The function of E2F6 in the Polycomb complex

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To Juan, Nicolás and all the Burronitos to come
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ABSTRACT

The E2F family of transcription factors are known cell cycle regulators that function at the G1/S transition. Unlike other E2Fs, E2F6 does not activate transcription and is not regulated by pocket protein binding. Instead, this protein appears to repress transcription through the recruitment of the Polycomb Group (PcG) complex. This complex is responsible for the maintenance of Hox gene expression patterns during development and thus ensures the correct anterior-posterior segmentation of the embryo. Genetic ablation of PcG proteins leads to posterior transformations of the axial skeleton as well as other developmental abnormalities such as hematopoietic, cerebellar and smooth muscle defects. The PcG complex has been implicated in cell cycle control since several of its members, including the oncoprotein Bmi1, appear to repress the transcription of p16INK4A and p19ARF. In order to determine the biological function of E2F6, we have generated and characterized E2f6−/− mice and mouse embryonic fibroblasts (MEFs). The mutant mice are viable and survive into adulthood with similar lifespan as their littermate controls. Furthermore, the E2f6 null MEFs are indistinguishable from wild-type MEFs in asynchronous proliferation, cell cycle re-entry from quiescence, senescence and E2F target genes expression levels. These findings suggest that E2F6 does not play a major role in cell cycle control or that its function can be compensated by the action of other factors. In fact, preliminary results from combined loss of E2f6 and Bmi1 suggest that E2F6 may take part in the Bmi1-mediated control of the cell cycle. Furthermore, we found that the loss of E2F6 results in posterior axial skeleton transformations that are reminiscent of the Bmi1-deficient mice defects. The study of the E2f6;Bmi1 compound mutant mice revealed a dosage-dependent synergism between E2F6 and Bmi1. These results indicate that E2F6 participates in segmentation during murine development. As a whole, our work has provided proof that E2F6 is a bona fide Polycomb Group protein and, at the same time, has opened the field to a number of interesting questions.

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Chapter One

Introduction
Part I: The E2F family of transcription factors

The E2F family of transcription factors has long been known to control the expression of genes that are critical for cell cycle entry and progression such as *Cyclin A*, *Cyclin E*, *cdc2*, *Cdc25A*, *E2fl*, *Rb*, *DHFR*, *thymidylate synthase*, *thymidine kinase*, *PCNA*, *ORC1*, *MCMs* and *cdc6* (DeGregori, 2002; Dyson, 1998; Helin, 1998; Nevins, 1998; Trimarchi and Lees, 2002). In recent years, however, microarray analysis in combination with chromatin immunoprecipitation revealed additional E2F-responsive genes that have roles beyond the G1/S transition such as DNA repair and recombination, apoptosis, mitosis, differentiation and development (Cam and Dynlacht, 2003; DeGregori, 2002; Muller et al., 2001; Stevaux and Dyson, 2002).

The E2F transcription factors can activate or repress their target genes in different contexts (Ferreira et al., 2001; Harbour and Dean, 2000b; Helin, 1998; Muller and Helin, 2000; Zhang and Dean, 2001). The activation of E2F target genes is believed to occur via chromatin modifications and the recruitment of the transcriptional machinery to promoters. Two models have been proposed for the E2F-mediated repression of transcription. In the first model, called “inhibition of activation”, pocket protein binding to the transactivation domain of E2F blocks its ability to stimulate the expression of target genes. In the second model, called “active repression”, E2Fs actively repress the expression of target genes through recruitment of chromatin-modifying complexes.

1. Discovery and characterization of E2F activity

Studies on the transcriptional activation of the adenovirus early promoters by Nevins and colleagues led to the discovery of the E2F transcription factors. The
adenovirus E1A (early region 1A) transforming protein activates the transcription of several viral and cellular promoters. Initially, Nevins and co-workers detected a cellular factor that was bound to the early E2 promoter in infected HeLa cells (Kovesdi et al., 1986a). This factor, called the E2 promoter binding factor (E2F), was later purified from extracts of not only infected cells but also of uninfected cells albeit at much lower concentrations (Kovesdi et al., 1986b). Consistent with this finding, E1A induces the E2 promoter binding activity after infection even in the presence of inhibitors of protein synthesis, demonstrating that the induction of E2F activity is post-translational (Reichel et al., 1988). In fact, it was demonstrated that E2F is found in complex with other cellular factors in uninfected cells and, upon infection, E1A dissociates these complexes, releasing the free E2F activity which can induce expression from the E2 promoter (Bagchi et al., 1990).

