. KHK pups were underrepresented in all litters examined in utero; however all KHK animals observed were alive, and the sample size examined was not statistically significant (Table I). Out of the 387 animals, we expected to see 48 KHK mice born. We observed that 34 of the 37 mice were born alive (Table I). This is slightly lower than expected, but the sample size examined was not large enough to be statistically significant. Most KHK
mice were born alive at close to expected numbers. There was a sharp decrease in viability after the first 48 hours of life as over half of the KHK pups failed to thrive past two days (Table I). KHK mice show a build up of fluid in the sinal cavity identical to the phenotype of E2f4−/− mice (data not shown and Humbert et al., 2000; Rempel et al., 2000). This drop in viability in the first few days of life could be due to accumulation of fluid in the sinuses associated with loss of E2F4 and not due to a novel phenotype resulting from further loss of repressive complexes.

Nine mice survived to one week of age, and of these animals only seven survived to two weeks of age, five to three weeks of age, and only one mouse was alive at six weeks (Table I). The cause of death of E2f4−/−;p107−/−;p130−/− mice is still under investigation. All of these mice displayed phenotypes not found in E2f4−/−, p107−/−, and p130−/− single or p107−/−;p130−/− mutant mice. The nature of these phenotypes are described below. Further analysis of all of these phenotypes are beyond the scope of this current study, but taken together they indicate that E2F•pocket protein repressive complexes are required for the development of a wide spectrum of tissues.

**KHK mice display severe growth retardation and defects in long bone formation, testicular maturation, neurological development, and white adipose tissue formation.**

As shown in Figure 2a, all E2f4−/−;p107−/−;p130−/− mice displayed severe growth retardation from birth. The bones of these animals also appeared to be thinner than their littermate counterparts; in correlation with this, many pups displayed tail fractures (data not shown). Male KHK mice at three weeks of age displayed defects in testicular
maturation. At three weeks of age the testes should have a layered appearance (Figure 2b top). In control three-week old animals, the bottom layer is comprised of dark appearing spermatogonia (S), the germ cells (Figure 2b top). As the spermatogonia enter meiosis I, they move out into the lumen and develop into primary spermatocytes (S₁), cells that are characterized by having a pale nuclei and a large cytoplasm. Once meiosis is completed the secondary spermatocytes quickly progress through meiosis II and become spermatids (S₂), that eventually mature into spermatozoa (as reviewed in Burkitt et al., 1993). Testes from KHK mice had a layer of spermatogonia and a layer of primary spermatocytes that occluded the lumen of the testes (Figure 2b bottom). The testes also contained abnormal looking cells and appeared to have fewer Leydig cells (Figure 2b bottom and data not shown). All animals surviving up to three weeks of age were male so that analysis of gamete maturation in female KHK mice could not be assessed. This gender bias could be because female animals are smaller and fail to compete effectively with their littermates for food.

All KHK mice that survived until three weeks of age clenched their limbs when lifted up by their tails (Figure 3a), behavior suggestive of a neurological defect (R. T. Bronson, personal communication). Many of the more severely effected pups of all ages developed ataxia and palsy. Analysis of brains from these animals revealed a defect in cerebellar development. The cerebellum is necessary for mediating motor control and coordination. Defects in the formation of the cerebellum could account for the neurological abnormalities observed in E2f4−/−; p107−/−; p130−/− mice (as reviewed in Bayer and J., 1991). The architecture of the cerebellum of the KHK mice appeared to be abnormal. The granular layer (GL) was thicker and the molecular layer (MolL) was
thinner than that of the control (Figure 3b). As shown in Figure 3b, three-week old KHK mice also have gaps in their Purkinje cell layer (PCL). The Purkinje cells are normally found in a continuous monolayer and are important for signaling between the spinal cord and the cerebral cortex (Bayer and J., 1991). The cerebellar abnormalities seen in the KHK mice could result in the clenching, tremors, and palsy observed.

KHK mice have a severe defect in the formation of white adipose tissue (WAT). As shown in Figure 4a, the subcutaneous fat pad is completely absent in all KHK mice examined. This loss of WAT accumulation is not due to defects in feeding as all animals had food in their stomachs when sacrificed (data not shown) which suggests that this phenotype is due to a defect in WAT accumulation. Histological analysis of KHK livers revealed the hepatocytes (the major cellular component of the liver) were cloudy (Figure 4b) and highly reminiscent of the descriptions of “fatty liver” phenotypes from other mouse models deficient for lipid accumulation and WAT production (Barak et al., 1999).

**Defects in KHK mice are due to Loss of E2F4 and p130.**

The developmental defects found in KHK mice could be due to the combined loss of E2F4, p130, and one allele of p107, or they could be attributed to the loss of E2F4 and p130 alone. In order to distinguish between these two possibilities, crosses between $E2f4^{−/−};p130^{−/−};p107^{−/−}$ mice were established. Though numbers for this cross have not reached statistical significance, Table II shows that $E2f4^{+/−};p130^{+/−}$ mice display a similar window of lethality to KHK mice. They are predominantly viable at birth, but a large number of double mutants fail to thrive during the first week of life. All $E2f4^{−/−};p130^{−/−}$ animals failed to live past three weeks of age. Analysis of these animals revealed defects
in WAT accumulation, long bone formation, and neurological development identical to those observed in the KHK mice (data not shown). Taken together, these data indicate that E2F4•p130 repressive complexes are necessary to proper development of a number of tissues. Further analyses of these defects are currently being conducted in our laboratory.

**E2F•pocket protein repressive complexes are required for neonatal and embryonic viability.**

Crosses to generate $E2f4^{−/−}; p107^{−/−}; p130^{−/−}$ (TKO) mice were also established (as shown in Figure 1), and the viability of these animals were analyzed. Out of 322 animals only three TKO pups were born alive, but no mutant animals survived past the day of birth (Table III). These data are consistent with previous results showing that p107 and p130 have overlapping roles essential for neonatal viability (Cobrinik et al., 1996). $p107^{−/−}; p130^{−/−}$ mice are viable until birth and do not exhibit inappropriate lethality during gestation. TKO mice, however, appear to not be fully viable during embryogenesis. Starting at 13.5 days postconitum (dpc) and continuing through 18.5 dpc, a low incidence of embryonic lethality was observed resulting in less than the expected number of embryos.
TKO mice display defects in long bone development.

As stated above, no TKO animals survived past the first day after birth, a phenotype highly reminiscent of \textit{p107}^{+/-};\textit{p130}^{+/-} mice. Since \textit{p107}^{+/-};\textit{p130}^{+/-} mice die from defects in endochondral ossification and long bone development, we wished to examine the formation of long bones in the TKO mutant mice. Embryos from the earliest day in gestation that this defect can be observed (16.0 dpc) and the day prior to birth (18.5 dpc) were fixed, cleared of soft tissue, and stained with dyes specific to bone (Alizaren red) and cartilage (Alcian blue). Analysis of forelimbs from 16.0 dpc TKO embryos (Figure 5ai and 5a ii) revealed a defect in endochondral ossification. The area of ossification in the radius, ulna, and humerus appeared to be reduced as compared to the control. This is similar to the staining pattern observed in \textit{p107}^{+/-};\textit{p130}^{+/-} mutant forelimbs (Figure 5aii). Forelimbs from 18.5 dpc TKO embryos also exhibit this defect, displaying a delay in the ossification of the digits (Figure 5b). The only difference between the phenotype of TKO and \textit{p107}^{+/-};\textit{p130}^{+/-} mice is that the bones of TKO embryos are thinner than those of the control while bones from \textit{p107}^{+/-};\textit{p130}^{+/-} mice are thicker. This shift in phenotype could arise from an increase in apoptosis, a decrease in proliferation, or a decrease in differentiated cells. TKO forelimbs do not display an increase in apoptosis as judged by TUNEL staining (data not shown). The difference between the phenotype of \textit{p107}^{+/-};\textit{p130}^{+/-} embryos and the TKO is still under investigation.

TKO mutant embryos display defects in hematopoiesis.

Defects in endochondral ossification cannot account for the incidence of embryonic lethality observed in \textit{E2F4}^{+/-};\textit{p107}^{+/-};\textit{p130}^{+/-} embryos. To investigate the cause
of death, full histological analysis was conducted in these embryos. TKO embryos exhibited (with varying degrees of penetrance) hypoplastic anemia, a severe decrease in the number of hematopoietic progenitors. Hematopoiesis first originates in the blood islands of the yolk sac at 7.5 dpc (Dzierzak and Medvinsky, 1995). At 10 dpc progenitor cells first begin to colonize the liver, the major site of hematopoiesis throughout gestation. Prior to birth (about 17.5 dpc), progenitors migrate to the primary site of hematopoiesis in adults, the bone marrow. Livers from 15.5 dpc TKO embryos show a sharp reduction in the number of darkly staining hematopoietic progenitor cells present (Figure 6aiii) as compared to the control (6ai). Hematopoietic activity is absent from the bone marrow of 18.5 dpc TKO embryos but is present in bone marrow from 18.5 dpc p107<sup>−/−</sup> embryos (Figure 6aii and 6aiv).

The phenotypes in these animals were not fully penetrant with 15.5-18.5 dpc embryos displaying varying degrees of severity. One potential explanation for this phenotype is that the progenitors exhibit defects in migration between the major sites of hematopoiesis. Evidence for this comes from one of the TKO embryos born alive. Its bone marrow was devoid of hematopoietic progenitors, but its liver still had high levels of hematopoietic activity (Figure 6b). There are several other potential explanations for this phenotype. The hypoplastic anemia could arise due to an absence of progenitors or defects in the ability of these cells to self renew, or terminally differentiate. This phenotype could also be due to non-cell autonomous defects in the liver or bone marrow environment.
The hypoplastic anemia observed in TKO embryos is due to a cell intrinsic defect.

To determine if the hypoplastic anemia observed in the TKO embryos arise from a cell autonomous defect, adoptive transfer experiments were performed. TKO or control 13.5 dpc fetal livers were used as a source of CD45.2-positive stem cells and were injected into lethally irradiated syngenic C57BL/6-CD45.1 wild type adults. The recipient animals were examined for the ability of the donor cells to reconstitute the hematopoietic compartment. Fetal liver cells taken from control embryos were able to reconstitute hematopoiesis in irradiated animals (Figure 7a) with an average of 86% (+/- 9.8%) of the hematopoietic cells present bearing the CD45.2 marker from the donor. Fetal liver cells derived from TKO embryos were not as efficient in reconstituting the hematopoietic compartment of irradiated mice (Figure 7b) with only an average of 53% (+/-32%) of the cells carrying the CD45.2 marker. This phenotype was not fully penetrant, and there was a large variation in the efficiency of reconstitution (Figure 7b). Out of the nine animals receiving TKO fetal liver cells four failed to thrive (as denoted by x’s) to the completion of the experiment.

All mice receiving fetal liver cells from TKO embryos developed all of the erythroid defects originally described for E2f4− mice (Humbert et al., 2000; Rempel et al., 2000). Animals that did survive for the duration of the experiment displayed a decrease in the contribution of TKO (CD45.2-positive) and an increased contribution of recipient (CD45.1-positive) cells to all myeloid and lymphoid lineages present in the bone marrow (Table IV). There also was a concomitant decrease in TKO donor derived mature B cells (B220 and IgM-double positive) and a complete absence of TKO derived Mac-1, Gr-1 double positive cells (granulocytes and macrophages) present in the
circulating blood (Figure 8a, b). All Gr-1, Mac-1 expressing cells have, in fact, derived from the CD45.1 positive recipient cells (Figure 8b). Taken together the data show that the hypoplastic anemia observed in E2f4−/−;p107−/−;p130−/− embryos is due to a cell intrinsic defect.

This data demonstrate a requirement for E2F4•pocket protein repressive complexes for the proper development of a number of tissues both during and after gestation. These results also show a clear need for E2F4•p107 and E2F4•p130 complexes in regulating the development of the hematopoietic compartment.
DISCUSSION

Loss of E2F4 and p130 leads to developmental defects.

In this study we wished to examine the role that E2F4-pocket protein repressive complexes play in differentiation and development. Our analysis has revealed a role for E2F4-p130 complexes in the development of a wide spectrum of tissues. As cells deficient for E2F4 and p130 have no apparent cell cycle defect in vitro, it is likely that the phenotypes in $E2f4^{-/-};p130^{-/-}$ and $E2f4^{-/-};p107^{-/-};p130^{-/-}$ mice arise due to a direct failure to develop appropriately (Landsberg et al., Submitted). It remains to be determined if the spectrum of defects are cell autonomous. As we previously reported, $E2f4^{-/-};p130^{-/-}$ MEFs are fully capable of differentiating into white adipose tissue with an efficiency equal to or greater than control cells (Landsberg et al., Submitted). This suggests that at least some of the phenotypes of the $E2f4^{-/-};p130^{-/-}$ and KHK mice arise from cell non-autonomous defects. Our lab is in the process of generating mice that carry tissue-specific targeted deletions of $p130$ and $E2f4$ to further characterize the nature of these phenotypes.

TKO embryos display defects in endochondral ossification.

Examination of mice deficient for E2F4, p107, and p130 reveal defects in endochondral ossification. It has been reported that $p107^{-/-};p130^{-/-}$ embryos have defects in endochondral ossification due to a failure of their chondrocytes to exit the cell cycle appropriately (Cobrinik et al., 1996; Rossi et al., 2002). The hypothesis that chondrocytes lacking p107 and p130 have a cell cycle defect is supported by the failure of $p107^{-/-};p130^{-/-}$ MEFs and 3T3s to withdraw from the cell cycle (Bruce et al., 2000; Classon et al., 2000; Hurford et al., 1997). TKO embryos display similar defects in
endochondral ossification to \( p^{107^-};p^{130^-} \) mice. This phenotype is also most likely due to defects in withdrawing from the cell cycle as \( E^{2f4-};p^{107^-};p^{130^-} \) MEFs have identical cell cycle exit defects as \( p^{107^-};p^{130^-} \) MEFs (Landsberg et al., Submitted). The decrease in bone thickness observed in TKO mutant limbs is a shift from the phenotype observed in \( p^{107^-};p^{130^-} \) limbs. This change does not appear to arise from a net increase in apoptotic cells. Bones of KHK and \( E^{2f4-};p^{130^-} \) mice also display a decrease in thickness but MEFs derived from these embryos do not exhibit cell cycle defects suggesting that this phenotypic shift might arise from defects in regulating differentiation.

**E2F•pocket protein repressive complexes are required for development of the hematopoietic system.**

TKO mutant embryos display a defect in generating a complete, functional hematopoietic system. In addition to a reduction of hematopoietic progenitors present \textit{in vivo}, the livers from these animals cannot effectively reconstitute the hematopoietic compartment of sub-lethally irradiated animals. Unlike the phenotypes of \( E^{2f4^-} \) and \( Rb^- \) mice in which only the erythroid lineages are affected, all hematopoietic cell types are affected in the TKO (Clarke et al., 1992; Humbert et al., 2000; Jacks et al., 1992; Lee et al., 1992; Rempel et al., 2000).

The hematopoietic defect is not fully penetrant which is most likely due to the fact that the \( p^{130} \) allele used in this study displays some residual activity (Rossi et al., 2002). In addition, there have been reports of strain specific modifiers of \( p^{130} \) activity (LeCouter et al., 1998). The experiments for this study were conducted in a
129/Sv:C57BL/6J mixed genetic background which could have an impact on the penetrance of this phenotype.

From the adoptive transfer experiments it appears that the hematopoietic phenotype is cell autonomous as opposed to arising from defects in the liver or bone marrow stroma. There are several potential explanations for this phenotype. The first possibility is that there are defects in the ability of progenitors to migrate between sites of hematopoiesis. Evidence for this comes from histology from TKO pups taken at later stages in gestation (as shown in Figure 6b) that lack hematopoietic activity in the bone marrow but show signs of hematopoiesis occurring in the liver. This may not explain the phenotype however as TKO fetal liver cells injected into sub-lethally irradiated mice can later be found in the bone marrow of the recipient (Table IV).

The second possibility is that there are defects in the ability of these cells to terminally differentiate into mature cells. This is supported by the reduction of granulocytes, macrophages and B cells produced from TKO derived donor cells (Figure 8 and Table IV). *In vitro* progenitor assays have been established and are currently being conducted to determine if there are any terminal differentiation defects.

The third possibility is that there is a defect in the ability of the progenitors to self-renew. Lethally irradiated mice receiving TKO fetal livers show a decrease in the number of cells derived from the donor (Figure 7 and Table IV). It is possible that the progenitors default-differentiate and irreversibly lose their potential to self-renew. The TKO-derived hematopoietic compartment might therefore only have a limited capacity to restore hematopoiesis in lethally irradiated mice. In the adoptive transfer experiments, only half of the recipients survived. Many of the survivors displayed compensation from
their own hematopoietic compartment (Figure 8 and Table IV). This model is also supported by previous work studying the ability of mouse embryonic fibroblasts (MEFs) derived from TKO embryos to differentiate in white adipose tissue (WAT). If given enough time, the entire population of TKO cells default-differentiates into WAT without the presence of formal inductive cues. It is possible that the hematopoietic progenitors display a similar phenotype. To test this phenotype, serial adoptive transfer experiments (lethally irradiated mice are reconstituted with the bone marrow from first generation recipients) are currently being established.

Taken together, this study has shown that E2F4•p107 and E2F4•p130 repressive complexes are required for proper development of many tissues. Future work will shed light into the mechanisms through which these complexes regulate these processes.

ACKNOWLEDGEMENTS

This work was in part supported by a grant from the National Institutes of Health to J.A.L. R.L.L. was supported in part by a fellowship from the Anna Fuller Fund.
Figure 1. Breeding strategy for generation of TKO and KHK mice.  