The transcriptional activation of viral and cellular promoters by E1A was dependent on binding of E2F to specific DNA sequences in these promoters (5’-TTTCCCGC-3’) (Trimarchi and Lees, 2002). DNase footprinting was performed to identify the precise binding site of E2F, revealing two distinct regions of binding at positions –33 to –49 and –53 to –71 of the E2 promoter (Kovesdi et al., 1987; Yee et al., 1987). Yee et al (1987) demonstrated that a purified E2F factor could induce transcription from a promoter containing two E2F-binding sites.

Simultaneously, La Thange and Rigby (1987) characterized a sequence-specific transcription factor, DRTF1 (differentiation regulated transcription factor 1), the activity of which is down-regulated during F9 embryonal carcinoma stem cells differentiation (La Thangue and Rigby, 1987). Soon it was clear that E2F and DRTF1 are the same factor.
E2F/DRTF1 was found in several protein complexes, one of which also contained the Retinoblastoma protein (pRB), a known E1A binding protein (Bandara and La Thangue, 1991; Chellappan et al., 1991; Kaelin et al., 1991; Partridge and La Thangue, 1991; Stevaux and Dyson, 2002; Whyte et al., 1988; Whyte et al., 1989). pRB had been previously identified as the tumor suppressor that when mutated leads to the development of several human tumors such as retinoblastoma, osteosarcoma, small cell lung carcinoma, breast and prostate cancer (Weinberg, 1992). In fact, pRB mutation or loss is detected in at least one third of human tumors. The finding that E1A causes the dissociation of pRB from E2F (Bagchi et al., 1990; Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991), suggested that E1A’s transforming capacity was a result of the release of E2F and that the pRB tumor suppressor functions in opposition by sequestering E2F. Interestingly, the pRB region that is required for E1A and E2F binding is a common site for naturally occurring inactivating mutations of the Rb gene, indicating that the tumors that arise from Rb mutation may be a consequence of E2F deregulation (Hu et al., 1990).

Several studies demonstrated that E2F regulates the expression of many cellular proteins that are necessary for cell cycle progression and S phase entry. A few examples are MYC (Hiebert et al., 1989; Thalmeier et al., 1989), DHFR (Blake and Azizkhan, 1989), thymidine kinase (Dou et al., 1992) and cdc2 (Dalton, 1992). Instead, pRB specifically inhibits the transcription of these genes and this repression is overridden by E1A (Zamanian and La Thangue, 1992). Taken together, these data supported a model in which free E2F promotes cell cycle progression and pRB negatively controls cell proliferation by sequestering E2F. However, it was later shown that the pRB-E2F
complex could also actively inhibit the transcription of target genes when bound to their promoter (Hamel et al., 1992; Weintraub et al., 1992).

2. The pRB/E2F pathway and cell cycle control

In order for a cell to divide and produce two identical daughter cells, it must first faithfully replicate its DNA and then segregate the replicated chromosomes into two separate cells. In addition, most cells double their mass and duplicate their cytoplasmic organelles in each cell cycle. To ensure that these events have been successfully carried out before dividing into two daughter cells, the cell is required to pass through a number of checkpoints that block cell cycle progression when the requirements for the next downstream process are not met. The major checkpoints known to date are a - the G1/S checkpoint (also called restriction point) which prevents entry into S phase in the absence of growth signals or when the cell has not reached a critical mass, b - the G2 checkpoint which ensures that the DNA is fully replicated, the environment is favorable and the cell is big enough before allowing the cell to enter mitosis, and c - the Metaphase checkpoint which blocks cell division when the replicated chromosomes are not properly aligned on the spindles.

The restriction point separates the G1 phase into two functionally different distinct parts. Progression through G1 before reaching the restriction point requires continuous stimulation by mitogenic signals and a high rate of protein synthesis. Once the cell passes through the restriction point, it becomes independent of mitogenic and inhibitory signals and is committed to S phase entry (Sherr, 1996; Zetterberg et al., 1995).

The role of E2F in the control of G1/S transition by stimulating the expression of
important cell cycle regulators, as well as components of the nucleotide biosynthesis and DNA replication machineries, has been well established (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). The indication that E2F is a major downstream target of pRB (Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991; Chittenden et al., 1991) has led to intense studies that identified a pathway in which E2F is regulated through the cell cycle by its interactions with pRB and other pocket proteins, p107 and p130 (Stiegler et al., 1998). Positive regulators, such as cyclin-dependent kinases (cdks), and negative regulators, such as the cdk inhibitors (CKIs), converge on this pathway and, in turn, regulate the activity of pRB and related proteins.

It has been clearly demonstrated that the pRB/E2F pathway mainly governs progression through the G1 restriction point. During quiescence (G0) and early G1, hypophosphorylated pRB binds to and negatively regulates E2F complexes, thereby blocking entry into S phase (Kato et al, 1993). Upon mitogenic stimulation, D-type cyclins (D1, D2 and D3) are induced and subsequently assembled into complexes with their catalytic partners, CDK4 and CDK6 (Kato et al., 1994). The resulting cyclin D-dependent kinases phosphorylate pRB late in G1, causing the release of free E2Fs, which in turn activates the transcription of cell cycle regulators and ensures S phase entry (DeGregori et al., 1995). Specific inhibitors of these kinases (the INK4 proteins, p21WAF1/CIP1, p27KIP1 and p57KIP2) can block cyclin D-dependent kinase activity and cause G1 arrest in response to several signals (Peter, 1997).

These events initiate a positive feedback loop that leads to progressive rounds of pRB phosphorylation and cell cycle progression. Upon E2F release, it transactivates the cyclin E gene (DeGregori et al., 1995; Ohtani et al., 1995) causing a rise in cyclin
E/CDK2 activity, which in turn hyperphosphorylates pRB. In addition, E2F1 is also an E2F target gene (Hsiao et al., 1994; Johnson et al., 1994b). Subsequently, the inactivation of pRB becomes mitogen-independent and can no longer be blocked by the cdk inhibitors. Once the cell enters S phase, cyclin E/CDK2 is inactivated by CDK2-mediated phosphorylation of cyclin E and it is subsequently degraded by ubiquitin-independent proteolysis (Clurman et al., 1996; Won and Reed, 1996).

Cyclin A- and cyclin B-dependent kinases are responsible for maintaining pRB hyperphosphorylation until mitosis. Cyclin A transcription is also regulated by E2F (DeGregori et al., 1995) and the cyclin A/CDK2 complex will begin to accumulate during S phase. This ensures that the E2F-mediated transcription is terminated at the end of S phase since cyclin A-CDK2 binds to the E2F complex and phosphorylates it thereby blocking DNA binding (Xu et al., 1994).

The importance of the pRB/E2F pathway in the restriction checkpoint is underscored by the findings that virtually all human tumors contain mutations that disrupt this pathway (Bartek et al., 1996; Sherr, 1996). The most common mutation found in sporadic human cancers is loss of function mutations of the Rb gene. However, inactivation of the p16^{INK4A} cyclin kinase inhibitor and amplification or translocation of the cyclin D gene are also naturally occurring mutations in several sporadic human tumors. These mutations are generally mutually exclusive since inactivation of a single step in this pathway is sufficient to achieve deregulation of E2F activity.
3. Members of the mammalian E2F family

To date, there are ten genes that encode components of the mammalian E2F transcriptional activity (Figure 1). These genes can be classified into two subfamilies, the E2F subfamily (E2f1 through E2f8) and the DP subfamily (Dp1 and Dp2). The endogenous E2F activity is achieved by heterodimerization of one E2F subunit and one DP subunit (Bandara et al., 1993; Huber et al., 1993), generating a basic helix-loop-helix transcription factor that recognizes the consensus DNA sequence 5’-TTTCGCGC-3’.

The DP proteins can associate with E2F1 through E2F6 interchangeably and this association is necessary for DNA binding. The functional specificity, however, is conferred by the E2F subunit.

The E2F subfamily can be subdivided into three groups based on their structure, transcriptional ability, expression patterns, pocket protein binding and functional properties. The first group, comprised of E2F1, E2F2 and E2F3a, are strong transcriptional activators, are expressed in a cell cycle-dependent manner and interact exclusively with pRB (Helin et al., 1992; Lees et al., 1993). Overexpression of these proteins is sufficient not only to induce quiescent cells to enter the cell cycle (Johnson et al., 1993; Lukas et al., 1996; Qin et al., 1994), but also to override cell cycle arrest in response to TGF-β, p16INK4A, p21CIP1 and p27KIP1 (DeGregori et al., 1995; Mann and Jones, 1996; Schwarz et al., 1995). Furthermore, in primary cells, overexpression of E2F1, E2F2 or E2F3a leads to transformation (Johnson et al., 1994a; Shan and Lee, 1994; Singh et al., 1994; Xu et al., 1995) whereas microinjection of antibodies against E2F3a
Figure 1. The mammalian E2F family of transcription factors.

The conserved domains of the E2F and DP proteins are indicated in the diagram. CA, Cyclin A binding domain. DBD, DNA binding domain. DIM, dimerization domain. MB, Marked box. TAD, transactivation domain. PP, Pocket protein binding domain.
causes cell cycle arrest. Finally, $E2f3$-deficient mouse embryonic fibroblasts (MEFs) display a delay in cell cycle re-entry after serum arrest (Humbert et al., 2000b) and MEFs lacking E2F1, E2F2 and E2F3 are unable to proliferate (Wu et al., 2001). These findings led to the belief that E2F1, E2F2 and E2F3a, called the activating E2Fs, are involved in the induction of cellular proliferation.