$E2f4^{+/+}; p130^{+/+}$ (*=unlinked) were bred with $p107^{-/-}$ mice; any resulting triple heterozygotes had to have undergone the cross-over event (L=linked), linking the E2F4 mutation with the p130 mutation. Further crosses to $p107^{-/-}$ mice yielded $E2f4^{+/+}; p107^{-/-}; p130^{+/+}$. These males were bred with triple heterozygous females to yield $E2f4^{+/+}; p107^{-/-}; p130^{-/-}$ (KHK) and $E2f4^{+/+}; p107^{-/-}; p130^{-/-}$ (TKO) at an expected frequency of one in eight.
Table I. Viability of \( E2f4^{-/-};p107^{+/+};p130^{+/+} \) (KHK) Mice

<table>
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<th>Expected</th>
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<th>Total # Animals</th>
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<tr>
<td>E13.5</td>
<td>21.9</td>
<td>13</td>
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<tr>
<td>Birth</td>
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<td></td>
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<tr>
<td>1 Week</td>
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<td>9</td>
<td>387</td>
</tr>
<tr>
<td>3 Weeks</td>
<td></td>
<td>5</td>
<td>387</td>
</tr>
</tbody>
</table>

( )=Dead
Figure 2. *E2f4<sup>−/−</sup>;p107<sup><sup>−/−</sup>;<p130<sup>−/−</sup> mice display growth retardation and defects in testicular maturation*. (A) *E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (KHK) mice at 1.5 weeks of age (left) are severely growth retarded as compared to control (right). (B) 40x magnification of hematoxylin and eosin (H&E) staining of testes from control (top) and KHK (bottom) mice at 3.5 weeks of age. S=spermatogonia; S<sub>1</sub>=primary spermatocytes; S<sub>2</sub>=spermatids. Arrow denotes gametes with abnormal meiosis.
Figure 3. $E2f4^{-/-};p107^{-/-};p130^{-/-}$ mice display neurological defects. (A) $E2f4^{-/-}p107^{-/-};p130^{-/-}$ (KHK) mice clench in response to being lifted by the tail (left). (B) 40x magnification of H&E staining of cerebellum from 3 week old control (left) and $E2f4^{-/-}p107^{-/-};p130^{-/-}$ (KHK) mice (right). Arrow denotes the gap in the Purkinje cell layer (PCL). MedL= Medullary Layer; GL=Granular Layer; MolL=Molecular Layer.
A. Subcutaneous Fat Pad

\[ \text{p}107^{+/-} \quad \text{E}2\text{f}4^{-/-};\text{p}107^{+/-};\text{p}130^{-/-} \]

B. Liver

\[ \text{p}107^{+/-} \quad \text{E}2\text{f}4^{-/-};\text{p}107^{+/-};\text{p}130^{-/-} \]

Figure 4. \textit{E}2\textit{f}4^{-/-};\textit{p}107^{+/-};\textit{p}130^{-/-} mice display defects in white adipose tissue accumulation. (A) 40x magnification of H&E stained subcutaneous fat pad from \textit{107}^{+/-} (right) and KHK mice (left). (B) 40x magnification of H&E stained liver from \textit{107}^{+/-} (right) and KHK mice (left).
Table II. Viability of \textit{E2f4}^{-/-};\textit{p130}^{-/-} Mice

<table>
<thead>
<tr>
<th>Age</th>
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<th>\textit{E2f4}^{-/-};\textit{p130}^{-/-}</th>
<th>Total # Animals</th>
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<tbody>
<tr>
<td>Birth</td>
<td>19</td>
<td>11(1)</td>
<td>76</td>
</tr>
<tr>
<td>1 Week</td>
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<td>4</td>
<td>76</td>
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<tr>
<td>3 Weeks</td>
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( )=Dead
Table III. Viability of E2f4-/-;p107-/-;p130-/- (TKO) Mice

<table>
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<td>322</td>
</tr>
<tr>
<td>e18.5</td>
<td>11</td>
<td>10(2)</td>
<td>93</td>
</tr>
<tr>
<td>e15.5-17.0</td>
<td>12</td>
<td>7(2)</td>
<td>93</td>
</tr>
<tr>
<td>e13.5</td>
<td>19</td>
<td>14(1)</td>
<td>170</td>
</tr>
</tbody>
</table>

( )=Dead
Figure 5. *E2f4*<sup>+/−</sup>*;*p107*<sup>+/−</sup>*;*p130*<sup>−/−</sup> embryos display defects in endochondral ossification. (A) Alizarin red (bone) and Alcian blue (cartilage) staining of forelimbs from 16.0 dpc (i) *107*<sup>+/−</sup> (ii) *E2f4*<sup>+/−</sup>*;*p107*<sup>+/−</sup>*;*p130*<sup>−/−</sup> (TKO), and (iii) *p107*<sup>−/−</sup>*;*p130*<sup>−/−</sup> embryos. R=radius; U=ulna; H=humerus. (B) Alizarin red and alcian blue staining of forelimbs from 18.5 dpc *107*<sup>+/−</sup> (top) and TKO (bottom) embryos. Arrows denote the area where ossification is delayed in TKO embryos.
Figure 6  *E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (TKO) embryos have hypoplastic anemia.  
(A) 40x magnification of H&E stained liver from 15.5 dpc and bone marrow from 18.5 dpc (i, ii) *p107<sup>−/−</sup>* and (iii, iv) *E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup>* (TKO) embryos.  
(B) 40x magnification of H&E stained liver (left) and bone marrow (right) from d1 TKO neonate.
Figure 7. *E2f4−/−;p107+/−;p130−/−* (TKO) hematopoietic progenitors are impaired in rescuing hematopoiesis in lethally irradiated mice. (A) Control and (B) *E2f4−/−;p107+/−;p130−/−* (TKO) fetal livers (CD45.2) were introduced into recipients (CD45.1) that were lethally irradiated. Cells from the peripheral blood were harvested and FACs-sorted based upon CD45 marker status.
Table IV. Contribution of Donor Cells to Hematopoietic Lineages

<table>
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<tr>
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<th>Control (Ctrl)</th>
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<td><strong>Total Cell #</strong></td>
<td>6.6x10^6</td>
<td>9.5x10^5</td>
</tr>
<tr>
<td></td>
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<td>Down 1/7 of ctrl</td>
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<td>6.6x10^4</td>
<td>4.6x10^5</td>
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<tr>
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<td>2.7x10^3</td>
</tr>
<tr>
<td></td>
<td>1.17x10^5</td>
<td>6x10^3</td>
</tr>
<tr>
<td>B220</td>
<td>9.7x10^5</td>
<td>3x10^4</td>
</tr>
<tr>
<td></td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Gr1/Mac1</td>
<td>2.85x10^6</td>
<td>3.5x10^4</td>
</tr>
<tr>
<td></td>
<td>6.3x10^3</td>
<td>1.26x10^5</td>
</tr>
<tr>
<td>CD90.2</td>
<td>1.8x10^5</td>
<td>2.6x10^5</td>
</tr>
<tr>
<td></td>
<td>5.5x10^4</td>
<td>3.0x10^5</td>
</tr>
</tbody>
</table>
A. "Control"

- **CD45.2 FITC (97.7%)**
  - 43.6%

- **CD45.1 FITC (1.8%)**
  - 1.5%

TKO

- **CD45.2 FITC (56.2%)**
  - 18%

- **CD45.1 FITC (43.2%)**
  - 0.4%

B.

- **CD45.2 FITC (97.7%)**
  - 9.6%

- **CD45.1 FITC (1.8%)**
  - 1.5%

- **CD45.2 FITC (56.2%)**
  - 0%

- **CD45.1 FITC (43.2%)**
  - 25%
Figure 8. *E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (TKO) hematopoietic progenitors display a reduced efficiency in contributing to circulating myeloid cells and lymphocytes. FACs analysis of (A) mature B cells or (B) granulocytes and macrophages present in the peripheral blood of irradiated host animals reconstituted with fetal liver stem cells derived from control (left) or *E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (TKO (right)) embryos. Cells were initially sorted by whether they were derived from CD45.2 positive donor cells (top) or CD45.1 positive host cells (bottom).
REFERENCES


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CHAPTER FOUR

CONCLUSIONS
This study has investigated the roles of E2F4, p107, and p130 in regulating cell cycle control, differentiation, and development. This study has served to emphasize the importance of these proteins in mediating withdrawal from the cell cycle and repressing the expression of E2F responsive genes (Chapter 2 and Appendix C). In addition, this study has examined the role of E2F4, p107, and p130 in regulating terminal differentiation both in vitro and in vivo. I have found that E2F4 repressive complexes function to inhibit the formation of adipocytes in culture (Chapter 2 and Appendix B). E2F4 appears to regulate this process, in part through a manner that is independent of its ability to interact with the pocket protein family and regulate E2F responsive gene expression (Chapter 2). I have also shown that E2F4•p130 repressive complexes are required for the development of a number of tissues in vivo (Chapter 3). Finally, E2F•pocket protein repressive complexes are required for the appropriate development of the hematopoietic system (Chapter 3). All of these findings will be discussed in detail below.

**The role of E2F and pocket proteins in the differentiation of white adipose tissue.**

In this study we wished to examine the role of E2F and pocket proteins in white adipose tissue (WAT) differentiation. The development of mature adipocytes in vivo does not occur until after birth, when the animal begins feeding (as reviewed in Rangwala and Lazar, 2000). Many of the compound mutant mice generated for this study (Chapters 2 & 3), however, are neonatal lethal. In order to address this question, in vitro differentiation assays using compound mutant mouse embryonic fibroblasts (MEFs) were
established. The process of adipogenesis in vitro has three distinct stages. First, the hormone insulin, dexamethasone (a glucocorticoid receptor agonist), and methylisobutylxanthine (a cAMP phosphodiesterase inhibitor) are added to confluence-arrested cells. The three chemicals stimulate the cells to re-enter the cell cycle and undergo one to two rounds of mitotic clonal expansion. Finally, the cells terminally arrest and differentiate into adipocytes (as reviewed in Rosen and Spiegelman, 2000). E2F4•p130 repressive complexes are present at confluence, disappear during clonal expansion, and return as cells permanently arrest (Richon et al., 1997). Previous studies using MEFs deficient for p107 and p130 showed that these cells could differentiate into white adipose tissue more efficiently than the controls. This suggested an inhibitory role for p107 and p130 in this process (Classon et al., 2000a). The enhanced ability of p107−/−;p130−/− MEFs to differentiate into adipocytes could not be separated from the ability of p107 and p130 to regulate the cell cycle, however, as these MEFs have defects in responding to G1 arrest stimuli (Bruce et al., 2000; Classon et al., 2000b; Hurford et al., 1997).

Cells deficient for E2F4, the major downstream target of p107 and p130, display an increase in adipogenesis in response to both hormone induction and confluence (Chapter 2 and Appendix B). The ability to differentiate into adipocytes in response to confluence alone requires the loss of E2F4 as cells maintaining wild type E2F4 such as p107−/−;p130−/− MEFs never spontaneously differentiate (Chapter 2). This phenotype was independent of the ability of E2F4 to regulate cell cycle progression, as E2f4−/− MEFs have no obvious cell cycle phenotype (Appendix A, Chapter 2, and Rempel et al., 2000).

The ability of E2F4 to repress adipogenesis is novel to this E2F family member. Loss of the activating E2Fs represses adipocyte formation (Appendix B). It is thought
that these E2Fs function to promote adipogenesis in part by stimulating cell cycle entry during the clonal expansion phase (Appendix B). Loss of the other repressive E2F, E2F5, had no effect on adipocyte formation in either a wild-type or E2f4−/− background (Chapter 2).

Loss of p107 and p130 in an E2F4 deficient background significantly enhanced adipogenesis. E2f4−/−;p107−/− MEFs displayed no changes in the ability to differentiate into adipocytes from E2f4−/− MEFs. Loss of p130 in an E2f4−/− background significantly enhanced adipogenesis (Chapter 2). These observations provide important insight into the relative roles of p107 and p130. To date, the analysis of both development and cell cycle defects in the p107;p130 compound mutant mice have largely highlighted the overlapping properties of these proteins (Cobrinik et al., 1996; Hurford et al., 1997; Lee et al., 1996). For example, loss of either p107 or p130 does not result in profound developmental defects, but p107−/−;p130−/− mice are neonatal lethal (Cobrinik et al., 1996; Lee et al., 1996). In contrast, our differentiation studies provide clear evidence for differential properties of p107 and p130. This result reflects the fact that p130 is predominant pocket protein in arrested cells, while p107 is at its highest levels in cycling cells (Moberg et al., 1996).

The increase in adipogenesis that occurs when p130 is lost in an E2F4 deficient background is independent of its role in regulating progression through the cell cycle since E2f4−/−;p130−/− MEFs do not have defects in maintaining a G1 arrest (Chapter 2). At this time it cannot be ruled out if E2F4 and p130 work through distinct pathways or if their effects on adipogenesis are synergistic. It is possible that E2F4 and p130 have both overlapping and distinct functions in regulating adipocyte differentiation. Evidence for
this model is that an E2F4 mutant lacking the pocket protein binding domain can significantly, but not completely, reduce the adipogenic potential of *E2f4^-/-* MEFs to that of the control (Chapter 2).

There is evidence that the inhibitory role of E2F4 in adipogenesis may not require the presence of E2F4 in pocket protein repressive complexes in the nucleus. E2F4 lacks a nuclear localization signal and is actively exported from the nucleus in a CRM1 dependent manner (Gaubatz et al., 2001; Lindeman et al., 1997; Verona et al., 1997). Chromatin immunoprecipitation studies in *p107^-/-;p130^-/-* MEFs reveal that E2F4 is not present at known E2F responsive promoters, suggesting that p107 and p130 might be required to bring E2F4 to the promoters of E2F responsive target genes (Rayman et al., 2002). The increase in differentiation observed when E2F4 is lost in a *p107^-/-;p130^-/-* background could therefore be due to a specific requirement for E2F4 to repress adipogenesis through interacting with proteins in the cytoplasm. There has been precedence for this mode of action. E2F4 has been found to interact with members of the Smad family. This interaction has been shown to repress expression from the *c-myc* promoter (Chen et al., 2002). It remains to be determined if E2F4 represses adipogenesis by interacting with cytoplasmic proteins. The allele of p130 used in this study has been shown to not be a complete null, which leaves open the possibility that residual p130 activity could bring E2F4 into the nucleus (Rossi et al., 2002). Future experiments conducting chromatin immunoprecipitation in the MEFs used for this study could resolve this issue.

Previous studies have shown that *Rb^-^-* MEFs are impaired in differentiating into adipocytes (Chen et al., 1996a). There have been two mechanisms proposed to describe
this defect. Loss of pRB has been shown to result in defects in withdrawing from the cell cycle and the regulation of E2F target gene expression (Herrera et al., 1996; Hurford et al., 1997). In addition, it has recently been proposed that pRB regulates adipogenesis in part by repressing the expression of PPARγ, an E2F responsive gene that is required for adipogenesis, by recruiting HDAC3 (Appendix B and Fajas et al., 2002). It has been proposed that the defects in forming adipocytes could be due to the inability of Rb+ cells to terminally withdrawal from the cell cycle. The second mechanism is that pRB regulates adipogenesis by functioning as a coactivator for C/EBPβ, a transcription factor necessary for adipocyte induction (Charles et al., 2001; Chen et al., 1996a; Chen et al., 1996b). Loss of E2F4 in an Rb− background completely suppresses the cell cycle phenotype but does not rescue the defect in adipocyte induction (Chapter 2 and Lee et al., 2002). It has previously been demonstrated that loss of E2F4 in an Rb+− background leads to repression of the activating E2Fs by p107 and p130, reducing the tumor spectrum observed (Lee et al., 2002). One could imagine a similar scenario in Rb−−;E2f−− MEFs that rescues the inappropriate proliferation observed upon loss of pRB. This represents the first evidence that the requirement for pRB in adipogenesis is independent of its role in cell cycle regulation. Moreover, it shows that pRB acts downstream of E2F4 in the regulation of adipogenesis.

Taken together this data is the first direct evidence for a direct role for E2F4 and the pocket protein family in the regulation of adipogenesis in a manner that is distinct from their known cell cycle inhibitory roles. A preliminary model for the timing of the action of these proteins during adipogenesis is shown in Figure 1. Given that data it appears that p107 and p130 are required for confluence arrest and for terminal cell cycle
exit as cell deficient for these two proteins fail to arrest appropriately. E2F4 appears to act to inhibit entry to the clonal expansion phase and upstream of pRB and C/EBPβ.

Future studies will be necessary to determine the nature and order of the precise mechanism(s) through which these proteins regulate adipogenesis. Though there is a known mechanism for pRB in promoting this process through interacting with the C/EBP family of transcription factors (Charles et al., 2001; Chen et al., 1996a; Chen et al., 1996b), it remains to be determined if E2F4, p107, or p130 could represses adipogenesis through a similar mechanism. One possible mechanism would be for E2F4, p107, and/or p130 to sequester C/EBP family members, preventing them from interacting with pRB and activating adipocyte specific gene expression. Additionally, it would be of interest in the future to use the compound and single mutant MEFs to extend the analysis begun in this study to investigate the role of E2F•pocket protein repressive complexes in regulating other differentiation processes. It is possible that E2F4, p107, and/or p130 repress the differentiation of other tissues in a manner similar to that observed for adipocytes.

**E2F•pocket protein repressive complexes are required for development**

A second focus of this study was to use E2f4−/−;p130−/−, E2f4−/−;p107−/−;p130−/− (KHK), and E2f4−/−;p107−/−;p130−/− (TKO) mice to investigate the role for E2F•pocket protein repressive complexes for development in vivo. Both p107−/− and p130−/− mice in a 129/Sv: C57BL/6J background do not have an apparent phenotype (Cobrinik et al., 1996; Lee et al., 1996). Mice deficient for both p107 and p130 are not viable at birth and exhibit defects in endochondral ossification (long bone formation) due to chondrocytes
that fail to appropriately withdraw from the cell cycle (Cobrinik et al., 1996; Rossi et al., 2002). E2F4 is the major downstream target of p107 and p130, but mice deficient for this E2F do not resemble p107−/− or p130−/− single or compound mutant mice. E2f4−/− mice exhibit growth retardation and an increase in susceptibility to rhinitis and otitis resulting from cranio-facial defects (Appendix A and (Rempel et al., 2000)). These mice also display a defect in end-stage erythropoiesis resulting in fetal macrocytic anemia and the presence of abnormally formed red blood cells (Appendix A and Rempel et al., 2000). The failure for E2f4−/− mice to resemble p107−/−;p130−/− mice could be due to compensation by E2F5. In order to dissect the roles of E2F•pocket protein repressive complexes in vivo, mice lacking E2F4, p130, and p107 were generated. Analysis of the compound mutant mice revealed a requirement for E2F4•pocket protein repressive complexes in the appropriate development of a number of tissues.

**Mice deficient for E2f4 and p130 display tissue specific defects.**

E2f4−/−;p107−/−;p130−/− and E2f4−/−;p130−/− mice displayed cranio-facial defects and defects in end stage hematopoiesis, phenotypes associated with loss of E2F4. An additional E2F4-related phenotype observed with increased severity in E2f4 and p130 deficient mice was growth retardation. E2f4−/−;p107−/−;p130−/− and E2f4−/−;p130−/− mice also exhibited a number of novel phenotypes that are not observed in any of the parental strains. E2f4−/−;p130−/− and E2f4−/−;p107−/−;p130−/− displayed defects in the formation of bone, testes, the cerebellum, and white adipose tissue (Chapter 2). Many of these defects will be the subject of future studies. It is important to note that the defect in white adipose tissue formation in these animals was the most striking. At the time of death,
mutant animals were observed with food in their stomachs but failed to have subcutaneous white adipose tissue fat pads (Chapter 3).