The second group, composed of E2F4 and E2F5, are weak transcriptional activators (Muller et al., 1997; Verona et al., 1997), are expressed constitutively throughout the cell cycle (Ikeda et al., 1996; Moberg et al., 1996) and interact with all members of the pocket proteins (Beijersbergen et al., 1994; Dyson et al., 1993; Hijmans et al., 1995; Vairo et al., 1995). Another difference with the activator E2Fs is that they are also regulated by their different subcellular localization during the cell cycle (Gaubatz et al., 2001; Magae et al., 1996; Muller et al., 1997; Verona et al., 1997). These E2Fs seem to function as repressors by recruiting pocket proteins and chromatin modifying enzymes to E2F target genes (Iavarone and Massague, 1999). Although E2F3b has not been characterized extensively yet, initial studies have led to its classification with the repressive E2Fs. First, the patterns of expression of E2F3b are reminiscent of E2F4 and E2F5 (He et al., 2000; Leone et al., 2000). Second, E2F3b has been recently described to act as a transcriptional repressor of the $p19ARF$ gene (Aslanian et al., 2004). Based on these observations, this group of proteins has been called the repressive E2Fs and are believed to play a role in exit from the cell cycle and terminal differentiation.

The third group of E2Fs, comprised by E2F6, E2F7 and E2F8, are pocket protein-independent repressors of transcription whose biological functions remain to be elucidated (Cartwright et al., 1998; de Bruin et al., 2003; Gaubatz et al., 1998; Logan et
al., 2004; Maiti et al., 2005; Morkel et al., 1997; Trimarchi et al., 1998). However, this is a more heterogeneous group. Although all three proteins appear to be expressed in a cell cycle-dependent manner, there are differences in the timing of peak expression. E2F6 reaches its highest level during mid-G1 whereas E2F7 and E2F8 peak during S phase (Dahme et al., 2002; de Bruin et al., 2003; Maiti et al., 2005). Furthermore, the structural analysis of these proteins also indicates that E2F7 and E2F8 are more closely related (Figure 1). E2F6 possesses conserved DNA binding, heterodimerization and marked box domains and lacks the pocket protein binding and transactivation domains (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998). In contrast, E2F7 and E2F8 possess two DNA binding domains and lack the rest of the E2F conserved domains (de Bruin et al., 2003; Logan et al., 2004; Maiti et al., 2005).

Despite the mounting evidence that the activating and repressive E2Fs have opposing functions in the regulation of gene expression, it is unclear if each E2F-responsive gene is regulated by all the different E2F complexes. Recently, it has been suggested that E2F target specificity may not be due to differential DNA binding but rather to the differential ability of E2F to interact with additional transcription factors (Gaubatz et al., 1998; Giangrande et al., 2003; Giangrande et al., 2004).

Both E2F and pRB are evolutionarily conserved with orthologs found in Drosophila melanogaster (Du et al., 1996; Dynlacht et al., 1994a; Ohtani and Nevins, 1994; Sawado et al., 1998; Stevaux et al., 2002), Caenorhabditis elegans (Ceol and Horvitz, 2001; Page et al., 2001), Xenopus laevis (Stevaux and Dyson, 2002) and several plant species (Albani et al., 2000; Magyar et al., 2000; Sekine et al., 1999; Suzuki and Hemmati-Brivanlou, 2000). Drosophila has two E2Fs (dE2F1 and dE2F2), one DP
(dDP) and two pRBs (RBF1 and RBF2). *Xenopus laevis* possesses two E2Fs (EFL1 and EFL2), one DP (DPL1) and one RB (LIN35).

**a. DP1 and DP2**

DP1 (dimerization partner 1 or DRTF1-binding protein) was isolated based on its ability to bind DRTF1/E2F (Girling et al., 1993). Subsequent cloning revealed a DNA binding domain that resembles that of E2F1 and recognizes the same E2F consensus DNA binding site (Girling et al., 1993; Helin et al., 1993). In fact, DP1 and E2F1 heterodimerize. This association results in stronger DNA binding and cooperative activation of an E2F-responsive promoter and is required for stable interaction with pRB (Helin et al., 1993; Krek et al., 1993). Several groups used different methods to clone the DP1 homolog, DP2 (Ormondroyd et al., 1995; Rogers et al., 1996; Wu et al., 1995; Zhang and Chellappan, 1995). Both DP1 and DP2 can heterodimerize with most E2F family members in vivo.

Mice deficient for *Dpi* die by embryonic day 12.5 due to failure of extraembryonic lineages to proliferate (Kohn et al., 2004). To study the role of DP1 in the embryo proper, chimeras were generated and the resulting embryos survive until mid-to late gestation suggesting that DP1 is dispensable for embryonic development (Kohn et al., 2004).

**b. E2F1**

The *E2f1* gene was simultaneously cloned by three different groups through cDNA expression library screening for pRB binding proteins (Helin et al., 1992; Kaelin...
et al., 1992; Shan et al., 1992). The E2F1 protein was found to interact exclusively with the pocket domain of pRB in vitro and in vivo and this interaction was competed by viral proteins known to disrupt the pRB-E2F association such as E1A. This protein co-purified with the E2F activity and could bind to E2F recognition sites causing a 10-fold activation of the adenovirus E2 promoter. Moreover, the E2F1 protein was found to be expressed in a cell cycle-dependent manner with highest levels during G1/S.

Sequence analysis of the E2f1 gene revealed regions that allowed DNA binding in a sequence-specific manner, DP dimerization, transactivation and pocket protein binding. The DP dimerization domain is composed of a hydrophobic heptad repeat (a putative leucine zipper) and a marked box motif, which has also been implicated in DNA bending (Cress and Nevins, 1996). Furthermore, E2F1 contains a cyclin A binding domain in the amino terminal region (Devoto et al., 1992; Krek et al., 1994; Mudryj et al., 1991; Pagano et al., 1992a; Xu et al., 1994). This domain allows specific binding to cyclin A/CDK2 and cyclin A/CDC2 complexes resulting in phosphorylation of the DP subunit and loss of E2F DNA binding and transactivation activities during S phase (Dynlacht et al., 1994b; Krek et al., 1995; Xu et al., 1994). There is also evidence that E2F1 is phosphorylated by cyclin A/CDK complexes resulting in its targeting for degradation (Adams and Kaelin, 1996; Peeper et al., 1995). Lastly, E2F1 possesses a nuclear localization signal and is, therefore, found exclusively in the nucleus (Muller et al., 1997; Verona et al., 1997).

Overexpression studies have shown that E2F1 can override the cell cycle arrest induced by serum deprivation or by growth arrest signals such as TGF-β or cdk inhibitors (DeGregori et al., 1995; Johnson et al., 1993; Kowalik et al., 1995; Mann and Jones,
1996; Schwarz et al., 1995; Shan and Lee, 1994) as well as transform primary fibroblasts (Johnson et al., 1994a; Shan and Lee, 1994; Singh et al., 1994; Xu et al., 1995). Finally, inhibition of E2F1 activity is necessary for differentiation of several lineages as ectopic expression of E2F1 can inhibit the differentiation of C2C12 myocytes (Wang et al., 1995), epidermal keratinocytes (Paramio et al., 2000), squamous keratinocytes (Wong et al., 2003) and chondrocytes (Wong et al., 2003). In agreement with this, E2F1 overexpression induces DNA synthesis in post-mitotic cerebellar neurons (Suda et al., 1994). However, E2F1 seems to promote differentiation of adipocytes through induction of PPAR-γ (Fajas et al., 2002), suggesting that the role of E2F1 in terminal differentiation might be tissue-specific.

E2F1 has also been implicated in the induction of apoptosis through p53-dependent and independent mechanisms as a result of overexpression or in response to DNA damage (DeGregori et al., 1997; Hiebert et al., 1995; Hsieh et al., 1997; Kowalik et al., 1995; Lissy et al., 2000; Phillips et al., 1999; Qin et al., 1994; Shan and Lee, 1994). Similarly, in flies, ectopic expression of dE2F1 in the imaginal discs also leads to apoptosis (Asano et al., 1996; Du et al., 1996). It is thought that the E2F induced apoptosis is blocked during normal cell cycle progression by pRB and the Ras-PI3 kinase signaling pathway (Hallstrom and Nevins, 2003; Harbour and Dean, 2000a; Lipinski and Jacks, 1999).

E2F1 is believed to trigger p53-mediated apoptosis partly through direct induction of p19^ARF, a known E2F target gene, which in turn inhibits the MDM2-mediated degradation of p53 (Bates et al., 1998). However, both through in vivo and in vitro experiments, p19^ARF was shown to be dispensable for the E2F1-mediated p53-dependent
apoptosis (Lindstrom and Wiman, 2003; Tolbert et al., 2002; Tsai et al., 2002), suggesting that there must be alternative mechanisms. One possibility is that E2F1 regulates p53 in a transcriptional independent manner (Hsieh et al., 2002; Nip et al., 2001; Phillips et al., 1999; Stanelle et al., 2003). For example, E2F1 could induce p53 stabilization by directly binding to it (Hsieh et al., 2002) or by inducing its phosphorylation (Rogoff et al., 2002).