The phenotype of E2f4 and p130 deficient mice is in direct contrast to the ability of E2f4⁺ⁿ; p130⁻⁺ MEFs to differentiate in adipocytes in vitro (Chapter 2). There are several potential explanations for this discrepancy. The first possibility is that there is a defect in the mesenchymal stem cell pool from which adipocytes arise in vivo. Our analysis of the ability of E2f4⁺ⁿ; p130⁻⁺ MEFs to differentiate into adipocytes revealed that when subjected to stimuli such as confluence or hormone induction, the cell population differentiates with increased efficiency (Chapter 2). It is possible that the mesenchymal stem cell population exhausts itself in vivo by default-differentiating during development instead of self-renewing. The loss or reduction of the stem cell populations would result in the failure to form adipocytes observed in E2f4⁺ⁿ; p130⁻⁺ and E2f4⁺ⁿ; p107⁻⁺; 130⁻⁺ mice. Additional support for this model is the observation that the bones of mice lacking E2F4 and p130 are abnormally thin. Osteoblasts, the cellular component of the bone responsible for producing matrix and maintaining its integrity, also arise from mesenchymal stem cells (as reviewed in Karsenty, 1999). Both adipocytes and osteoblasts arise later in development than skeletal muscle, another tissue derived from the mesenchyme (Karsenty, 1999). Skeletal muscle histologically appears normal in E2f4⁻⁻; p107⁻⁻; p130⁻⁻ and E2f4⁻⁻; p130⁻⁻ mice (our unpublished data). Future experiments examining the differentiation of osteoblasts and myocytes in vivo will be informative in examining the ability of these mesenchymally-derived cells to differentiate normally. Additionally, it would be useful to study the ability of MEFs deficient for E2F4 and p130 to differentiate into osteoblasts and myocytes in vitro.
The second potential explanation for the phenotype of both $E2f4^{-/};p107^{+/};p130^{-/}$ and $E2f4^{-/};p130^{-/}$ mice is that E2F4 and p130 influence the development of these tissues in a non-cell autonomous manner. For example, loss of E2F4 and p130 could affect the production of one or more hormones resulting in all of the phenotypes observed in these animals. To address this issue in the future, chimeras could be generated using embryonic stem cells deficient for E2F4 and p130. If $E2f4^{-/-};p130^{-/-}$ mice fail to form white adipose tissue due to a non-cell autonomous defect, we would expect that cells lacking E2F4 and p130 should still be able to contribute to white adipose tissue fat pads. If the phenotype of the $E2f4^{-/-};p130^{-/-}$ mice is due to defects in the ability of the mesenchymal stem cells to self-renew, the chimera should have a reduction in the contribution of $E2f4^{-/-};p130^{-/-}$ cells to mesenchymally derived lineages.

An alternative way to investigate the defects observed in $E2f4^{-/-};p107^{+/};p130^{-/-}$ and $E2f4^{-/-};p130^{-/-}$ mice would be to rederive the E2F4 mutation in $p130^{-/-}$ embryonic stem cells by flanking the $E2F4$ gene with lox sites. $E2f4^{lox/lox};p130^{-/-}$ mice can be crossed to mice carrying CRE driven off of tissue specific promoters. This would catalyze tissue specific loss of E2F4 in a p130 deficient background. These mice would be critical for addressing the requirement for E2F4•p130 repressive complexes in regulating the development of not only white adipose tissue but also of bones, testes, and the cerebellum.
E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (TKO) mice exhibit hypoplastic anemia and defects in endochondral ossification.

The requirement for E2F4•pocket protein repressive complexes was investigated by examining mice deficient for E2F4, p107, and p130. E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (TKO) mice fail to thrive at birth and exhibit a low level of embryonic lethality. The neonatal lethality can be attributed to a defect in endochondral ossification that is very similar to that observed in p107<sup>−/−</sup>;p130<sup>−/−</sup> embryos (Chapter 3 and Cobrinik et al., 1996). This phenotype (in the TKO) is most likely due to hyperproliferative chondrocytes. This is supported by the fact that TKO MEFs have the same defect in maintaining an arrest in G<sub>1</sub> as p107<sup>−/−</sup>;p130<sup>−/−</sup> MEFs (Chapter 2). The difference in the phenotype of E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> mice is that the bones of their limbs appear thinner while the bones of p107<sup>−/−</sup>;p130<sup>−/−</sup> mice are thicker than those of the control (Chapter 3 and Cobrinik et al., 1996). As discussed above, E2f4<sup>−/−</sup>;p130<sup>−/−</sup> have thinner bones than their littermate controls. This shift in phenotype could be due to a requirement for E2F4 and p130 containing repressive complexes in mediating osteoblast differentiation.

Mice deficient for E2F4, p107, and p130 also display a low incidence of embryonic lethality that can most likely be attributed to hypoplastic anemia, a severe reduction of the hematopoietic compartment (Chapter 3). This phenotype is not fully penetrant, and displays varying degrees of severity. Adoptive transfer experiments performed show that this phenotype is cell autonomous, as fetal liver taken from E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> embryos do not contribute as efficiently to the hematopoietic compartment of the donor animals (Chapter 3). There are three possible explanations for this
phenotype. The first is that there is a defect in the ability of \( E2f4^{-/-};p107^{-/-};p130^{-/-} \) (TKO) hematopoietic cells to migrate between sites of hematopoiesis. Late stage \( E2f4^{-/-};p107^{-/-};p130^{-/-} \) embryos have been found that lack hematopoiesis in the bone marrow (the site of hematopoietic activity at the end of gestation) but have progenitors present in the liver (Chapter 3). This mechanism however, may not explain the phenotype in TKO mice as cells derived from \( E2f4^{-/-};p107^{-/-};p130^{-/-} \) donors are present in the bone marrow of recipients.

The second possibility is that loss of E2F4, p107, and p130 leads to an inability of hematopoietic cells to appropriately differentiate into mature cells. This would suggest that E2F4•p107 and E2F4•p130 repressive complexes are required for mediating terminal cell cycle withdrawal and creating conditions favorable for terminal differentiation. There is data from this study that supports this model. Irradiated mice that have received donor cells from \( E2f4^{-/-};p107^{-/-};p130^{-/-} \) embryos display a striking reduction in donor derived mature granulocytes, natural killer cells, macrophages, and B cells (Chapter 3).

A defect in the production of mature hematopoietic lineages should lead to an increase in the population of immature cells. This is not observed in mice receiving donor cells derived from \( E2f4^{-/-};p107^{-/-};p130^{-/-} \) embryos. Instead, there is a reduction in number of donor derived hematopoietic cells from all stages of maturity (Chapter 3). It is also possible that the immature cells undergo apoptosis in failing to terminally differentiate successfully. In this scenario the hematopoietic cells continue to proliferate inappropriately after the differentiation program has begun causing an apoptotic response. There is precedence that loss of p107 and p130 containing repressive
complexes could lead to inappropriate apoptosis when cells continue to proliferate in the presence of arrest signals. I have found that MEFs deficient for p107 and p130 undergo apoptosis in response to conditions of low serum (Appendix C). It therefore is possible that a similar response occurs in E2f4−/−;p107−/−;p130−/− hematopoietic cells. This mechanism might not account for the phenotype as the apoptosis documented in MEFs is due to the loss of p107 and p130 and is not enhanced by subsequent loss of E2F4 (Appendix C). Mice deficient for both of these pocket proteins, however, have not been documented to exhibit hypoplastic anemia (our unpublished data and Cobrinik et al., 1996). It might be possible that, in the hematopoietic compartment, subsequent loss of E2F4 in a p107−/−;p130−/− background could create the conditions under which inappropriate apoptosis would be favored.

Assays to detect apoptosis performed in E2f4−/−;p107−/−;p130−/− embryos have not shown that there are aberrant levels of apoptosis in any tissues (Chapter 3). It has yet to be determined if E2f4−/−;p107−/−;p130−/− derived donor cells apoptose in the recipient animals from adoptive transfer experiments. Future experiments focusing on performing assays to detect apoptosis in lethally irradiated mice receiving E2f4−/−;p107−/−;p130−/− fetal livers will be informative in accepting this hypothesis. An additional experiment that could be of help in determining if this model can account for the phenotype observed is to perform in vitro progenitor assays. If colonies of mature hematopoietic cells fail to form in this assay it would suggest that this phenotype arises from a direct defect in end-stage differentiation.
The final possibility is that there is a defect in the ability of the hematopoietic progenitors from $E2f4^{-/-};p107^{-/-};p130^{-/-}$ animals to self-renew. Many of the lethally irradiated animals receiving livers from $E2f4^{-/-};p107^{-/-};p130^{-/-}$ mice did not survive. Those animals that lived to the completion of the experiment displayed compensation from their own hematopoietic compartment (Chapter 3). Therefore, one could imagine that in the absence of E2F4, p107, and p130 the progenitor cells are predisposed to differentiating instead of self-renewing. *In vitro* white adipose tissue differentiation assays using $E2f4^{-/-};p107^{-/-};p130^{-/-}$ MEFs revealed that these cells would almost completely differentiate into white adipose tissue in response to confluence alone (Chapter 2). As stated above, white adipose tissue only forms in wild type cells when they are hormonally induced. This ability for the entire population to spontaneously default-differentiate could also explain the phenotype of the hematopoietic compartment. This would suggest that E2F4, p107, and p130 function to prevent inappropriate differentiation.

To test if this third mechanism explains the phenotype observed in $E2f4^{-/-};p107^{-/-};p130^{-/-}$ mice serial adoptive transfer experiments can be performed. In this experiment the hematopoietic compartment of lethally irradiated mice would be reconstituted with fetal liver cells from a donor animal. The bone marrow of these mice will be flushed and used to reconstitute the hematopoietic component of a second lethally irradiated mouse. If there is a defect in the ability of the hematopoietic compartment to self-renew, the second recipient of $E2f4^{-/-};p107^{-/-};p130^{-/-}$ derived cells should not be able to survive without displaying compensation for their own hematopoietic compartment.
Taken together, my results show that E2F·pocket protein repressive complexes are required for appropriate cell cycle exit. My data also reveals a role for E2F·p130 complexes in regulating differentiation and development independently from their well-characterized roles in controlling the cell cycle. Finally, the data has demonstrated that E2F·p130 and E2F·p107 repressive complexes are required for the development of the hematopoietic compartment. Future experiments will be informative in determining the exact mechanisms for these proteins in regulating terminal differentiation and development.
Figure 1. Proposed role of pRB pathway in adipogenesis. Possible mechanism for action of E2Fs and pocket proteins in regulating adipocyte formation.
REFERENCES


APPENDIX A

E2F4 is essential for normal erythrocyte maturation and neonatal viability

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Author’s contribution : Figure 1H


ABSTRACT

The retinoblastoma protein (pRB) plays a key role in the control of normal development and proliferation through the regulation of the E2F transcription factors. We generated a mutant mouse model to assess the in vivo role of the predominant E2F family member, E2F4. Remarkably, loss of E2F4 had no detectable effect on either cell cycle arrest or proliferation. However, E2F4 was essential for normal development. E2f4−/− mice died of an increased susceptibility to opportunistic infections that appeared to result from craniofacial defects. They also displayed a variety of erythroid abnormalities that arose from a cell autonomous defect in late stage maturation. This suggests that E2F4 makes a major contribution to the control of erythrocyte development by the pRB tumor suppressor.
INTRODUCTION

The E2F transcription factors are key regulators of cell cycle progression that control the expression of genes required for the G1/S transition (reviewed in Dyson 1998; Helin 1998). The activity of E2F is regulated by the retinoblastoma protein (pRB), a tumor suppressor that is functionally inactivated in most human tumors (reviewed in Dyson at al. 1998). pRB binds to E2F during the G1 phase of the cell cycle. This inhibits the transcriptional activity of E2F and the resulting complex actively represses E2F-responsive genes through the recruitment of histone deacetylases (reviewed in Brehm and Kouzarides 1999). Repression is relieved at the G1/S transition through pRB phosphorylation by the cyclin dependent kinases. In this manner, E2F participates in both the repression and activation of E2F responsive genes.

pRB belongs to a family of proteins, called the pocket proteins, that includes two additional members, p107 and p130 (reviewed in Dyson 1998). p107 and p130 can also bind to E2F and mediate the transcriptional repression of E2F-responsive genes (Starostik et al. 1996; Zwicker et al. 1996; Iavarone and Massague 1999). However, mutant mouse models have revealed dramatic differences in the biological roles of the pocket proteins (reviewed in Mulligan and Jacks 1998). As expected, Rb<sup>+</sup> mutant mice are highly tumor prone. Moreover, Rb<sup>−/−</sup> mice die in utero between day 13.5 and 15.5 of gestation as a result of extensive apoptosis of hepatocytes and erythroid progenitors. In stark contrast, mice deficient for p107 or p130 are viable, healthy, and show no increase in tumor incidence. Mice deficient for p107 and p130
have a defect in chondrocyte proliferation that impairs long bone formation and results in neonatal lethality.

There are significant changes in the regulation of E2F-responsive genes in mouse embryonic fibroblasts (MEFs) lacking various pocket proteins. pRb-deficient MEFs prematurely express both *cyclin E* and *p107* whereas the combined mutation of *p107* and *p130* causes the inappropriate activation of the *DHFR, B-myb, cdc2, E2F-1, TS, RRM2* and *cyclin A2* genes during G₀/G₁ (Herrera et al. 1996; Hurlford et al. 1997; Mulligan et al. 1998). These studies suggest that p107 and p130 together regulate a subset of E2F-responsive genes that are distinct from the pRB-responsive targets.

To date, eight genes encoding components of E2F have been cloned (reviewed in Dyson 1998). Their protein products can be subdivided into two groups, the E2Fs (1 through 6) and the DPs (1 and 2) that heterodimerize to generate functional E2F activity. Although the DP subunit is critical for activity, the biological properties of the E2F•DP complex is determined by the E2F moiety. The E2F family can be divided into three distinct subgroups, based on differences in both their transcriptional properties and their interaction with the pRB-family members.

E2F4 and E2F5 represent one of the E2F subclasses. These E2Fs lack a nuclear localization signal and the E2F4•DP, and E2F5•DP complexes are primarily cytoplasmic in vivo (Lindeman et al. 1997; Muller et al. 1997; Verona et al. 1997). Consistent with this finding, these complexes are poor transcriptional activators and cannot induce quiescent cells to enter S-phase. Nuclear localization of E2F4 and E2F5 seems dependent upon their interaction with pRB-family members. E2F5 binds primarily to p130 in G₀ cells (Moberg et al. 1996). In
contrast, E2F4 is expressed throughout the cell cycle and it accounts for most of the endogenous pRB-, p107- and p130-associated E2F activity (Moberg et al. 1996). These findings suggest that E2F4 and E2F5 are primarily involved in the repression of E2F-responsive genes through the recruitment of pocket proteins and associated histone deacetylases (reviewed in Dyson 1998).

In this study, we have generated E2f4 mutant mouse strains to investigate the role of E2F4 in both development and tumorigenesis. We show that E2F4 is fully dispensable for the control of cellular proliferation but it plays a critical role in terminal differentiation. In particular, E2F4 is essential for end stage maturation of erythroid cells highlighting the importance of the pRB/E2F pathway in the control of erythropoiesis in vivo.
MATERIALS AND METHODS

Construction of E2f4 targeting vectors

We isolated genomic clones encompassing exons encoding the E2f4 start codon, DNA binding domain and dimerization domains from a 129/Sv muring library using standard techniques. An engineered linker containing STOP codons in all three reading frames, and then EcoRI, AccI and SpeI restriction sites was introduced into a SmaI site located at codon 4 of E2f4 coding sequence. A 4.7 kb SpeI fragment was subcloned into the disruption vector pPNT, to form the 5’ targeting arm. A 3.2 kb EagI-HpaI genomic fragment (containing sequences 3’ of the E2F4 dimerization domain exons) was subcloned into the NotI site of this vector to generate the E2F4 targeting vector, pE2f4-neo. The puromycin-resistance targeting vector, pE2f4-puro, was generated using the same 5’ and 3’ E2f4 genomic sequences and a 1.7 kb SalI fragment derived from the pPGKpuro library that contained the PGK selection cassette.

Generation of targeted ES cells and production/maintenance of E2f4 mutant mice

D3 ES cells were electroporated with 50 μg of NotI linearized pE2f4-neo and then selected for resistance to G418 (300μg/ml) and Gancyclovir (0.5μg/ml). DNA from double resistant ES cell clones were digested with EcoRI and analyzed by Southern blotting using as 3’ probe a 1.1 kb Rsal DNA fragment located outside the 3’ targeting arm genomic region. Of the 212 clones analyzed, 22 had a novel 7.1 kb band corresponding to a correctly 3’ targeted ES cell clone (WT, 10.5 kb). AccI digested DNA from these 22 clones was then probed with a 370 bp SpeI DNA fragment outside of the 5’ targeting arm genomic region. 20 of the 22 clones had a
novel 5.8 kb band (WT 7.9 kb) corresponding to a correctly targeted 5’ end and a single integration of the E2F4 targeting vector (as judged by probing with the neo gene). These ES cell clones were injected into 3.5 day C57BL/6 blastocysts and the resulting chimeric mice mated to C57BL/6 females. The targeted E2f4 allele was detected in agouti pups by Southern blotting of tail DNA. PCR of mouse ear punch DNA was then used for subsequent genotyping using the common primer 5’ AACCCTGAACCTATCAAGCCTC, the wild type E2f4-specific primer 5’-ACTGATTTCAGTCGCTGCTC, and the targeting vector specific primer, 5’-CTTCCATTTCGTCACGTCTGC. Homozygous null E2f4 ES cells were generated by electroporating E2f4+/− ES cell clones with 50 μg of NotI linearized pE2f4-puro and then selecting for resistance to puromycin (5 μg/ml), G418 (300 μg/ml) and Gancyclovir (0.5 μg/ml). We identified 3/107 triply-resistant clones that had undergone correct homologous recombination at the remaining wild-type allele.

Viable E2f4−/− mice were generated by treating E2f4+/− mothers with sulfamethoxazole and trimethoprim oral suspension (Berre National Inc, Baltimore) during the last week of pregnancy until weaning.

MEF isolation and analysis

The generation analysis of MEFs was exactly as described by Humbert et al. (2000).

Hematopoietic cell analysis.

Blood samples were collected from avertin or methoxyflurane anesthesized adult animals by retro-orbital bleeding using heparinized glass capillaries and Potassium-EDTA coated sample
collection tubes (Sarstedt, Germany). Peripheral blood counts and erythrocyte parameters were determined with a Bayer H*3 flow cytometer (Bayer Diagnostics, Tarrytown, NY). Newborn and embryonic blood was collected using micro-hematocrit tubes (Microcaps, Drummond Scientific) and 2 μl samples were used for hematocrit determination, blood smears and the calculation of erythrocyte parameters. Blood smears were air dried and stained with Wright-Giemsa stain. Reticulocyte counts were determined both manually and automatically using supravital stains.

In vitro hematopoietic progenitor colony assays were conducted using fetal livers from E14.5-E15.5 embryos. Single cell suspensions were plated in methylcellulose media (MethoCult M3230, Stem Cell Technologies, Vancouver, Canada) containing 20% FCS supplemented with one or more of the following recombinant growth factors: 2 U/ml erythropoietin (Epo), 50 ng/ml rat c-kit ligand (KL), 10 ng/ml IL-3, 0.5 μg/ml human granulocyte colony stimulating factor (G-CSF), 5 ng/ml granulocyte-macrophage stimulating factor (GM-CSF) and 0.5 μg/ml human thrombopoietin (Tpo).

Adoptive transfer assays were performed as described previously (Horwitz et al., 1997). Briefly, fetal livers were genotyped by PCR and 10⁶ cells injected into the tail vein of irradiated host animals (800 rad followed by 400 rad irradiation two hours later). Host animals were treated with antibiotics for 4 weeks and then analyzed for reconstitution of the hematopoietic system using FACS analysis. C57BL/6-CD45.1 mice (NCI; B6-Ly5.2/Cr) were utilized to discriminate host from donor derived cells. Cells of mixed origin (129/sv and C57BL/6) were
distinguished from C57BL/6-CD45.1 cells using a FITC-conjugated CD45.2 antibody (Pharmingen).

Hematopoietic lineages were identified using α-Ter-119-biotin, α-Mac-1-PE, α-GR-1-biotin, α-Thy-1.2-biotin, α-B220-PE, α-CD4, α-CD8 and α-CD25 (Pharmingen).

**Histology and Bone analysis**

For histology, embryos and tissues were fixed in 10% formalin or Bouin’s solution, embedded in paraffin blocks and 6 μm sections were stained with hematoxylin and eosin. For bone analysis, E18.5 embryos were cleared of soft tissue by alkali treatment and stained for cartilage and bone using alcian blue and alizarin red respectively as described in (Kessel and Gruss, 1991). Age and sex matched littermates were radiographed while in left lateral and dorsal recumbancy using a Transworld 325V single tube X-ray machine.
RESULTS

Generation of E2f4 mutant mice.

To establish the role of E2F4 in cell cycle control and development, we functionally inactivated the E2f4 gene in embryonic stem (ES) cells by introducing termination codons in all three open-reading frames after codon 4 and replacing the genomic sequences encompassing the DNA binding and dimerization domains (amino acids 5-192) with a neomycin resistance gene (Figure 1A). After electroporation and G418 selection, correctly targeted E2f4+/− ES cell lines were used to generate chimeric animals. Two independent cell lines (F4-1-61 and F4-1-96) transmitted the mutation into the germline. The following data was obtained primarily from mice and cells derived from the F4-1-61 ES clone but the two mutant strains had identical phenotypes.

The loss of E2F4 dramatically reduces the level of cellular E2F activity.

Since E2F4 accounts for the majority of the endogenous E2F DNA-binding activity, we wanted to establish the cellular consequences of E2F4-loss. We derived wild-type, E2f4+/− and E2f4−/− mouse embryonic fibroblasts (MEFs) from littermate E13.5 embryos and also generated E2f4−/− ES cells by re-targeting the parental E2f4+/− ES cell line with a puromycin-resistant E2f4 targeting vector. Western blotting confirmed that the E2f4+/− MEFs and ES cells no longer expressed the E2F4 protein (Figure 1B). To determine the impact of E2F4 loss on total E2F-DNA binding activity, we performed gel retardation assays using whole cell extracts of asynchronously growing MEF and ES cell cultures (Figure 1C). In wild-type MEFs, E2F4 comprised up to 80% of the endogenous DNA-bound E2F complexes and it was evenly
distributed between “free” and pocket protein E2F complexes (Figure 1C, compare lanes 6 and 15). [Note that antibody-bound supershifted “free” E2F complexes migrate similarly to pocket protein-bound E2F in the absence of antibodies]. In wild-type ES cells, E2F exists almost exclusively in the “free” form and the anti-E2F4 antibody supershifted almost 95% of this activity (Figure 1C, compare lanes 3 and 12). The remaining activity was generated by E2F5 (data not shown).

The level of E2F4 complexes was reduced by approximately 50% in the E2f4+/− MEFs and ES cells and was completely abolished in the E2f4−/− cell lines (Figure 1C). Despite these changes, we did not observe any increase in the level of the other E2F family members at the level of either expression (data not shown) or DNA binding activity (Figure 1C). Consequently, the loss of E2F4 results in the loss of the majority of total cellular E2F activity.

**E2F4-deficient cells show normal proliferation and cell cycle control**

Despite the dramatic reduction in total cellular E2F activity, E2f4+/− MEFs proliferated at the same rate as wild-type or E2f4−/− littermate controls under either low (Figure 1D) or high (Figure 1E) density culture conditions. The cell cycle distribution of asynchronously growing MEFs was also unaffected by the E2f4 mutation (Figure 1F). Similarly, we did not detect any difference in the proliferative capacity of wild-type or E2f4−/− ES cells (data not shown). Thus, E2F4 is not required for the control of proliferation of cells continuously dividing in culture.

The spectrum of E2F complexes is significantly different in quiescent versus actively dividing cells and E2F4 is believed to be required for proper exit from the cell cycle. To test this hypothesis, we compared the ability of wild-type and E2f4−/− MEFs to respond to the withdrawal
and re-addition of serum. Significantly, E2F4-deficient MEFs arrested as efficiently as either wild-type or E2f4−/− littermate controls in low serum conditions (data not shown). Furthermore, upon serum re-addition, the wild-type and E2f4−/− mutant cell lines re-entered the cell cycle with similar kinetics (Figure 1G). Consistent with these findings, we could detect no difference in the cell cycle regulation of splenic B cells and lymph node T cells derived from either wild-type or E2f4−/− adult littermates (data not shown). Therefore, although E2F4 accounts for the majority of the endogenous E2F complexes, it is fully dispensable for the regulation of cell cycle arrest and proliferation in a variety of different cell types.

**Normal expression of E2F-responsive genes in E2F4-deficient MEFs**

Since E2F4 accounts for the majority of the pocket protein-associated E2F activity, we wished to determine whether E2F4-loss would affect the regulation of E2F-responsive genes in a similar manner to that observed in either Rb−/− or p107−/−:p130−/− MEFs. We therefore compared the kinetics of expression of E2F responsive genes in wild-type or E2f4−/− MEFs in response to serum-starvation/re-stimulation (Figure 1H). Compared to the loading control (ARPP P0), we were able to detect a subtle (2 fold) upregulation in the levels of cdc2 and PCNA mRNA during the G1 stage of the cell cycle in the E2f4−/− MEFs. However, this deregulation was far smaller than that observed in the p107−/−:p130−/− MEFs (Hurford et al. 1997). Furthermore, we did not detect any consistent, quantifiable changes in the expression pattern of any other E2F-responsive genes including cyclin E1, cyclin A2, B-myb or DHFR (Figure 1H and data not shown). Thus, consistent with the normal proliferation of E2f4−/− MEFs, E2F4 is fully dispensable for the correct cell cycle regulation of most E2F responsive genes.
Loss of E2F4 causes neonatal lethality

To determine the role of E2F4 in the whole animal, we intercrossed the E2f4<sup>−/−</sup> animals. E2f4<sup>−/−</sup> pups arose at the expected frequency (Table I) but they were pale and their birth weight was 20-30% lower than either wildtype or E2f4<sup>+/−</sup> littermate controls. Most of these E2f4<sup>−/−</sup> animals died within 3-4 days of birth and more than 85% failed to reach weaning age (Table 1). A general histological analysis indicated that the moribund E2f4<sup>−/−</sup> neonates were dehydrated and the most severely affected showed a degeneration of the intestinal tract that was consistent with starvation. Apart from these lesions, we did not detect any obvious morphological defects in the major organs of these animals. These data suggest that the E2f4<sup>−/−</sup> neonates die of dehydration and starvation.

E2F4 loss results in fetal anemia and the production of abnormal erythrocytes

We examined E2f4<sup>−/−</sup> embryos to determine the time of onset of the runted and pale appearance. The growth retardation was readily detected at E13.5 and persisted through subsequent embryonic stages (Figure 2A and data not shown). The small percentage of surviving E2f4<sup>−/−</sup> adults were also growth retarded and consistently weighed 50-65% of littermate controls (Figure 2B and C).

Significantly, there seemed to be a strong correlation between the onset of runting and the pale appearance of the E2f4<sup>−/−</sup> embryos. The difference between wild-type and E2f4<sup>−/−</sup> embryos was relatively subtle at E13.5 but by E15.5-E16.5 it was striking (Figure 2A and data not shown). To establish whether the pallor was due to anemia, we compared the red blood cell packed volume (hematocrit) of the peripheral blood of wild-type, E2f4<sup>+/−</sup> and E2f4<sup>−/−</sup> mutant
littermates at E13.5, E14.5, E16.5 or E18.5 (Figure 2D and data not shown). At each of these stages, the E2F4-deficient embryos showed a 30 to 40% decrease in hematocrit indicating that they were anemic. Furthermore, peripheral blood analysis revealed numerous abnormalities in the red blood cells (RBCs) of the $E2f4^{-/}$ embryos and neonates (Figure 2E and data not shown). In E16.5-E18.5 embryos, the $E2f4^{-/}$ erythrocytes showed a significant variation in size (anisocytosis) and a marked increase in mean cell volume (macrocytosis; Figure 2E). There was a significant increase in the number of morphologically abnormal RBCs and a very high frequency of erythrocytes containing Howell-Jolly bodies, nuclear remnants leftover from improper enucleation (Figure 2E). We also detected an increased presence of reticulocytes, the last stage of immature erythrocytes. All of these defects persisted until at least day 3 postpartum. In addition, the peripheral blood of $E2f4^{-/}$ neonates contained nucleated RBCs (Figure 2E). The increased number of reticulocytes and the presence of nucleated RBCs indicate that stress erythropoiesis is ensuing in the $E2f4^{-/}$ embryos and neonates. Consistent with this notion, we observed a 2 to 3 fold increase in the number of fetal liver erythroid progenitors (CFU-E and BFU-E) in the $E2f4^{-/}$ embryos (Figure 2F). Taken together, these data indicate that the loss of E2F4 causes marked anemia during embryonic development that is associated with increased erythropoiesis, a common physiological response to anemia.

**Erythrocyte maturation defects in adult E2F-4-deficient animals**

To determine whether E2F4 was required for the development and maintenance of the adult hematopoietic system we also examined $E2f4^{-/}$ adult survivors. The $E2f4^{-/}$ adults had a normal hematocrit (Figure 2D) and near normal mean cell hemoglobin levels (14.8 ± 0.7, n=16
for $E2f4^{-/}$ animals compared to $15.9 \pm 0.5$ g/dl, n=8, for wild-type littermates). Therefore, in contrast to $E2f4^{-/-}$ embryos and neonates, the $E2f4^{-/-}$ adult animals were not anemic. Consistent with this finding, there was no increase in the reticulocyte numbers in the $E2f4^{-/-}$ adults indicating that there was no stress erythropoiesis. Despite the lack of anemia, $E2f4^{-/-}$ adult animals displayed a number of defects in the red blood cell compartment. There was a 20 to 30% decrease in the number of peripheral blood RBCs (Figure 2G). As observed in the $E2f4^{-/-}$ neonates, we also detected a significant macrocytosis (Figure 2E and G). FACS analysis confirmed that this was due to a general increase in the RBC size and not the presence of an aberrant RBC population (data not shown). There was no change in the osmotic fragility of the $E2f4^{-/-}$ RBCs, indicating that the increased cell size is not due to osmotic imbalance (data not shown). Finally, as in the $E2f4^{-/-}$ embryos, we also observed a very high frequency of erythrocytes containing Howell-Jolly bodies. Taken together, these data are consistent with a defect in the maturation of the $E2f4^{-/-}$ erythrocytes. In the adult animals, these RBC abnormalities arise in the absence of anemia and consequent stress erythropoiesis suggesting that they are the primary defect.

**The erythroid defects of E2F4-deficient animals arise from an intrinsic defect of the hematopoietic compartment.**

The presence of Howell-Jolly bodies is often indicative of splenic dysfunction. We therefore wished to establish whether or not the RBC defects were cell autonomous. To address this issue, we used wild-type or E2F4-deficient fetal livers as source of CD45.2 stem cells to reconstitute the hematopoietic system of lethally-irradiated C57BL/6-CD45.1 wild-type (WT)
adult hosts. The E2f4\(^+\) fetal liver successfully reconstituted the hematopoietic system of the irradiated animals and gave rise to all of the mature hematopoietic cell subsets, as judged by the use of CD45.1 and CD45.2 markers to distinguish donor from host cells (data not shown). However, animals reconstituted with E2f4\(^+\) hematopoietic cells displayed all of the RBC defects, including decreased RBC numbers, macrocytosis and Howell-Jolly bodies, present in the adult E2f4\(^+\) mice (Figure 2H and data not shown). Moreover, transplantation of the wild-type stem cells into the E2f4\(^+\) host was sufficient to rescue all of these erythroid defects including the presence of Howell-Jolly bodies (Figure 2H and data not shown). Thus, E2F4 is essential for the normal development and maintenance of the red blood cell compartment in a cell autonomous manner. Given the lack of any detectable defect in the generation of immature erythrocytes and the increased presence of abnormal mature red blood cells, we conclude that E2F4 is critical for the late stages of erythrocyte differentiation.

The E2f4\(^+\) mice have an increased susceptibility to bacterial infections.

The small proportion of surviving E2f4\(^-\) animals emitted a distinctive chirping sound suggestive of respiratory constraint. We therefore conducted a full histological analysis of the head of the E2f4\(^-\) adult and neonate animals (Figure 3A). In both cases, the nasal passages (Figure 3A, i, ii, v and vi) and middle ear (Figure 3A, iii and iv) were completely obstructed by a purulent exudate indicating that these animals were suffering from chronic rhinitis and otitis media. Higher magnification of the nasal cavities showed high levels of macrophages and neutrophils (Figure 3A, vii and viii), suggesting that the E2f4\(^-\) animals were responding to an infectious agent. Consistent with this hypothesis, nasal swabs from the E2f4 mutant animals
revealed the high levels of the infective agent *Pasteurella pneumotropica*, a common opportunistic pathogen. To establish the effect of this pathogen, we treated *E2f4<sup>−/−</sup>* mothers with an antibiotic determined to be effective against *Pasteurella pneumotropica*. Remarkably, this treatment rendered the *E2f4<sup>−/−</sup>* neonates predominantly viable (Table I). Despite the severe rhinitis and otitis, there was no evidence of pulmonary infections or generalized septicemia in the *E2f4<sup>−/−</sup>* animals. This suggests that the neonatal lethality of the *E2f4<sup>−/−</sup>* animals is caused by chronic rhinitis that results in impaired feeding and thereby dehydration and starvation.

**An intrinsic hematopoietic cell defect cannot account for the increased susceptibility to infections of the *E2f4<sup>−/−</sup>* mice.**

Given the presence of cell intrinsic defects within the *E2f4<sup>−/−</sup>* erythroid cells, we wished to establish whether the susceptibility to bacterial infections resulted from a defect in the immune cells of the *E2f4<sup>−/−</sup>* animals. Surprisingly, we were unable to detect any difference in the number or distribution of neutrophils, macrophages or mature B and T cells between wild-type and *E2f4<sup>−/−</sup>* adult littermates (Figure 3B and data not shown). Furthermore, there was no significant difference in the proliferative response of wild-type or *E2f4<sup>−/−</sup>* splenic B cells (LPS, IgM or anti-CD40) and thymic T cells (ConA, anti-CD28 or anti-CD3) to a variety of stimulatory agents (data not shown). As an alternative approach, we conducted a full histological examination of our lethally irradiated adoptive transfer mice three months after cessation of antibiotic treatment (Figure 3C). As expected, there was no evidence of either rhinitis or otitis in any of the wild-type or *E2f4<sup>−/−</sup>* animals reconstituted with wild-type hematopoietic stem cells. However, the wild-type immune system was unable to rescue the susceptibility to infection of the *E2f4<sup>−/−</sup>* hosts.
Moreover, the $E2f4^{-/-}$ immune system did not confer susceptibility to infection on the wild-type host. Although we cannot rule out the possibility that there is a subtle defect in the immune compartment that contributes to the susceptibility to upper respiratory tract infections, these data strongly suggest that it results primarily from non-hematopoietic cell defect.

The susceptibility to infections seems to arise from craniofacial defects in $E2f4^{-/-}$ animals.

To further address the cause of the increased susceptibility to infections, we used blastocyst transfer to re-derive the $E2f4$ mutant mice into a Pasteurella pneumotropica-free environment. In this specific pathogen-free (SPF) facility, the E2F4-deficient mutant mice are almost fully viable in absence of antibiotic treatment (data not shown). Consistent with this observation, histological examination did not detect the presence of any bacteria or macrophage infiltrates in the nasal passages or middle ears of the $E2f4^{-/-}$ animals (Figure 3A, ix-xii). However, both cavities contained pools of eosinophilic fluid suggestive of a proteinaceous exudate that was completely absent in either wild-type or $E2f4^{+/+}$ controls. Moreover, the nasal turbinates of the adult animals were highly atrophic, characterized by a flat and unconvoluted structure and the olfactory epithelium was also diffusely atrophic (Figure 3A, xi and xii). These defects are indicative of high pressure resulting from the lack of fluid drainage. Together with adoptive transfer studies, these data suggest that the susceptibility to bacterial infections of the $E2f4^{-/-}$ animals arises from the aberrant accumulation of proteinaceous exudates. Consistent with this hypothesis, the $E2f4^{-/-}$ mice have abnormal facial features with a characteristic bull-like appearance (see Figure 2B). X-ray analysis detects significant craniofacial abnormalities in the $E2f4^{-/-}$ mice including a dramatic shortening and broadening of the snout (Figure 4A). Thus, loss
of E2F4 causes craniofacial defects that result in the aberrant accumulation of proteinaceous secretions, increased susceptibility to bacterial infections and thereby neonatal lethality.

Given the presence of craniofacial defects within the E2f4− mice and our previous finding that E2F4 accounts for the majority of the p107- and p130-associated E2F activity (Moberg et al. 1996), we examined the E2f4− neonates for any evidence of the long bone defects that have been observed in the p107−:p130− mice (Cobrinik et al., 1996). However, there were no detectable differences in either the skeleton or cartilage of wild-type and E2F4-deficient embryos (E18.5) beyond that due to their size difference (Figure 4B). There was also no decrease in the length or thickness of the ulna, radius and humerus of the forelimbs of E2f4− embryos (Figure 4C). Taken together, these data suggest that loss of E2F4 specifically affects the formation of craniofacial structures and does not give rise to any of the bone developmental defects that result from the loss of p107 and p130.

The erythroid and embryonic growth defects of the E2f4− animals do not result from their increased susceptibility to bacterial infections.

We next tested whether the erythroid or growth defects of the E2f4− animals might arise as a secondary consequence of their infection by Pasteurella pneumotropica. Importantly, antibiotic treatment did not rescue any erythroid defects described above. We also observed a similar degree of growth retardation in E2f4− embryos derived from treated versus untreated mothers. In contrast, we did observe a partial rescue of the growth retardation in E2f4− adults that were generated in the presence of antibiotics or a Pasteurella pneumotropica-free environment (from 50% of wild-type controls to 65-80%). Together, these data indicate that the
erythroid defects and the embryonic growth retardation arise completely independently of the
bacterial infection of the $E2f4^+$ animals. Moreover, the increased growth of the adult animals
directly supports our hypothesis that the chronic rhinitis results in impaired feeding and
starvation.