Several mechanisms have been proposed for the p53-independent apoptosis triggered by E2F1. First, p73 has been shown to be transactivated by E2F1 and required for the E2F1-mediated apoptosis in cells lacking p53 (Irwin et al., 2000; Lissy et al., 2000; Stiewe and Putzer, 2000). Second, E2F1 can inhibit anti-apoptotic signals such as NF-kappa B (Phillips et al., 1999) and Mcl-1 (Croxton et al., 2002; Elliott et al., 2001). Finally, recent studies have shown that E2F1 can also induce propaptotic genes such as Apaf-1 (Moroni et al., 2001), caspases (Nahle et al., 2002), BH3-only proteins (Nahle et al., 2002) and SIVA (Nahle et al., 2002).

Lastly, a clear link between E2F1 and DNA damage control has also been established. Upon DNA damage, E2F1 protein levels rise, probably due to phosphorylation and stabilization of the protein (Lin et al., 2001; Stevens et al., 2003), leading to either apoptosis (Huang et al., 1997; Lin et al., 2001; Stevens and La Thangue, 2003) or cell cycle arrest through inhibition of E2F1 functions by TopBP1 (Liu et al., 2003). Furthermore, E2F1 associates with the Mre11 recombination and repair complex (Maser et al., 2001).

Mutant mice for E2f1 have been generated and have supported the in vitro findings demonstrating a role for E2F1 in cell proliferation and apoptosis (Yamasaki et
al., 1996). These mice are viable and fertile but exhibit testicular atrophy, exocrine
dysplasia, impaired pancreatic growth and increased tumorigenesis as well as increased T
cell numbers and splenomegaly (Fajas et al., 2004; Lissy et al., 2000; Yamasaki et al.,
1996). This suggests that E2F1 functions both as a tumor suppressor, probably through
its capacity to induce apoptosis, and as an oncogene, through its ability to stimulate cell
proliferation. Consistent with this notion, Pierce et al (Pierce et al., 1998a; Pierce et al.,
1998b; Pierce et al., 1999; Pierce et al., 1998c) have shown that the overexpression of
E2F1 in tissue-specific transgenic mice both predisposes the mice to a variety of
spontaneous tumors and prevents the formation of skin tumors following a carcinogenesis
protocol. Loss of E2f1 in the Rb heterozygous background reduces the frequency of
pituitary and thyroid tumors demonstrating that E2F1 deregulation is an important factor
in the pRB-related tumorigenesis (Yamasaki et al., 1998). However, E2f1−/− MEFs
display normal proliferative properties suggesting that the loss of E2F1 is compensated
by the activities of the other activating E2Fs (Humbert et al., 2000b).

c. E2F2 and E2F3

Electrophoretic mobility shift assays suggested that E2F1 could not account for
all the E2F DNA binding activity. Therefore, a cDNA library was screened at low-
stringency hybridization with an E2F1 probe and two clones that encoded proteins with
high degree of sequence conservation to E2F1 were isolated (Ivey-Hoyle et al., 1993;
Lees et al., 1993). The conservation was observed throughout the protein including the
cyclin A binding, nuclear localization signal, DNA binding, dimerization, transactivation
and pRB binding domains. These E2F-like proteins, called E2F2 and E2F3, are very
similar to E2F1 not only in structure but also in function and regulation. They are expressed in a cell cycle-dependent manner, are found exclusively in the nucleus and bind cyclin A/CDK complexes (Devoto et al., 1992; Leone et al., 2000; Mudryj et al., 1991; Muller et al., 1997; Pagano et al., 1992b; Verona et al., 1997). E2F2 and E2F3 can bind wild-type but not mutant E2F recognition sites and activate the transcription of E2F-responsive genes. They also bind exclusively to pRB and are strong transcriptional activators. In fact, they behave in a similar manner to E2F1 in overexpression assays by inducing cell cycle progression and transformation of primary fibroblasts (DeGregori et al., 1997; Lukas et al., 1996; Xu et al., 1995).

In contradiction to initial studies (DeGregori et al., 1997; Kowalik et al., 1998; Leone et al., 2001; Lissy et al., 2000), recent findings show that, like E2F1, E2F2 and E2F3 are also capable of inducing apoptosis although this is still in debate in the field (Vigo et al., 1999; Ziebold et al., 2001).