**Analysis of adult $E2f4$ mutant mice.**

Through the antibiotic treatment of $E2f4^{+/+}$ mothers, we were able to generate a large
cohort of wild-type, $E2f4^{++}$ and $E2f4^-^+$ littermate animals and monitor the phenotypic
consequences of E2F4-deficiency throughout their lifespan. We did detect a high level of male
(80%) and female (90%) sterility in the $E2f4^-^+$ animals (data not shown). Histological analysis
did not detect any obvious defects in the testes or ovaries of the $E2f4^-^+$ animals so the underlying
basis is currently unclear. Apart from this sterility, we did not observe any significant
differences between the $E2f4^{+/+}$, $E2f4^{++}$ and $E2f4^-^+$ mutant mice. The mean lifespan of the $E2f4^-^+$
mice was indistinguishable from that of wild-type and $E2f4^{++}$ controls (Figure 5) and these mice
died of a similar spectrum of defects (data not shown). In particular, although E2F4 accounts for
the majority of the transcriptionally repressive pocket protein complexes, there was no difference
in either the spectrum or incidence of tumors between the wild-type, $E2f4^{++}$ and $E2f4^-^+$ mice. We
therefore conclude that E2F4 plays a key role during embryonic development but is not required
for the suppression of tumor formation in the adult animal.
DISCUSSION

The E2F transcription factor plays a critical role in controlling the transcriptional program that regulates S phase entry and cell cycle progression (reviewed in Dyson 1998, Helin 1998). We have used an E2f4 mutant mouse strain to establish the consequences of E2F4-deficiency in both cell cycle regulation and the whole animal. Loss of E2F4 removes the majority of the endogenous E2F-DNA binding activity with little or no compensation by the other E2F family members. Contrary to expectations, this has little or no impact on the cell cycle-dependent regulation of known E2F-responsive genes. Moreover, E2F4 was fully dispensable for either the cell cycle arrest or proliferation of a number of cell lineages, including mouse embryonic fibroblasts, ES cells and mature B and T cells. Despite these findings, we demonstrate that E2F4 is essential for normal development. Although E2F4-deficient animals are born at the expected frequency, they display multiple developmental defects including fetal anemia, growth retardation and an increased susceptibility to bacterial infections. Our analysis of one affected cell type, the red blood cells, demonstrates a critical role for E2F4 in terminal maturation of this lineage.

The role of E2F4 in the control of erythropoiesis

Defects in the terminal maturation of E2F4-deficient erythrocytes cause severe macrocytic anemia in the E2f4- embryos. Anemia could occur through the decreased production of red blood cells and/or the loss of these cells by either inappropriate destruction (hemolysis) or hemorrhage. The increased numbers of erythroid progenitors and reticulocytes observed in the
$E2f4^+$ embryos and neonates respectively strongly suggests that the anemia is not due to ineffective erythropoiesis, that is, insufficient RBC production. Importantly, no qualitative or quantitative abnormalities were detected by microscopic examination of fetal liver or bone marrow preparations, nor was there any histological evidence for inappropriate blood loss in the $E2f4^+$ embryos. It remains to be determined whether the $E2f4^+$ RBCs exhibit a shortened lifespan in the embryo, perhaps as a result of the maturation defect.

The morphological defects of the RBCs persist in the $E2f4^+$ adults but these animals are no longer anemic. In this regard, the $E2f4$ mutant mouse joins the "flexed" and the $STAT5a^+$;$STAT5b^+$ mutant mice in a growing family of mutant mouse strains that display fetal anemia and normal steady state adult erythropoiesis (Russell and Bernstein 1966; Socolovsky et al. 1999). This supports the emerging view that the erythropoietic reserve capacity, that is the animal's ability to increase erythroid output, is minimal in the embryo compared with the adult animal (Moritz et al. 1997; Palis and Segel 1998).

Importantly, the anemia and sustained hypoxia of the $E2f4^+$ embryos seems to adversely affect other aspects of fetal development. First, there is a tight correlation between both the timing of onset and the degree of the growth retardation and the timing of onset and severity of the fetal anemia. In addition, we observed a consistent 4-fold decrease in the number of megakaryocytic progenitors in $E2f4^+$ fetal liver (see Figure 2F). Significantly, Cullen and McDonald (1989) have reported that hypoxia can cause a decrease in murine megakaryocytic progenitors. This strongly suggests that the growth retardation and reduction in megakaryocytic
progenitors arise as a secondary consequence of the anemia and sustained hypoxia of E2f4−/− embryos.

**The mortality of the E2F4-deficient animals.**

Loss of E2F4 also results in a high level of neonatal lethality that is due to an increased susceptibility to bacterial infections. Significantly, the infection seem to be restricted to the nasal passages and middle ear of the E2f4−/− animals and adoptive transfer experiments strongly suggest that this results from an intrinsic defect in the body, and not the immune system, of the E2f4−/− animals. The analysis of E2f4 mutant mice in the SPF facility provides considerable insight into the cause of this defect. In the absence of either infection or ensuing immune response, we see the accumulation of proteinaceous exudates in the nasal cavities and inner ears of the E2f4−/− neonates. The observed dilation of the nasal cavities and the flat, unconvoluted profile of the turninate structures suggest that the build-up of fluid creates considerable pressure. Importantly, in a number of human diseases, chronic rhinitis has been shown to be a common secondary effect of craniofacial bone anomalies (Bollerslev and Mosekilde 1993; Beighton 1995).

Consistent with this observation, E2F4-deficient animals have a shortened and broadened snout and X-ray analysis reveals abnormal bone structure in the muzzle of these animals. Together, these data suggest that the increased infection susceptibility of the E2F4-deficient mice arises from craniofacial bone anomalies that prevent the drainage of proteinaceous exudates and the clearing of the infectious agent.

Through the use of antibiotics and the SPF facility, we have been also able to address the relationship between the increased susceptibility to infections and the other phenotypes of the
E2F4-deficient animals. Although these treatments almost completely rescue the neonatal lethality, the E2F4-deficient neonates still display the characteristic anemia, growth retardation and abnormal nose structure. Thus, these three defects arise completely independently of the upper respiratory tract infections. In fact, we believe that the craniofacial defects, and the resulting susceptibility to infection, may arise as a secondary consequence of the fetal anemia. Previous studies have shown that the population of migrating neural crest cells responsible for establishing the craniofacial structures are particularly sensitive to environmental insults such as hypoxia (Sulik et al. 1988). In this manner, the hypoxic stress of the anemic E2f4−/− embryos could cause the craniofacial abnormalities through the inappropriate apoptosis of these migrating neural crest cells.

**Implications for the relative roles of the E2F and RB family members.**

Mutant mouse models have been generated previously for E2f1 (Field et al. 1996; Yamasaki et al., 1996), E2f3 (Humbert et al. 2000) and E2f5 (Lindeman et al. 1998). Comparison of their phenotypes with those arising in the E2f4 mutant mice underscores the emerging view that there are general similarities in the properties of E2Fs within a given E2F subgroup. E2f1 (Pan et al. 1998; Tsai et al. 1998; Yamasaki et al. 1998) and E2f3 (Humbert et al. 2000) play a critical role in controlling the proliferation of normal and/or tumor cells. In contrast, E2f4 (this study) and E2f5 (Linderman et al. 1998) are required for correct terminal differentiation and development. However, it is now clear that each one of the E2F mutant mice has a different spectrum of phenotypes. Thus, even members of the same E2F subgroup have
distinct biological roles that must result from differences in their expression, activity and/or regulation in vivo.

E2F4 accounts for the majority of endogenous E2F DNA binding activity and is thought to play a key role in mediating the transcriptional repressive properties of pRB, p107 and p130. Consequently, comparison of the phenotypes of E2f4 and Rb, p107 and p130 mutant mice provides considerable insight into the biological roles of the E2F-pocket protein complexes. Mice deficient for p107 and p130 have a defect in long bone development that results from the failure of chondrocytes to arrest and differentiate at the correct developmental stage (Cobrinik et al. 1996). In addition, p107<sup>-/-</sup>:p130<sup>-/-</sup> mutant MEFs enter S-phase prematurely and this correlates with the dramatic deregulation of many E2F-responsive genes (Hurford et al., 1997; Mulligan et al. 1998). These data support the notion that p107•E2F and p130•E2F complexes play a key role in mediating the repression of E2F-responsive genes during G<sub>S</sub>/G<sub>1</sub>. However, our data indicate that loss of E2F-4, and therefore the vast majority of the p107•E2F and p130•E2F complexes, does not cause any defect in long bone formation or cell cycle regulation and it has little or no effect on the expression of E2F-responsive genes. Thus, loss of E2F4 is not equivalent to loss of p107 and p130. These findings support two alternative models of p107/p130 action. First, correct cell cycle regulation could be dependent upon the formation of transcriptionally repressive p107•E2F and p130•E2F complexes but E2F5 is sufficient to mediate this effect in the absence of E2F4. Alternatively, loss of p107 and p130 could lead to the deregulation of E2F-responsive genes and thereby S-phase entry through the inappropriate release of “free”, transcriptionally active E2F4 and E2F5 complexes. This would be entirely analogous to the
finding that ectopic S phase entry and aberrant apoptosis in Rb-deficient embryos or tumors is largely due to the inappropriate activation of E2F1, a pRB-specific E2F (Pan et al. 1998; Tsai et al. 1998; Yamasaki et al. 1998). However, it is inconsistent with the current belief that, at least in normal cells, E2F4 and E2F5 make little or no contribution to the activation of E2F-responsive genes (Verona et al. 1997).

In contrast to p107 and p130, we have found significant similarities in the roles of E2F4 and pRB in vivo. pRb-deficient mice die at E13.5 from ineffective erythropoiesis (reviewed in Mulligan and Jacks 1998). This phenotype is complex and results from defects in both RBCs and hepatocytes. However, there is evidence to support the presence of a cell-autonomous component to the erythroid defect. First, embryos with high Rb+ ES cell contribution display abnormal nucleated erythrocytes peaking at E16.5 of development (Maandag et al., 1994). Second, pRB-deficient erythroid progenitor cells can repopulate the bone marrow and periphery of wild-type adoptive transfer animals but there is a high frequency of nucleated erythrocytes and these animals develop anemia within a year (Hu et al., 1997). In this study, we now show that E2F4 also plays a critical role in normal erythroid maturation in a cell intrinsic manner. Taken together, these data suggest that E2F4 makes a major contribution to the regulation of erythropoietic system development by the RB tumor suppressor.

ACKNOWLEDGEMENTS

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Socolovsky, B. Paw, N. Trede, P. Vyas, M. Fleming, J. Sevigny, S. Robson for helpful discussions and B. Weinberg, T. Jacks, F. Gertler and the Lees lab for comments on the manuscript. P.H. was supported by fellowships from the Anna Fuller Fund and Merck. This work was supported by grants from the NIH and Amgen to J.A.L.
Figure 1. Cell cycle and E2F-responsive gene regulation in the E2f4 mutant MEFs. (A) The E2f4 gene was inactivated in embryonic stem cells using the indicated targeting construct. The asterisk (*) indicates novel restriction sites inserted for screening purposes. A, AccI; E, EcoRI; Sm, Sma I; N, Not I; X, Xba I. (B) The expression of E2F4, was determined by western blot analysis of whole cell extracts generated from wild-type, E2f4+/+ or E2f4−/− MEFs or ES cells. (C) The composition of E2F complexes in asynchronously dividing wild-type, E2f4+/+ or E2f4−/− MEFs or ES cells was determined by gel shift analysis. E2F4 complexes were identified by the addition of antibodies to E2F4 (LLF4-2). The growth rate of wild-type, E2f4+/+ or E2f4−/− MEFs (D) plated at low density (2 x 10⁴ cells/6cm dish) and followed for 10 days or (E) maintained at high density for 38 days by trypsinizing, counting and replating (2 x 10⁵ cells/6cm dish) every three days. For the high density experiment, the plot shows the calculated cumulative growth. (F) Cell cycle distribution of asynchronous E2f4-mutant MEFs. (G) [H]-Thymidine incorporation of MEFs following synchronization and serum release. (H) Northern blot analysis of E2F target genes in E2F4-deficient MEFs.
Table 1. Genotype distribution of progeny from heterozygous matings

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Figure 2. Loss of E2F4 results in cell-autonomous erythroid defects, fetal anemia and growth retardation. (A) The E16.5 E2f4\textsuperscript{-/-} embryo (right) is pale and growth retarded compared to the wild-type littermate control (left). (B) At 5 weeks old, the E2F4-deficient mice (right) is still substantially smaller than the wild-type littermate (left). (C) Representative weight curve of E2f4\textsuperscript{+/-}, E2f4\textsuperscript{+/+} and E2f4\textsuperscript{-/-} female littermate mice. (Similar data was obtained for male animals). (D) Hematocrit of peripheral blood from E14.5, E16.5, E18.5 and adult E2f4\textsuperscript{+/-}, E2f4\textsuperscript{+/+} and E2f4\textsuperscript{-/-} littermates. Each circle represents data derived from a single animal. (E) Wright-giemsa staining of E18.5 embryo and adult blood smears reveals morphological defects in the E2f4\textsuperscript{-/-} erythrocytes. Howell-Jolly bodies are indicated by the arrowhead. (F) In vitro differentiation of wild-type, E2f4\textsuperscript{+/-} or E2f4\textsuperscript{-/-} E15.5 fetal liver cells into CFU-E, BFU-E, megakaryocyte or macrophage/granulocyte lineages. Each bar represents data derived from a different animal. (G) RBC count and mean cell volume (MCV) of peripheral blood from E2f4\textsuperscript{+/-} (n=10), E2f4\textsuperscript{+/+} (n=11) and E2f4\textsuperscript{-/-} (n=14) adult littermates. Each circle represents data derived from a single animal. (H) Analysis of the peripheral blood of the indicated adoptive transfer donor-recipient combinations. Each circle represents data derived from a single animal.
A. $+/+$ ii $-/-$

B. $+/+$ $-/-$

C. Incidence of opportunistic infections in reconstituted adoptive transfer animals

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Figure 3: *Chronic rhinitis and otitis in E2F4-deficient adults and neonates.*

(A) (i-viii) Hematoxylin- and eosin-stained coronal sections of *E2f4* wild-type and mutant animals maintained in the absence of any antibiotic treatment in the normal animal facility. (i and ii) Adult nasal passages. (iii and iv) Adult middle ear. (v - viii) Neonatal (three day postpartum) nasal passages at low (v and vi) or high (vii and viii) magnification. (ix - xii) Hematoxylin- and eosin-stained coronal sections of *E2f4* wild-type and mutant animals maintained in the SPF facility in the absence of antibiotics. (ix and x) Neonatal (three day postpartum) nasal passages. (xi and xii) High magnification of turbinate structures of adult nasal passage. The arrowhead indicates the purulent exudate and the eosinophilic fluid in animals maintained in the normal or SPF facilities respectively. ns, nasal septum; m, malleus; tm, tympanic membrane; nc, nasal cavity, ne, nasal epithilium. (B) FACS analysis of splenic B cells (upper two panels) and thymic T cells (lower two panels) from 6 month old wild-type or *E2f4* littermates. (C) Incidence of opportunistic infections in the lethally-irradiated adoptive transfer animals.
Figure 4: E2F4-deficiency results in craniofacial defects.

(A) X-ray radiography reveals shortened and broadened nasal bones in \( E2f4^{−/−} \) adult mice (right, arrow head) relative to wild-type adult littermate control (left). (B) Alcian blue and Alizarin red staining of cleared E18.5 embryos to highlight cartilage and bone structures, respectively. (C) The humerus (h), radius (r) and ulna (u) were normally developed in \( E2f4^{−/−} \) embryos as compared to wild-type embryos.
Figure 5: *E2F4*-deficiency does not reduce the lifespan of adult survivors. The average lifespan of *E2f4*<sup>-/-</sup> mice (n=26) is similar to that of wildtype (n=12) and *E2f4*<sup>+/+</sup> (n=12) littermate controls.
REFERENCES


APPENDIX B

E2Fs regulate adipocyte differentiation

Lluis Fajas, Rebecca L. Landsberg, Yolande Huss-Garcia, Claude Sardet, Jacqueline A. Lees, and Johan Auwerx

Author's contribution Figure 4c, 5a


ABSTRACT

When preadipocytes re-enter the cell cycle, PPARγ expression is induced coincident with an increase in DNA synthesis suggesting the involvement of the E2F family of cell cycle regulators. We show here that E2F1 induces PPARγ transcription during clonal expansion whereas E2F4 represses PPARγ expression during terminal adipocyte differentiation. Using a combination of experiments in knock out and chimeric animals, and studies based on differentiation of ES cells, and embryo fibroblasts deficient in the various E2Fs we demonstrate that the absence of E2F1 impairs, whereas depletion of E2F4 stimulates adipocyte differentiation. The finding that E2Fs are upstream regulators of PPARγ underscores the role of these proteins in the control of both cell proliferation and differentiation.
INTRODUCTION

Studies with preadipocyte cell lines, such as 3T3-L1 cells, have been instrumental to unravel the molecular mechanisms controlling adipocyte differentiation (Green and Kehinde, 1975). Upon reaching confluence, proliferating preadipocytes become growth arrested by contact inhibition. Those contact inhibited preadipocytes re-enter cell cycle after hormonal induction, arrest proliferation again and finally undergo terminal adipocyte differentiation. Peroxisome proliferator activated receptor gamma (PPAR\(\gamma\)) has been shown to be crucial in the control of terminal adipocyte differentiation (Fajas et al., 1998b; Rosen et al., 2000). PPAR\(\gamma\), upon activation by either fatty acid derivatives or antidiabetic thiazolidinediones, drives the expression of several adipocyte specific genes such as the fatty acid binding protein (aP2) (Tontonoz et al., 1994a) or lipoprotein lipase (LPL) (Schoonjans et al., 1996). Ectopic expression of PPAR\(\gamma\) furthermore induces adipocyte differentiation (Tontonoz et al., 1994b). This primordial role of PPAR\(\gamma\) in adipocyte differentiation was further highlighted by the phenotype observed in humans with mutations in the PPAR\(\gamma\) gene and by the characterization of PPAR\(\gamma\) deficient mice (reviewed in (Fajas et al., 2001).

Whereas much effort has been directed towards the understanding of the terminal stages of adipocyte differentiation, the molecular mechanisms underlying the transition between cell proliferation and differentiation of preadipocytes remain largely elusive. Re-entry into cell cycle is one of the key events taking place in early adipogenesis, since inhibition of DNA synthesis at this stage blocks differentiation (Patel and Lane, 2000; Reichert and Eick, 1999). Like in most cells, the entry of growth arrested preadipocytes into S-phase depends on the activation of the G1 cyclins/cdk5 and the retinoblastoma protein pRB-E2F pathway that controls the G1/S transition of the cell cycle. E2F transcription factors are the effectors of this pathway and they control the
expression of genes involved in cell cycle progression, apoptosis, and DNA synthesis (for review see (Helin, 1998; Sardet et al., 1997; Trimarchi and Lees, 2002).

E2F activity is the result of the heterodimerization of two proteins belonging to the E2F family (E2F1 to 6) and the DP family (DP1 and 2), respectively (Dyson, 1998; Gaubatz et al., 1998). When bound to DNA, this heterodimeric complex exist either as free E2F/DP, or forms a larger complex that contains a member of the retinoblastoma protein family (pRB, p107, p130). pRB associates with all E2Fs except for E2F5 and E2F6, whereas p130 associates specifically with E2F4 and E2F5, and p107 complexes exclusively with E2F4 (Cobrinik et al., 1993; Sardet et al., 1995). E2F complexes can activate (free heterodimers) or repress (large complexes) the transcription of E2F-responsive genes. Such repression is mediated through the recruitment of histone deacetylases, which interact with proteins of the pRB family (reviewed in Harbour and Dean, 2000). Information about the role of the individual E2F family members has been derived from both over-expression studies and the analysis of E2F-deficient mice and cells (reviewed in Dyson, 1998; Trimarchi and Lees, 2002). These studies show that E2F1-3 play a key role in the activation of E2F-responsive genes and therefore the induction of cellular proliferation. In contrast, E2F4 and E2F5 appear to be primarily involved in the repression of target genes and are particularly relevant for the transition between cell proliferation to differentiation (Gaubatz et al., 2000; Humbert et al., 2000a; Rempel et al., 2000).

The E2F and pRB family members appear to participate in the regulation of cell cycle events that are required for adipogenesis. In growth arrested preadipocytes, E2F4 and E2F5 are complexed with p130 leading to repression of its target genes (for review see (Dyson, 1998)). Upon re-entry into cell cycle of these growth arrested preadipocytes, p130, as well as the other members of the retinoblastoma family, are phosphorylated by the cyclin/cdk holoenzymes, releasing the E2F complex, resulting in the activation of the E2F target genes (Richon et al.,
1997). After several rounds of DNA synthesis, the cyclin dependent kinase inhibitors such as p21, p27, or p18 are induced and mediate cell cycle exit and maintain the irreversible growth arrest characteristic of terminal adipocyte differentiation (Morrison and Farmer, 1999). PPARγ and C/EBPα have been shown to contribute to this permanent cell cycle exit by inhibiting the E2F DNA-binding activity and up-regulating the levels of p21, respectively (Altio et al., 1997; Porse et al., 2001; Timchenko et al., 1996). There is also evidence that pRB plays a positive role in adipocyte differentiation through association and activation of C/EBPα (Chen et al., 1996).

In this study, we show that the E2F proteins play a direct role in the regulation of early adipocyte differentiation. E2F1 and 3 trigger the expression of PPARγ during the early stages of adipogenesis, whereas E2F4 represses expression of PPARγ at the terminal stage of adipocyte differentiation.
MATERIALS AND METHODS

Materials and oligonucleotides. The oligonucleotides used for various experiments in this manuscript are the following in (5' to 3' orientation): E2 consensus: gcctattcggctcttctcag; E2F- PPARγ1 site: ggaaccgggaaagccggctgc; E2F-PPARγ1 mut.: ggaaccgggacggcagctgctcc; ChIP PPARγ1 forward: ggaaccgggaaagccggctgc; ChIP PPARγ1 reverse: gggggagccggccggctcgg; ChIP PPARγ3 forward: gttaagattgaagaagccggcaac; ChIP PPARγ3 reverse: ggcctttcatgaggtatttgta; ChIP cyclin D1 forward: cagcgcggccgctcagggatgc; ChIP cyclin D1 reverse: gccgctgtaagttactgttgta. Rosiglitazone (BRL 49,653) was a kind gift of Dr. R. Heyman of Ligand pharmaceuticals (San Diego, CA). All chemicals, except if stated otherwise, were purchased from Sigma (St-Louis, MO). Antibodies were purchased from Santa Cruz Biotechnology (Sta Cruz, CA) except for the anti-BrdU antibody which was from Sigma.

Plasmids and probes. The PPARγ, aP2, and 36B4 probes are described previously (Fajas et al., 1997). For the construction of the expression vector pcDNA-E2F4, a BamHI/EcoRI fragment from pBabe-E2F4 (a kind gift of Dr. Helin) was inserted in the same sites of the pcDNA3 vector (Stratagene, La Jolla, CA). The CMV-DP1, CMV-E2F1, and p130 expression vectors were a gift from Dr. L. Le Cam. The PPARγ1, PPARγ2 and PPARγ3 promoter-reporter vectors have been previously described (Fajas et al., 1997; Fajas et al., 1998a). The PPARγ1 promoter-reporter vector with a mutation in the E2F binding site was constructed by site directed mutagenesis using the indicated oligonucleotides.

Cell culture, protein extracts, retroviral infection, transfections, and implantation of preadipocytes. E2F1-/-, E2F3 -/-, and E2F4 -/- and wild type MEFs were described (Humbert et al., 2000a; Humbert et al., 2000b). NIH-3T3 and 3T3-L1 cells were grown in DMEM, 10% foetal calf serum (FCS). Human primary preadipocytes were purchased from Zen-Bio (Research Triangle, NC) and maintained under the conditions specified by the provider. Cells were
differentiated with DMEM, 10% serum, 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX), 10 μg/ml insulin and 1μM dexamethasone for 2 days. From the day 3 on, cells were incubated with DMEM, 10% serum, 10 μg/ml insulin, and 1 nM BRL 49,653. Oil-red-O staining and quantification is described elsewhere (Ramirez-Zacarias et al., 1992). Nuclear and whole cell extracts were prepared as described (Fajas et al., 1997). All transfections were performed using the Lipofectamine Plus reagent (GIBCO Life Technologies, Rockville, MD). Luciferase activity was measured as described (Fajas et al., 1997).

**ES cells differentiation, chimeric and knock-out mice.** Generation and maintenance of E2F4 +/+, +/-, and -/- ES cells was described previously (Humbert et al., 2000a). ES cells were differentiated according to Rosen et al. (Rosen et al., 1999). For generation of chimeric mice, wild type or E2F4 -/- ES cells were injected into 4 day blastocysts from C57BL/6 mice and reimplanted into pseudopregnant wild type mice. At 7 weeks of age, animals were sacrificed, and the organs were harvested in 50mM Tris-HCl, 0.1% Triton X-100. All materials for glucose phosphate isomerase (GPI) analysis were purchased from Helena Labotratories (Beaumont, Texas). GPI analysis was performed as previously described (Nagy and Rossant, 1999). Briefly, cells derived from the injected 129 D3 ES cells (E2F4 +/+ and E2F4 -/-) will have a different GPI isoform with different electrophoretic mobility than the cells derived from the C57BL/6 recipient blastocysts. Quantification of the electrophoretic bands after an enzymatic colorimetric reaction, will be indicative of the relative composition of the analysed tissue. E2F1 -/- and +/+ mice were purchased from Jackson Laboratories (Main, Maine, U.S.A.) and fed a high fat diet (58% Fat, 25% carbohydrates, 16% protein) during 8 weeks.

**Electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP).**

Cell extracts were incubated for 15 min at 21 °C in a total volume of 20 μl binding buffer (10 mM Tris-HCl (pH 7.9), 40 mM KCl, 10% glycerol, 0.05% Nonidet P-40 and 1 mM DTT, 1μg
poly(dI:dC)) in presence of 1 ng of a T4-PNK end-labeled double-stranded oligonucleotide probe. For gel-supershift analysis, 2 μg of antibody was added to the reaction. DNA-protein complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.25 x TBE buffer at 21 °C and 10V/cm. Chromatin immunoprecipitation assays were performed as described previously (Takahashi et al., 2000). Briefly, proteins were formaldehyde cross-linked to DNA in confluent human primary preadipocytes before induction of differentiation or in cells induced with differentiation medium for 3 days. Proteins were then immunoprecipitated using the indicated antibodies, DNA was extracted from the immunoprecipitates and PCR amplification was performed using promoter-specific oligonucleotide primers.

**Immunofluorescence.** For all immunofluorescence experiments, cells were grown on coverslips. For BrdU incorporation, cells were incubated 4h in the presence of BrdU and an additional treatment of the cells with 1.5N HCl for 10 minutes at 21 °C was performed. After fixation and permeabilization with methanol, cells were incubated with the indicated antibodies. Preparations were then incubated with a combination of Texas Red-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG.

**Northern and western blot analysis.** SDS-PAGE and electrotransfer was performed as described (Schoonjans et al., 1996). The membranes were blocked overnight in blocking buffer (20 mM Tris, 100 mM NaCl, 1% Tween-20, 10% skim milk). Filters were first incubated 4h at 21 °C with the indicated antibody, and then for 1h at 21 °C with a peroxidase conjugate secondary antibody. The complex was visualized with 4-chloro-1-naphtol as reagent. Northern blots were hybridized with PPARγ, αP2, and 36B4 cDNAs and visualized as described (Schoonjans et al., 1996).
RESULTS

PPAR\(\gamma\) expression coincides with re-entry into cell cycle

When hormonally stimulated, confluent 3T3-L1 preadipocytes re-enter cell cycle before they undergo differentiation (Green and Kehinde, 1975). We correlated the expression of some cell cycle regulators and known E2F target genes with the expression of PPAR\(\gamma\) during differentiation of 3T3-L1 preadipocytes. Protein levels of cyclin A, E2F1, and E2F4 were increased after 1 day of differentiation (Figure 1A). Whereas levels of cyclin A and E2F1 were not detected during the later stages of adipocyte differentiation, E2F4 protein levels remained elevated for at least 5 days after induction of differentiation. At this time point, adipogenic markers, such as the PPAR\(\gamma\) protein, were strongly expressed (Figure 1A, bottom panel). Interestingly, PPAR\(\gamma\) expression was switched on at the same time point as cyclin A and E2F1.

Consistent with re-entry into cell cycle, p130 became and remained phosphorylated during clonal expansion (Figure 1A). At a later stage during differentiation, p130 became hypophosphorylated, and thus active (Figure 1A). Total pRB protein levels remained almost stable from day 1 of differentiation onwards (Figure 1A). Most of the pRB protein was hyperphosphorylated (Figure 1A).

Next, 3T3-L1 cells at day 1 of differentiation were incubated with BrdU to evaluate DNA synthesis. Visualization of the cells by fluorescence microscopy demonstrated that cells which had incorporated BrdU also stained positive for PPAR\(\gamma\) protein (Figure 2B), indicating that PPAR\(\gamma\) expression coincided with active DNA synthesis in these differentiating 3T3-L1 cells. A similar experiment was performed using an anti-cyclin A antibody. Again, PPAR\(\gamma\) was coexpressed in the nucleus with cyclin A, a marker of the S phase of the cell cycle (Figure 2C). These results suggest that expression of PPAR\(\gamma\) might be under the control of the same factors that induce re-entry into the cell cycle, i.e. the E2F family of transcription factors.
E2Fs bind in vitro to the PPARγ1 promoter

Computer-assisted sequence analysis of the regulatory regions of the human PPARγ gene demonstrated the presence of a consensus E2F binding site at position -215 in the PPARγ1 promoter. The sequence of the E2F site in the PPARγ1 promoter was identical to the E2F binding site of the c-Myc promoter and highly similar to the binding sites in the cyclins E and D1 or the adenoviral E2 promoters (Figure 2A). Binding of E2F to the PPARγ1 promoter was tested by electrophoretic mobility shift assay (EMSA). Typical E2F complexes were bound to the radiolabeled PPARγ1-E2F site when whole cell extracts prepared at different times of the adipocyte differentiation process were used. At day 0, the predominating DNA-binding complex (Figure 2B, day 0) was composed of E2F4 and p130 (Figure 2C; lanes 1-4). One day after induction of differentiation, two different complexes were detected: a slower migrating band and a faster migrating double complex (Figure 2B, day 1). Faster migrating complexes contained the different free E2F species (mainly E2F4), whereas E2F4, p107, and cyclin A/cdk2, formed the slow-migrating complex (Figure 2C; lanes 5-8 and data not shown). The pattern of E2F DNA-binding observed at this time, suggested that most of the cells have already re-entered cell cycle. Between days 2 to 4 of differentiation free E2F remained predominant, whereas the composition of the slow-migrating E2F complex changed from E2F/p107 to E2F/p130 (Figure 2B, days 2-5). At day 5 after induction of differentiation, the free E2F complex decreased in intensity, but now the predominant complex was again composed of E2F4 and p130 (Figure 2B and 2C, lanes 9-12). These results suggest hence, that E2F is able to bind in vitro to the PPARγ1 promoter. Furthermore, northern blot analysis showed a good correlation between the presence of free E2F complexes bound to the PPARγ1 E2F response element and PPARγ mRNA levels during adipocyte differentiation of 3T3-L1 cells (Figure 2D). This suggests that E2F could be
responsible for triggering PPARγ expression early during adipogenesis.

**E2Fs bind in vivo to the PPARγ1 promoter and transactivate its expression.**

In order to demonstrate the occupancy of the PPARγ1 promoter by E2F proteins *in vivo* in human cells, chromatin immunoprecipitation assays (ChIP) were performed using either specific antibodies (anti-E2F4, E2F1, or an anti-acetylated H4 antibody) or preimmune serum. Whereas no amplification of the PPARγ1 promoter was observed when non-specific antibodies were used (Mock, Figure 3A, lanes 5 and 10), a specific fragment, corresponding to the PPARγ1 promoter, was amplified when chromatin of cells that were stimulated to differentiate was immunoprecipitated with either the anti-E2F4 (Figure 3A, lane 7) or the anti-E2F1 (Figure 3A, lane 9) antibody. No amplification product was observed when immunoprecipitated chromatin from confluent non-differentiated human primary preadipocytes was used as template (Figure 3A, lanes 2 and 4). Strikingly, the same pattern of E2F1 and E2F4 binding was observed when oligonucleotides corresponding to the cyclin D1 promoter were used to amplify the immunoprecipitated chromatin (Figure 3A, lanes 2, 4, 7, and 9). A different fragment from the PPARγ gene, covering the proximal PPARγ3 promoter (Fajas et al., 1998a), could not be amplified either from confluent or differentiated cells when the anti-E2F1 or the anti-E2F4 antibody was used (Figure 3A, lanes 2, 4, 7, and 9). The results of these ChIP assays hence prove that E2F1 and E2F4 specifically bind to the PPARγ1 promoter in differentiating human adipocytes. ChIP assay also demonstrated the presence of acetylated histone H4 on the PPARγ1 and cyclin D1 promoters (Figure 3A, lane 8), suggesting that the binding of E2Fs results in the activation of these promoters. Interestingly, when using an anti-acetylated histone H4 antibody, the PPARγ3 promoter could also be amplified, indicating that at this stage of differentiation the
PPARγ3 promoter was also activated, although not directly by binding of E2F (Figure 3A, lane 8).

To assess whether E2Fs can directly regulate the human PPARγ1 promoter activity, we co-transfected expression plasmids coding for either E2F1, E2F4, DP1, pRB, or p130 together with the pGL3γ1E2_wt luciferase reporter vector which contains the human PPARγ1 promoter (Fajas et al., 1997). When NIH-3T3 cells were cotransfected with either E2F1 (Figure 3B), or E2F4 (Fig. 3C), luciferase activity increased 5- and 4-fold respectively relative to the basal activity of the hPPARγ1 promoter. This induction was substantially enhanced (up to 8-fold respectively) upon cotransfection with the heterodimeric partner of E2F, DP1. As expected for an E2F target gene, when a constitutively active form of pRB or p130 were cotransfected with E2F1/DP1, the stimulatory effect on the PPARγ1 promoter was abrogated (Figure 3B and C). This repressive effect of E2Fs of the PPARγ1 promoter was clearly dependent on the presence of the pocket proteins pRB and p130, since cotransfection of the adenoviral protein E1A 13S resulted in the abrogation of the repression (Fig. 3B and C). Similar results were observed when an E2F3 (data not shown) or expression vector was used. Finally, to demonstrate that it is through binding to the PPARγ1-E2F site, that E2Fs modulate the activity of the hPPARγ1 promoter, we substituted four bases in the PPARγ1-E2F site (from GCGGGAAA to GCGGCGGC) to generate the pGL3γ1E2_mut reporter plasmid. Cotransfected E2F1/DP1 was unable to stimulate the mutated pGL3γ1E2_mut reporter vector in NIH-3T3 cells (Figure 3B black bars). In the same experiment, the wild-type promoter was induced by 5-fold (data not shown).

**E2F1 and E2F3 positively regulates adipogenesis**

We have shown that E2F1 and PPARγ1 are expressed at the same time in the differentiation process and E2F1 binds and activates the PPARγ1 promoter. To determine
whether the activating E2Fs, E2F1 and E2F3, play a positive role in adipogenesis, we analysed
the effects of E2F1 or E2F3 depletion either in mice or in primary mouse embryonic fibroblasts
(MEFs). Consistent with a role for E2F1 in triggering PPARγ expression and adipogenesis,
E2F1/- mice were resistant to obesity induced by feeding a high fat diet for eight weeks,
whereas E2F1+/+ mice increased their weight significantly upon this high fat diet (Figure 4A).
The difference in weight could be entirely attributed to differences in fat mass (Figure 4B). No
significant differences were observed in food intake between the E2F1/- and the E2F1+/+ mice
(Figure 4B). The phenotype of the E2F1/- animals suggested a problem in adipose tissue
homeostasis. To test this notion, we compared the capacity of MEFs deficient in the various
E2Fs to differentiate into adipocytes in vitro in response to hormone stimulation. Adipocytes
were scored using Oil-red-O staining to detect lipid droplets. Hormonally stimulated E2F1/-
and E2F3/- MEFs showed a slightly, but consistently reduced capacity to differentiate into
adipocytes in vitro, when compared to wild type MEFs (Figure 4C). Taken together, these data
suggest that E2F1-3 stimulate adipogenesis through activation of PPARγ.

**E2F4 negatively regulates adipogenesis.**

In addition to E2F1, the ChIP experiments showed that E2F4 associated with the PPARγ
promoter in vivo. Our transient transfection assays showed that E2F4 activates the pGL3γ1E2_wt
luciferase reporter and this is repressed by the co-expression of p130 (Fig. 3C), confirming that
E2F4 can regulate the PPARγ promoter. However, since E2F4 behaves as an activating E2F
when it is over-expressed, this assay did not reveal whether the endogenous E2F4 regulates
PPARγ expression, and therefore adipogenesis, positively (通过 free E2F/DP heterodimers) or
negatively (through recruitments of pRB-family members and associated HDACs). To address
this issue, we determined how the absence of E2F4 affected adipogenesis. The E2F4/- mice are
highly susceptible to bacterial infections and the resulting abnormal feeding pattern precludes their use for analysis of adipose tissue homeostasis in vivo (Humbert et al., 2000a; Rempel et al., 2000). As an alternative approach, we first evaluated the ability of E2F4-/- MEFs (Figures 4C and 5A) and ES cells (Figure 5B) to differentiate into adipocytes in vitro. In both cases, we saw a significant increase in the number of Oil-red-O positive cells in the E2F4-/- versus the E2F4 +/- monolayers (Figure 4C and 5A, B). This suggests that E2F4 exerts a negative effect on adipogenesis.

To further explore this hypothesis, E2F4 +/- or E2F4-/- 129 D3 ES cells were injected into C57BL/6 blastocysts, which were then reimplanted in pseudopregnant wild type mice in order to create chimeric progeny. Twelve mice of each genotype considered highly chimeric were sacrificed and several organs were harvested and analyzed for the contribution of the ES cells to the composition of the tissues, using GPI as a marker. Tissue analysis revealed only a minor contribution of the injected 129 D3 E2F4 +/- ES cells to the development of white adipose tissue of the chimeric mice (Figure 5C and D). In contrast, white adipose tissue from chimeric mice resulting from injection of 129 D3 E2F4 -/- cells was composed of at least 40% of the mutant cells (Figure 5C and D). Importantly, no significant differences in contribution between E2F4 +/- and E2F4 -/- chimeras were observed in some other tissues, such as kidney or pancreas (Figure 5D, and data not shown). Together with the in vitro studies of the E2F4-/- cells, the analysis of these chimeras mice proves that E2F4 has a negative effect on adipose tissue development.