Recently, it was shown that the E2f3 locus encodes for two different proteins: the original E2F3, now called E2F3a, and the novel E2F3b (He et al., 2000; Leone et al., 2000). An intronic promoter within the first intron of the E2f3 locus allows expression of E2F3b from an alternative exon 1. The result is a protein that differs from E2F3a in that the 122 residues of the N-terminus are replaced by 6 distinct amino acids while the rest of the protein remains identical. Thus, E2F3b contains all the conserved domains described for E2F3. Two pieces of data suggest that E2F3b possesses distinct functional characteristics from E2F2 and E2F3a and is therefore classified with the repressive E2Fs. First, E2F3b is expressed throughout the cell cycle with peak levels in G0 when it is associated with pRB. Second, E2F3b has recently been shown to participate in the
transcriptional repression of the p19ARF gene (Aslanian et al., 2004). However, unlike E2F4 and E2F5, E2F3b seems to associate primarily with pRB during quiescence (Leone et al., 2000).

Despite the characterization of E2F2 as an activator E2F, homozygous loss of the \( E2f2 \) gene in mice provides evidence for its role in transcriptional repression of genes necessary for cell proliferation in T lymphocytes. This leads to an accumulation of autoreactive effector/memory T lymphocytes that are responsible for the late-onset autoimmune disease in \( E2f2 \) mutant mice (Murga et al., 2001). Furthermore, compound mutants for \( E2f1 \) and \( E2f2 \) are highly predisposed to tumor formation and exhibit some signs of autoimmunity (Zhu et al., 2001). These results suggest that E2F2 may also function to negatively regulate cellular proliferation at least in certain tissues.

Mice deficient for E2F3 were generated and they have demonstrated a role for E2F3 in normal embryonic development as well as in normal cell proliferation in response to mitogenic signals (Humbert et al., 2000b). It is important to note that these mice lack both E2F3a and E2F3b making it difficult to assess the contribution of each of these proteins. Only 25% of expected numbers of \( E2f3 \) mutant mice are born and MEFs lacking E2F3 are not able to induce the expression of several E2F-responsive genes in response to mitogens. The \( E2f3 \)-deficient adult mice develop congestive heart failure but in contrast to E2F1, \( E2f3 \)-deficiency does not lead to increased tumor formation. Interestingly, loss of E2F3 in the \( Rb \) heterozygous background suppresses the pituitary tumors like E2F1 but enhances the thyroid carcinomas metastasis (Ziebold et al., 2003). Almost all the defects observed in mice lacking either E2F1 or E2F3 were exacerbated in the compound mutant (Cloud et al., 2002). Thus, it appears that E2F1 and E2F3 have
distinct roles as well as overlapping functions. The overlapping role of all the activating E2Fs in cell cycle entry is underscored by the cell proliferation block observed in MEFs deficient for all three activating E2Fs (Wu et al., 2001).

d. E2F4 and E2F5

The discovery of p107 and p130 suggested that E2F1, E2F2 and E2F3, which only bind pRB, could not account for all of the cellular E2F activity (Cobrinik et al., 1993; Shirodkar et al., 1992). This led several laboratories to embark on a search for the missing E2F(s) and, as a result, E2F4 and E2F5 were cloned (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Sardet et al., 1995). These proteins have significant homology to E2F1 (approximately 50%) but much higher homology to each other (78% similarity). They are truncated at the N-terminus and therefore lack the cyclin A binding domain as well as the nuclear localization signal (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Leone et al., 2000; Sardet et al., 1995). Instead, E2F4 contains two leucine/isoleucine-rich nuclear export signals, which trigger the export of E2F4 from the nucleus when it is not bound to pocket proteins (Gaubatz et al., 2001). Both proteins require DP for efficient DNA binding to promoters containing E2F sites. E2F4 associates with all members of the pocket proteins whereas E2F5 preferentially associates with p130. Although E2F4 and E2F5 lack the cyclin A binding domain, they are phosphorylated by cyclin/CDK complexes through p107- and p130-mediated interactions (Devoto et al., 1992; Faha et al., 1992; Ginsberg et al., 1994; Lees et al., 1992; Shirodkar et al., 1992). The expression patterns of E2F4 and E2F5 are very different than those of the activating E2Fs as they are constitutively expressed throughout
the cell cycle with peak levels in mid-G1 phase before the activating E2Fs are detectable.