Coincidence of PPARγ and E2F1 or E2F4 expression in the nucleus is dependent on the stage of adipogenesis
Significantly, our analysis of the endogenous E2F complexes shows that E2F4 associates with the hypophosphorylated, and thus active form, of p130 prior to induction of adipogenesis and the disappearance of this complex coincides with the induction of PPARγ expression on day 1 of differentiation (compare Figures 2B and 1A). This suggests that E2F4 inhibits adipocyte differentiation by repressing PPARγ expression through its association with p130. It has been previously shown that the free E2F4/DP complexes are predominantly nuclear whereas association with the p130 and pRB relocalizes these complexes in the cytoplasm (Magaes et al., 1996; Muller et al., 2001; Verona et al., 1997). We therefore compared the expression and subcellular localization of PPARγ, E2F1 and E2F4 in 3T3-L1 cells and human primary preadipocytes at different stages of adipogenesis by immunofluorescence microscopy (Figure 6 and data not shown). Confluent, growth arrested 3T3-L1 preadipocytes displayed high levels of nuclear E2F4 whereas E2F1 and PPARγ were expressed at low to undetectable levels (Figure 6A and 6B, 0h). Twenty four hours after induction of differentiation, a significant fraction of the cells expressed PPARγ (Figure 6A and B, 24h). Significantly, the PPARγ-positive cells all expressed high levels of nuclear E2F1 and E2F4 protein. This is consistent with the idea that E2F1 activates PPARγ expression (Figure 3B). Moreover, since the p130 protein has largely dissociated from E2F4 at this time (Figure 2B), it suggests that free E2F4 may also contribute to the activation of PPARγ. During the terminal differentiation phase (96h), nuclear PPARγ protein is expressed in most but not all of the cells (Figure 6A and B). By this stage, E2F1 is undetectable. This is consistent with the end of the clonal expansion phase and the declining expression of E2F1 (Figure 1A). In contrast, E2F4 is still expressed in all cells (Figure 6A). However, at this stage, the majority of these cells have cytoplasmic rather than nuclear E2F4 (Figure 6A, arrowheads). Significantly, we saw a perfect correlation between the localization of E2F4 and the expression of PPARγ: E2F4 was cytoplasmic in PPARγ-positive cells and nuclear
in PPARγ-negative cells (Figure 6A, arrowhead). Thus, at late stage of differentiation, expression of PPARγ can occur in the complete absence of either free E2F1 or free E2F4 complexes. Moreover, the presence of nuclear E2F4 complexes correlates with the repression of this responsive gene.
DISCUSSION

Differentiation of preadipocytes into adipocytes requires that growth arrested preadipocytes re-enter the cell cycle before undergoing terminal differentiation. This particular situation is underscored by the fact that adipocyte differentiation is essentially triggered by proliferative stimuli, such as insulin or cAMP. In this study, we elucidate the molecular mechanisms operating at the transition between cell proliferation and adipocyte differentiation. Consistent with the previous observation that a block of DNA synthesis during the clonal expansion phase of adipocyte differentiation inhibits adipogenesis (Patel and Lane, 2000; Reichert and Eick, 1999), we have demonstrated that clonal expansion is accompanied by changes in the E2F complexes. Within 1 day of hormonal induction, the suppressive E2F4/p130 complex is completely lost and there is a major induction of the expression of the activating E2Fs, particularly E2F1. These changes correlated closely with the induction of E2F-responsive genes, like cyclin A, that are known to be required for cellular proliferation. Significantly, the master regulator of adipogenesis, PPARγ, is induced at the same time and our data strongly suggest that this is a bona-fide E2F target gene. First, the E2F proteins bind to a consensus E2F DNA-binding site in the PPARγ1 promoter both in vitro (EMSA) and in vivo (ChIP). Second, reporter assays confirm that the E2Fs can regulate transcription from the PPARγ1 promoter in a manner that is entirely dependent upon the identified E2F binding site.

Consistent with the hypothesis that the E2F proteins play a direct role in the regulation of PPARγ expression, we also present genetic evidence that the individual E2F proteins have profound effects upon adipocyte differentiation in vitro and in vivo. E2F1- and E2F3-deficient cells have a reduced capacity to differentiate into adipocytes and E2F1/-/- mice have limited fat accretion upon high fat feeding. Thus, these activating E2Fs play a positive role in adipogenesis. In contrast, E2F4 has a negative influence on the differentiation process. E2F4-deficient MEFs
and ES cells have an increased propensity to undergo adipogenesis. Moreover, E2F4/- ES cells contribute at a significantly higher level to the adipose versus the other tissues of chimeric mice.

Based on the appearance/disappearance and subcellular localization of the individual E2F complexes during differentiation and the phenotypes of the E2F-deficient mice and cells, we propose the following model of E2F action (Figure 7). In confluent preadipocytes, the E2F4/p130 complex acts to repress the transcription of E2F-responsive genes including PPARγ. Hormonal stimulation results in the loss of this repressive complex and the induction of E2F1. This leads to the coordinate expression of the classic E2F target genes, and the activation of clonal expansion, and the expression of PPARγ, and thereby activation of differentiation. Our data strongly suggest that free E2F1/DP is involved in the transcriptional activation of PPARγ. During the late stages of differentiation, the expression of PPARγ clearly occurs in the absence of nuclear E2F species. This is entirely consistent with previous observations that that PPARγ expression is regulated by other transcription factors, including C/EBPs and SREBP (Fajas et al., 1999; Saladin et al., 1999). Importantly, at this stage the E2F4/p130 complex reappears and the presence of nuclear E2F4 is clearly incompatible with PPARγ expression. This raises the possibility that the reformation of E2F4/p130 might facilitate the “switching-off” of PPARγ that occurs in terminally differentiated adipocytes.

Significantly, the opposing roles of E2F1-3 versus E2F4 in the regulation of PPARγ expression, and therefore adipogenesis, are entirely consistent with the prevailing view that these proteins are involved in the activation or repression of classic E2F-responsive genes respectively (reviewed in (Trimarchi and Lees, 2002)). Moreover, our model fits with the previous demonstration that adipogenesis is promoted by the combined loss of p107 and p130, the major E2F4-associated proteins (Classon et al., 2000). However, a number of issues remain to be resolved. First, since E2F4 remains in the nucleus during clonal expansion, we cannot rule out
the possibility that free E2F4/DP complexes actually contribute to the activation of PPARγ. At least in transient transfection assays, E2F4 can transactivate the PPARγ promoter in the absence of p130. The phenotype of the E2F4-deficient mice and cells simply shows that E2F4’s repressive activity is dominant over any role it might play in activation. Second, the E2F and pocket proteins have now been shown to regulate adipogenesis through a number of different mechanisms that appear to affect cell cycle regulation and/or the differentiation process. For example, pRB appears to essential for both cell cycle exit and to cooperate with the C/EBP family members in the activation of key transcriptional targets (Chen et al. G+D. 1996; Higgins et al. J. Virol. 1996; Hanson et al. JBC. 1999; Classon et al. PNAS, 2000). Thus, it will important to establish the relative importance of these various mechanisms and determine how they are coordinated in vivo. Finally, it is currently unclear why PPARγ is not expressed along with other E2F-responsive genes in actively cycling cells. However, we envisage several possibilities. First, chromatin remodeling could account for the silencing of the PPARγ gene in proliferating cells, as has been shown for other genes (Krebs et al., 2000; Krebs et al., 1999). Second, other repressive protein complexes may specifically inhibit the expression of the PPARγ gene in cycling cells. GATA-2, GATA-3 and AEBP1 are all good candidates for such a role (Tong et al., 2000) (He et al., 1995). Alternatively, signaling through the Wnt-10b and Pref-1 (notch-like) pathway could render the PPARγ gene refractory to induction by E2Fs (Ross et al., 2000; Smas and Sul, 1993). Interestingly, the induction of PPARγ expression has been reported in a number of proliferative disorders such as colon cancer (DuBois et al., 1998), prostate adenocarcinoma (Mueller et al., 2000), and breast cancer cells (Mueller et al., 1998; Samid et al., 2000). It is therefore tempting to speculate that the mechanism that normally blocks PPARγ expression in cycling cells is somehow abrogated.
In summary, our data show that E2F1-3 and E2F4 have opposing effects on adipocyte differentiation that appears to be largely attributable to their differential regulation of PPARγ expression. This provides direct support for the idea that the E2F and pRB family members play a central role in coordinating the transition between cell proliferation and terminal differentiation. Modulation of the activity of E2Fs might open up new perspectives in the control of adipogenesis and metabolic diseases.

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Figure 1: Expression of PPARγ and cell cycle related proteins during 3T3-L1 differentiation.

A. Western blot analysis of whole cell extracts prepared at different days of adipocyte differentiation of 3T3-L1 cells. The proteins detected with specific antibodies are indicated.

B. Analysis of PPARγ protein expression and BrdU incorporation by immunofluorescence in post-confluent 3T3-L1 cells stimulated with differentiation medium for 1 day as described under experimental procedures. Cells expressing PPARγ are labeled in green (FITC) whereas cells, which have incorporated BrdU, are labeled in red. Nuclei were stained with the Hoechst reagent (blue staining).

C. Analysis of cyclin A and PPARγ co-expression by immunofluorescence in 3T3-L1 cells induced to differentiate for 1 day. PPARγ expressing cells are labeled in red whereas cyclin A expressing cells are in green. Nuclei were visualized with Hoechst staining.
Figure 2: In vitro binding of E2F to the PPARγ1 promoter.

A. Schematic representation of the genomic structure of the 5' region of the human PPARγ gene. Transcription initiation sites are indicated by arrows and labeled γ1, γ2, and γ3. Shaded boxes indicate exons. The E2F binding site in the PPARγ1 promoter is depicted as a striped box. Comparison of this PPARγ E2F site with the E2F binding site of classical E2F target genes is illustrated. Arrows indicate the DNA fragments amplified in ChIP analysis.

B. In vitro binding of E2F to the PPARγ1 promoter. EMSA analysis of the radiolabeled E2F DNA-binding site of the PPARγ1 promoter incubated with nuclear extracts of 3T3-L1 cells at the indicated times of differentiation. Composition of the different complexes is indicated. Double-stranded cold oligonucleotides representing either the PPARγ1-E2F site (γ1-E2F), the mutated PPARγ1-E2F site (γ1-E2Fmut), or the E2F consensus binding site from the adenoviral E2 promoter (E2Fcons), were included in the competition assays (lanes 7-9). Only the retarded complexes are shown.

C. EMSA demonstrating the composition of the retarded complexes in nuclear extracts of 3T3-L1 cells at day 0 (left panel, lanes 1-4), day 1 (middle panel; lanes 5-8), or day 5 (right panel; lanes 9-12) of induction of differentiation. The antibodies used for the gel-supershift are indicated.

D. Northern blot analysis of PPARγ, aP2, and 36B4 mRNA expression at different times after induction of differentiation of 3T3-L1 preadipocytes.
Figure 3: *In vivo* binding of E2F4 to the PPARγ1 promoter and transactivation assays.

A. Chromatin immunoprecipitation (ChIP) assays demonstrating binding of E2F1 and E2F4 to the PPARγ1 promoter. Cross-linked chromatin from either confluent (left panel) or human primary preadipocytes differentiated during 3 days (right panel) was incubated with antibodies against E2F4 (lanes 2 and 7), E2F1 (lanes 4 and 9), acetylated histone H4 (lanes 3 and 8), or without any antibody (mock, lanes 5 and 10). Immunoprecipitates were analyzed by PCR using primers specific for the indicated promoters (see figure 2A). As a control, a sample representing 1% of the total chromatin was included in the PCR (input, lanes 1 and 6).

B-C. E2Fs modulate the PPARγ1 promoter. Relative luciferase activity as determined after transfection of NIH 3T3 cells with the reporter constructs pGL3γ1E2<sub>wt</sub> and pGL3γ1E2<sub>mut</sub>. Cells were either transfected with an empty expression vector, with expression vectors for E2F1, E2F4, DP1, p130, pRB, E1A 13S, or with combinations of these vectors as indicated. Values are the mean of 3 independent experiments. An asterisk depicts statistically significant differences.
Figure 4: E2F1-3 stimulate adipogenesis.

A. Weight gain curves of E2F1 -/- (black squares) and E2F1 +/- (black triangles) mice fed with a high fat diet. Each group was composed of ten animals. Animals were weighed every week for a period of 8 weeks.

B. Food intake and weight of the epididymal fat pads (expressed as % of total body weight) of the animals used in the experiment described in A. Statistically significant differences (p<0.05) are indicated by an asterisk.

C. Quantification of lipid incorporation by measuring the intensity of oil-red-O staining of either wild type (WT), E2F1 +/-, E2F3 +/-, or E2F4 +/- MEFs stimulated to differentiate into adipocytes. An asterisk indicates statistically significant differences (p< 0.05).
**Figure 5: E2F4 inhibits adipogenesis.**

A. Oil red-O staining comparing lipid accumulation in E2F4 +/+ or E2F4 -/- MEFs induced to differentiate into adipocytes.

B. Oil red-O staining comparing lipid accumulation in E2F4 +/+ or E2F4 -/- ES cells induced to differentiate into adipocytes as described in material and methods. Quantification of lipid incorporation by measuring the intensity of oil-red-O staining is indicated at the right.

C. Protein electrophoresis followed by colorimetric detection of the two different GPI isoforms in adipose tissue (WAT) of chimeric mice. The lower band, indicated by an open arrowhead, corresponds to the GPI isoform contributed by recipient C57Bl/6 blastocyst cells. The upper band, indicated by a closed arrowhead, corresponds to the GPI isoform derived from microinjected E2F4+/+ (upper panel) or -/- 129 D3 ES cells (bottom panel).

D. Quantification of the relative contribution of the microinjected ES cells (WT, E2F4 -/-) in the development of either adipose tissue (WAT) or kidney.
Figure 6: Comparative analysis of PPARγ, E2F4, and E2F1 expression by immunofluorescence at different times of the adipocyte differentiation program.

Confluent 3T3-L1 cells (A) or human primary preadipocytes (B) were stimulated to differentiate as described. At the indicated time points, cells were fixed and incubated with both anti-PPARγ and anti-E2F4 (A) or anti-E2F1 (B) antibodies. PPARγ protein was detected with a texas-red labeled secondary antibody (red labeling) whereas E2F4 and E2F1 proteins were detected with a FITC labeled secondary antibody (green labeling). Nuclear localization was assessed by Hoechst staining of the nuclei. The white arrow in panel A highlights that E2F4 nuclear localization in differentiating cells coincides with the absence of PPARγ expression.
Figure 7: Model of PPARγ regulation by E2Fs during adipogenesis in the presence or absence of E2F4.

In growth arrested preadipocytes (day 0) the PPARγ gene is silenced by E2F4-p130-HDAC complex. During the clonal expansion phase, at least two pools of cells can be distinguished. Cells in which E2F1-3 and possibly E2F4 trigger the expression of PPARγ, and cells in which E2F4 is associated with a p107-HDAC repressor complex. During the terminal differentiation phase, in most cells E2F4 is exported to the cytoplasm, allowing that other transcription factors, such as C/EBPs or ADD1/SREBP1, sustain the expression of PPARγ, resulting in the differentiation of the cells into adipocytes. In other cells, however, E2F4 remains in the cell nucleus repressing the expression of PPARγ, through association with p130/p107, which recruits HDACs to the PPARγ promoter. These cells remain undifferentiated preadipocytes. Undifferentiated preadipocytes are indicated by gray cells, whereas the differentiated adipocytes are in orange.
REFERENCES


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Saladin, R., Fajas, L., Dana, S., Halvorsen, Y. D., Auwerx, J., and Briggs, M. (1999). Differential regulation of peroxisome proliferator activated receptor γ1 (PPARγ1) and


APPENDIX C

p107 and p130 repress apoptosis under low mitogenic conditions

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Author’s contribution: Figures 1, 2, and 3

ABSTRACT

E2F-pocket protein repressive complexes have been shown to be critical for regulating cell cycle progression. Loss of two members of the pocket protein family, p107 and p130, results in a failure to withdraw from the cell cycle in response to G1 arrest stimuli such as serum starvation and confluence. We previously have shown that loss of E2F4, the predominant repressive E2F in vivo does not enhance or suppress the cell cycle phenotype of confluent p107−/−;p130−/− mouse embryonic fibroblasts (MEFs). This study describes the additional characterization of the ability of E2f4−/−;p107−/−;p130−/− (TKO) MEFs to respond to a serum withdrawal mediated G1 arrest as compared with DKO MEFs. We have surprisingly discovered a shift in the phenotype of MEFs lacking both p107 and p130 as both p107−/−;p130−/− (DKO) and TKO MEFs exhibit only subtle deregulation of E2F responsive gene expression in response to low mitogenic conditions. Instead of exhibiting elevated levels of E2F-responsive gene expression, both DKO and TKO exhibited high levels of apoptosis when placed into low serum conditions. This change in phenotype is most likely due to the residual activity from the p130 allele used in this study. The activity of this allele could be affected by the presence of strain specific modifiers of p107 and p130.
INTRODUCTION

The members of the pocket protein family (pRB, p107, and p130) are critical regulators of the $G_1$ to $S$ transition of the cell cycle. The cell cycle inhibitory effect of these proteins is largely due to the repression of their major downstream target, the E2F transcription factor. E2F•pocket protein repressive complexes control cell cycle progression through the regulation of E2F responsive genes that are required for DNA synthesis and S-phase entry (Dyson, 1998; Trimarchi and Lees, 2002). In addition to blocking the transcriptional activity of E2F, pRB can actively repress E2F target gene expression through recruitment of histone deacetylase and methylase activities (Bannister et al., 2001; Brehm et al., 1998; Luo et al., 1998; Nielsen et al., 2001).

Eight genes encoding components of the E2F transcription factor have been identified to date. E2F is a heterodimeric factor composed of one DP subunit, of which there are two family members (DP-1, DP-2), and one of the seven E2F family members (as reviewed in Trimarchi and Lees, 2002). The E2F family can be divided into three subgroups based upon structural and functional homology. E2F6, the sole member of one subgroup, does not interact with any of the pocket protein family members (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Ogawa et al., 2002; Trimarchi et al., 1998). Instead of directly regulating the cell cycle, E2F6 plays a role in vertebrate patterning through interaction with the mammalian polycomb complex (Storre et al., 2002; Trimarchi et al., 2001).

E2F1, E2F2, E2F3a, and E2F3b are members of the second subgroup and are repressed only by pRB. Analysis of cells lacking or overexpressing members of this subgroup have shown that E2F1, E2F2, and E2F3a are necessary for activating E2F
target gene transcription, DNA synthesis, and S-phase progression (Helin, 1998; Humbert et al., 2000b; Wu et al., 2001). E2F3b was recently identified as a second product of the E2F3 gene (Adams et al., 2000; He et al., 2000; Leone et al., 2000). The function of this family member is currently under investigation.

The last subgroup consists of E2F4 and E2F5, the E2Fs responsible for mediating active transcriptional repression through interaction with the pocket protein family. While E2F5 interacts only with p130, E2F4 interacts with all three of the pocket protein family members and comprises the majority of the E2F–pocket protein repressive complexes in vivo (Hijmans et al., 1995; Moberg et al., 1996; Vairo et al., 1995).

Mutant mice lacking one or more members of the pocket protein family have shown that repressive complexes are required for development. Rb−/− embryos die in utero exhibiting high levels of inappropriate proliferation and apoptosis in the central and peripheral nervous systems, liver, and lens (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The proliferation and apoptosis observed are in part due to the inappropriate activity of E2F. Loss of either E2F1 or E2F3 in a Rb−/− background suppresses the inappropriate proliferation and apoptosis in the CNS and, in the case of E2F3, partially suppresses the proliferation and apoptosis in the PNS (Tsai et al., 1998; Ziebold et al., 2001). These data supported evidence from in vitro experiments that suggested high levels of activating E2Fs unbound to the pocket protein family could lead to inappropriate apoptosis (as reviewed in Dyson, 1998). Analysis of Rb−/− mouse embryonic fibroblasts (MEFs) also confirmed that loss of pocket protein repression led to cell cycle defects as these cells exhibited a decrease in the length of time required to
transverse from G1 to S and an increase in the expression of two E2F responsive genes, *cyclin E* and *p107* (Herrera et al., 1996; Hurford et al., 1997).

Loss of p107 and p130 also leads to developmental abnormalities and loss of cell cycle control. *p107<sup>-/-</sup>;p130<sup>-/-</sup> (DKO) mice die shortly after birth and display defects in long bone development. These defects appear to arise from deregulated chondrocyte proliferation as opposed to a direct impairment in differentiation (Cobrinik et al., 1996; Rossi et al., 2002). Consistent with this model, mouse embryonic fibroblasts (MEFs) derived from these embryos cannot arrest appropriately in G1 and exhibit deregulation of a large number of E2F responsive genes (Bruce et al., 2000; Hurford et al., 1997; Landsberg et al., Submitted).

Mutant mouse models have been generated for the repressive E2Fs: E2F4 and E2F5. Both E2F4 and E2F5 single mutant mice have specific defects in development and differentiation (Humbert et al., 2000a; Lindeman et al., 1998; Rempel et al., 2000). MEFs derived from these mice do not have any apparent cell cycle phenotypes, indicating that E2F4 and E2F5 alone are dispensable for cell cycle regulation but are required for the development of specific tissues. It could not be ruled out, however, that elimination of one repressive E2F leads to compensation by the other. To determine the developmental and cell cycle consequence of loss of all E2F•pocket protein repressive complexes, compound mutants have been generated. *E2f4<sup>-/-</sup>;E2f5<sup>-/-</sup>* mice are not viable at birth, and MEFs derived from these embryos are defective in arresting in G1 (Gaubatz et al., 2000).

Recently, we have described the characterization of MEFs deficient for E2F4, p107, and p130. These cells lack E2F•pocket protein repressive complexes, but
regulation of the activating E2Fs by pRB remains intact (Landsberg et al., Submitted).

E2f4<sup>−/−</sup>;p107<sup>−/−</sup>;p130<sup>−/−</sup> (TKO) MEFs, like p107<sup>−/−</sup>;p130<sup>−/−</sup> (DKO) MEFs inappropriately initiate S-phase and exhibit deregulation of E2F target gene expression under confluent conditions. This study describes additional characterization of the ability of TKO MEFs to respond to a serum withdrawal mediated G<sub>1</sub> arrest as compared with DKO MEFs. DKO and TKO MEFs display only subtle increases in E2F responsive gene expression but exhibit a striking number of apoptotic cells. Inappropriate E2F transcriptional activity has been shown to lead to apoptosis (as reviewed in Ginsberg, 2002). Therefore, we believe that we fail to see increased levels of E2F responsive genes because the p107 and p130 deficient cells used in this study might be undergoing apoptosis in response to inappropriate E2F activity.
MATERIALS AND METHODS

Cell Culture and Analysis. All mouse embryonic fibroblasts (MEFs) were generated at day 13.5 as previously described (Humbert et al., 2000b) and maintained in Dulbecco’s modified eagle media (DMEM) containing 10% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine (MEF media). For each experiment two to four independently derived, passage 4 MEF lines were analyzed. Confluent monolayers were fed every other day with MEF media as previously described (Landsberg et al., Submitted). All serum starvation experiments were performed as previously described (Humbert et al., 2000b). Cell cycle progression was determined by northern blotting or incubation for two hours in media containing 5μCi of 3H-thymidine or 3mg/ml BrdU and 0.3 mg/ml FdU. Analysis of BrdU and 3H-thymidine incorporation was performed as previously described (Humbert et al., 2000b).

Annexin V Staining. Passage 4 MEFs were plated in triplicate onto 6cm dishes at 5x10^5 cells/dish and arrested by serum starvation or confluence as described above. For each time point the media was pulled off and collected, the cells were washed with PBS, and trypsinized. The collected cells were pool with the media and PBS wash, spun down, and washed twice with cold PBS. Cell pellets were resuspended in cold 10mM Hepes/NaOH, pH 7.4, 140mM NaCl, 2.5mM CaCl2 (Binding Buffer) for a final concentration of 2x10^6 cells/mL. 1x10^5 cells were incubated with anti-Annexin V-FITC (1:20, Pharmingen International) and 0.5μg propidium iodide (PI) in PBS for 15 minutes in the dark at 25°C. The reaction was diluted 1:5 with Binding Buffer and analyzed by flow cytometry.
**zVAD Treatment.** Passage 4 MEFs were plated as described above for Annexin V staining. Cells were treated with either 25 μmol/L of the pan-caspase inhibitor zVAD in DMSO or DMSO alone for two hours. Cells were serum starved as described above in the presence of either 25 μmol/L zVAD or DMSO alone. For each day of the experiment 25 μmol/L zVAD (or DMSO) was added directly into the cell culture. Annexin V analysis was performed as described above.

**Viral Expression and Immunoblotting.** pBabe-Bcl-xL was generated by subcloning human Bcl-xL into the pBabe vector. 293 T cells were infected at 60% confluence with 1μg pBabe vector and 1μg of pCL-Eco packaging construct (Naviaux et al., 1996) using FuGENE 6 (Roche Diagnostics). The media was replaced 24 hours later, and supernatants were harvested at 48 and 72 hrs, filtered and used to infect MEFs with 8 μg polybrene. Infected cells were selected by culturing in 2μg/mL puromycin for 48 hrs. Western blotting assays were performed using 50μg whole cell lysates and probed with anti-Bcl-xL (1:500 dilution, Signal Transduction Laboratories).
RESULTS

We have previously described the initial characterization of mouse embryonic fibroblasts (MEFs) deficient for E2F4, p107, and p130 (Landsberg et al., Submitted). $E2f4^{-/-};p107^{-/-};p130^{-/-}$ (TKO) and $p107^{-/-};p130^{-/-}$ (DKO) MEFs exhibited defects in appropriately exiting the cell cycle in response to confluence. Previous work has shown that DKO MEFs exhibit deregulation of E2F responsive gene expression when released from a serum withdrawal mediated G$_1$ arrest (Hurford et al., 1997). $E2f4^{-/-}$ MEFs, however, do not display any defects in responding to a serum withdrawal mediated arrest (Humbert et al., 2000a; Rempel et al., 2000). We have extended our initial analysis to investigate if TKO MEFs also exhibit an increase in E2F-responsive gene expression in response to a serum withdrawal mediated arrest.

**DKO and TKO MEFs exhibit defects in responding to a serum withdrawal mediated arrest.**

We were interested in comparing E2F target gene expression in TKO MEFs that were released from a serum withdrawal mediated arrest to that of expression in DKO and wild-type MEFs. Briefly, cells were washed twice and then maintained in media containing 0.1% serum for three days. After this time the MEFs were released from this arrest by being fed with media containing 10% serum. Rather surprisingly, expression of E2F target genes such as $cdc2$, $cyclin A2$, proliferating cell nuclear antigen (PCNA), $thymidylate synthase (TS)$, dihydrofolate reductase (DHFR), and B-myb were only slightly upregulated in both DKO and TKO as compared to the control (Figure 1 and data not shown). The deregulation observed was not to the extent observed in DKO and TKO.
MEFs arrested at confluence or that was previously reported for DKO MEFs (Hurford et al., 1997; Landsberg et al., Submitted). The magnitude of $^3$H-thymidine incorporation for both DKO and TKO MEFs was reduced as compared to the control (Figure 2a and data not shown). The decrease in $^3$H-incorporation did not appear to reflect a primary defect in the ability of cells to enter S-phase as the kinetics of S-phase entry as judged by BrdU incorporation revealed that both TKO and DKO MEFs could enter S-phase in an identical manner to the control (Figure 2b and data not shown).

**DKO and TKO MEF undergo apoptosis in response to low serum conditions.**

The discrepancy between the phenotypes of the DKO and TKO MEFs labeled with BrdU versus $^3$H-thymidine incorporation could be because BrdU labeling quantitates the kinetics of S-phase entry on a cell by cell basis but $^3$H-thymidine incorporation reflects the status of the entire cellular population. The reduction in $^3$H-thymidine incorporation observed could reflect a reduction in the cellular population as opposed to a direct defect in entering S-phase. A high level of cellular debris appeared in the media of DKO and TKO MEFs after 60-72 hours of serum withdrawal suggesting that there was a reduction in the cell population due to an increase in cell death. The presence of apoptotic cells was monitored by performing Annexin V/propidium iodine (PI) double staining that revealed an increase in apoptosis in both DKO and TKO MEFs in response to low serum conditions. DKO and TKO showed a 15-30% decrease in viability after 60 hours of serum withdrawal (Figure 2c). This sensitivity to apoptosis did not extend to other forms of arrest since confluence arrested MEFs of both genotypes did not exhibit a decrease in viability as compared to the control (Figure 2d).
Previously we have shown that DKO and TKO MEFs exhibit inappropriate BrdU incorporation in response to a confluence mediated G₁ arrest (Landsberg et al., Submitted). We did not observe aberrant BrdU incorporation in either DKO or TKO MEFs under low serum conditions (Figure 2b and data not shown). The lack of inappropriate BrdU incorporation and absence of severe deregulation of E2F responsive gene expression gives rise to the possibility that DKO and TKO MEFs initiate apoptosis in response to failing to arrest appropriately in low serum conditions. The lack of BrdU positive cells and the absence of severe deregulation of E2F responsive genes could be due to loss of cells that attempt to cycle to apoptosis. In order to determine if this was the explanation for this phenotype, we investigated the nature of the apoptotic response elicited when MEFs lacking p107 and p130 are placed under low serum conditions.

**Apoptosis in DKO and TKO MEFs is not due to a p53 responsive pathway.**

Deletion of E2F4 does not appear to enhance or suppress either the apoptotic response or expression of E2F responsive genes (Figure 1, 2c). Therefore the apoptosis observed in both DKO and TKO MEFs appear to be a phenotype associated with the loss of p107 and p130. This implicates a shared role for these pocket protein family members in inhibiting apoptosis. Inappropriate E2F activity has been shown to result in apoptosis, and deregulated E2F activity could explain the apoptosis observed (as reviewed in Ginsberg, 2002). E2F has been shown to be involved in activating p53 responsive apoptosis (Hiebert et al., 1995; Qin et al., 1994). The expression of p53 responsive genes was analyzed to investigate if p53 activation accounts for the apoptosis observed when DKO and TKO MEFs are deprived of serum. Expression of p21, bax, GADD45, and
$mdm2$ (Figure 3a and data not shown) was regulated normally suggesting that the apoptosis observed when DKO and TKO MEFs are placed in low serum is not due to the activation of p53.

**Apoptosis in DKO and TKO MEFs is not due to a Bcl-xL responsive pathway.**

E2F has been shown to be involved in activating apoptosis through non-p53 responsive pathways (as reviewed in Ginsberg, 2002). We wished to determine if the apoptosis observed in DKO and TKO MEFs was due to a pathway that could be blocked by Bcl-xL expression. Bcl-xL is a member of the Bcl-2 family of anti-apoptotic proteins that function by blocking mitochondrial disruption and cytochrome c release (as reviewed in Adrain and Martin, 2001). This prevents the formation of the apoptosome, whose function is to activate a family of cysteine proteases (caspases) that function to degrade cellular structures (as reviewed in Adrain and Martin, 2001). A retroviral vector containing human Bcl-xL (pBabe-xL) was generated and introduced via retroviral infection into control, DKO and TKO MEFs (Figure 3b and data not shown). After 48 hours of puromycin selection the MEFs were exposed to media containing low levels of serum for 60 hours and the level of apoptosis was analyzed by Annexin V/PI staining. Expression of Bcl-xL did not appear to rescue the apoptosis in DKO and TKO MEFs (Figure 3c and data not shown). This indicates that the apoptosis observed occurs through a pathway that does not require apoptosome formation.
The apoptosis observed in DKO and TKO MEFs can be rescued by the pan-caspase inhibitor zVAD.

To determine if the apoptosis observed in DKO and TKO MEFs could be rescued, the cells were treated with the pan-caspase inhibitor zVAD. DKO and TKO MEFs placed into low serum with zVAD for 24 hours showed a reduction in apoptosis as compared to untreated cells (Figure 3c left). The maximal levels of apoptosis, however, are not observed until after 60 hours of serum starvation. After 24 hours of drug treatment, both control and mutant MEFs exhibited levels of apoptosis higher than the non-treated cells (Figure 3c right). This is most likely due to detrimental effects of prolonged treatment with zVAD.

These results show that cells deficient for p107 and p130 exhibit an increase in sensitivity to apoptosis in response to a reduction of mitogens. This apoptosis has not been attributed to a specific pathway. The data suggests that loss of pocket protein mediated repression leads to activation of an apoptotic program in response to low serum conditions possibly through inappropriate E2F activity.
DISCUSSION

In this study we have extended our analysis of the function of E2F-pocket protein repressive complexes in mediating cell cycle withdrawal. Our previous analysis demonstrated that p107<sup>−/−</sup>:p130<sup>−/−</sup> (DKO) and E2f4<sup>−/−</sup>:p107<sup>−/−</sup>:p130<sup>−/−</sup> (TKO) MEFs fail to arrest appropriately in response to confluence (Landsberg et al., Submitted). Loss of E2F4 in a p107<sup>−/−</sup>:p130<sup>−/−</sup> background did not significantly impact the ability of the cells to arrest by confluence in G<sub>1</sub>. A previous study had reported that p107<sup>−/−</sup>:p130<sup>−/−</sup> MEFs displayed as these cells display high levels of E2F responsive gene expression in response to a serum withdrawal mediated arrest (Hurford et al., 1997). We extended our analysis to determine if loss of E2F4 impacts the ability of MEFs lacking p107 and p130 to respond to a serum withdrawal mediated arrest. Our results demonstrate that loss of E2F4 does not alter the p107<sup>−/−</sup>:p130<sup>−/−</sup> phenotype. Surprisingly, the phenotype of MEFs lacking p107 and p130 in this study is different from that previously reported.

When placed in media containing low levels of serum both DKO and TKO MEFs only display slight deregulation of E2F target gene expression but exhibited a sharp increase in programmed cell death. This finding differs from earlier studies that reported p107<sup>−/−</sup>:p130<sup>−/−</sup> MEFs have highly elevated levels of E2F target gene expression when released from a serum withdrawal mediated arrest (Hurford et al., 1997). This phenotypic difference in phenotype is most likely due to the high levels of apoptosis elicited when p107<sup>−/−</sup>:p130<sup>−/−</sup> MEFs are placed in low serum. The conditions that, in previous studies, have caused an increase in expression of E2F responsive genes now appear to cause a decrease in viability. This suggests that the DKO and TKO MEFs used

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in this study have a lower threshold for tolerating inappropriate E2F activity, leading to the apoptosis observed.

There are several potential explanations for this difference in phenotype. The p130 allele used in this study has been shown to be weakly functional (Rossi et al., 2002) and modifiers of p130 activity have been described (LeCouter et al., 1998). Our analysis and others have used MEFs generated from mice of mixed genetic backgrounds (129/Sv;C57BL/6J), and it is possible that the two separately derived MEF populations might have differences in the balance between the two genetic backgrounds. One can envision a scenario in which, depending on the activity of the residual p130 the cell is pushed towards apoptosis in low serum conditions as opposed to increased E2F responsive gene expression.

The loss of pocket protein repressive complexes in p107<sup>-/-</sup>;p130<sup>-/-</sup> MEFs could lead to apoptosis potentially through the inappropriate activity of E2F. The apoptosis observed in cells lacking p107 and p130 was not p53 dependent, nor could the apoptosis be rescued by Bcl-xL expression. E2F has been shown to activate apoptosis through a number of mechanisms, not all of which are dependent on p53 and Bcl-xL (as reviewed in Ginsberg, 2002). Failure to attribute either of these pathways directly to the apoptosis observed does not rule out E2F involvement. Future experiments will be necessary to confirm E2F’s involvement in the apoptosis. These experiments could include retrovirally-infecting cells with p73 or dominant negative Dp.

Our data demonstrate that p107 and p130 containing repressive complexes function to repress activation of E2F responsive gene expression and serve to protect the cell from inappropriate apoptosis. The exact nature of the apoptosis observed has yet to
be determined, however, the likely cause is inappropriate E2F activity. Future studies will be necessary to confirm that it is the activity of E2F that induces apoptosis in p107−/−;p130−/− deficient cells. This is the first study to show that p107 and p130 have overlapping roles in protecting against apoptosis.

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Fig. 1. E2F responsive gene regulation in serum starved DKO and TKO MEFs.

$p107^{+/+}$, $p107^{+-}$, $p107^{-/-};p130^{-/-}$ (DKO), and $E2f4^{+/+};p107^{+-};p130^{-/-}$ (TKO) were serum starved, released, and analyzed at 0, 3, 6, 9, 12, and 24 hours after serum stimulation for expression of E2F responsive genes. Gene expression was quantitated using the loading control ($ARPP P0$).
Fig. 2. Cells deficient for p107 and p130 apoptosis in response to serum starvation.

(A) \( p107^{+/+} \), and two different lines of \( E2f4^{-/-};p107^{+/+};p130^{-/-} \) (TKO) MEFs were serum starved, released and analyzed for \(^3\)H-thymidine incorporation at the indicated time points. (B) \( p107^{+/+} \) and \( E2f4^{-/-};p107^{+/+};p130^{-/-} \) (TKO) MEFs were serum starved, released and pulsed with BrdU for two hours at 0, 12, 16, and 20 hours after serum stimulation. At least 200 dapi positive nuclei were counted for each time point. (C) At least three independent preparations of \( p107^{+/+}, E2f4^{-/-}, p107^{+/+};p130^{-/-} \) (DKO), and \( E2f4^{-/-};p107^{-/-};p130^{-/-} \) (TKO) were serum starved for 60 hours and labeled with Annexin V/PI. (D) \( p107^{+/+}, E2f4^{-/-}, p107^{-/-};p130^{-/-} \) (DKO), and \( E2f4^{-/-};p107^{-/-};p130^{-/-} \) (TKO) were confluence arrested for 4 days and labeled with Annexin V/PI.
Fig. 3. Analysis of apoptosis in cells deficient for p107 and p130. (A) p107+/− and E2f4+/−;p107−/−;p130−/− (TKO) MEFs were serum starved and analyzed at 0, 1, 2, and 3 days after serum withdrawal for expression of p53 responsive genes. (B) Bcl-xL was constructed in the pBabe vector (Bcl-xL). This plasmid and the empty vector (pBabe) were introduced into p107+/− and E2f4+/−;p107−/−;p130−/− (TKO) MEFs by retroviral infection. Infected cells were serum starved for 60 hours and labeled with Annexin V/PI. (C) p107−/− and E2f4+/−;p107−/−;p130−/− (TKO) MEFs were treated with either DMSO (D) or zVAD (Z) and serum starved for 24 (left) or 60 (right) hours and labeled with Annexin V/PI.
REFERENCES