E2F4 and E2F5 are much less effective than the activating E2Fs in releasing cells from quiescence when overexpressed (DeGregori et al., 1997; Lukas et al., 1996). Consistently, overexpression of the repressive E2Fs cannot overcome a p16\(^{INK4A}\)-induced cell cycle arrest (Mann and Jones, 1996). Furthermore, reporter assays using mutants of the E2F sites within the promoters of E2F-responsive genes revealed an increase in reporter activity during G0/G1 suggesting that while E2Fs are responsible for activation of transcription during G1/S, they also negatively regulate the expression of target genes during G0/G1 (Dalton, 1992; Lam and Watson, 1993). In fact, E2F4-p130 complexes were bound to promoters during G0/G1 and are thought to be the main complex that represses the transcription of E2F-responsive genes during quiescence and early G1 (Takahashi et al., 2000). This repression appears to be achieved by the recruitment of HDAC1 and mSin3B corepressor complexes (Rayman et al., 2002). Taken together, these data suggest that E2F4 and E2F5 function to repress E2F target genes during G0.

The generation of \(E2f4\) and \(E2f5\) mutant mice uncovered specific functions for these proteins in development. Mice lacking E2F5 displayed increased numbers of cerebrospinal fluid-producing epithelial cells in the choroids plexus and as a result developed hydrocephalia (Lindeman et al., 1998). In contrast, loss of E2F4 lead to craniofacial defects, hematopoietic abnormalities due to defects in maturation and a reduction in the thickness of the gut epithelium (Humbert et al., 2000a; Rempel et al., 2000). The analysis of \(E2f4;E2f5\) compound mutant revealed that the repressive E2Fs also perform overlapping functions in development since loss of both proteins results in neonatal lethality (Gaubatz et al., 2000). Surprisingly, mutation of the repressive \(E2f4\) in
the Rb heterozygous background suppresses the development of pituitary and thyroid tumors as well as the inappropriate gene expression and proliferation (Lee et al., 2002). The tumor suppression appears to occur through release of p107 and p130 molecules, which in the absence of pRb, can bind and inhibit the deregulated activating E2Fs.

While it is clear that the repressive E2Fs play a role in terminal differentiation, depending on the context the regulation can be positive or negative. For example, loss of E2F4 leads to spontaneous adipogenesis in a pocket protein-independent manner (Landsberg et al., 2003) but E2F4 overexpression promotes the differentiation of the P12 neuronal precursors and keratinocytes (Paramio et al., 2000; Persengiev et al., 1999).

In contrast to the obvious role of the repressive E2Fs in development and differentiation, studies of the mutant MEFs have shown that E2F4 and E2F5 are dispensable for normal cell cycle progression (Gaubatz et al., 2000; Humbert et al., 2000a; Lindeman et al., 1998). However, E2f4;E2f5 double mutant MEFs failed to arrest in G1 in response to p16INK4A, indicating that they are necessary for pocket protein-mediated arrest (Gaubatz et al, 2000).

e. E2F6, E2F7 and E2F8

E2F6, E2F7 and E2F8, the most recently identified E2Fs, are repressors of transcription that act independently of pocket proteins. However, this is not a cohesive group since there are significant differences between these proteins. E2F7 and E2F8, share unique structural features that distinguish them from all other E2F members (de Bruin et al., 2003; Logan et al., 2004; Maiti et al., 2005). These proteins possess two DNA binding domains and lack the heterodimerization, marked box, pocket protein
binding and transactivation domains. Modeling studies suggest that, in order to bind DNA, the two DNA binding domains must interact probably mimicking the E2F/DP heterodimer structure. This organization resembles the E2F-like proteins from *Arabidopsis thaliana* (Vlieghe et al., 2005). Thus, E2F7 and E2F8 associate with E2F consensus recognition sites in a DP-independent manner.

Both proteins are expressed in a cell cycle dependent manner with peak levels during S phase, at least in part, through E2F regulation. Furthermore, they display similar patterns of expression in adult tissue with highest expression in skin and testes. At least E2F7 has been shown to associate with a subset of E2F-responsive promoters during S phase and overexpression of E2F7 or E2F8 delays the proliferation of MEFs. However, their biological functions remain to be determined.

In contrast, E2F6 is much more conserved to the rest of the E2F family. This protein was identified by several groups based on the sequence conservation of the E2F DNA binding domain (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998). However, once the full-length sequence was obtained, it was clear that the homology extended also to the dimerization domain, composed of the leucine zipper and the marked box, as well as to the nuclear localization signal (Figure 1). Yet, E2F6 is truncated at the C-terminus and therefore lacks the pocket protein binding and transactivation domains. Consistent with this structure, it has been shown that E2F6 is a nuclear protein that binds DP but not pocket proteins and that the E2F6/DP heterodimer is able to associate with DNA at E2F binding sites. It appears that the E2F6/DP complex has a preference for a 5’-TTTCCCCGC-3’ E2F recognition site instead of the E2F consensus site from the E2 promoter (5’-TTTCGCGC-3’). In addition, E2F6
overexpression fails to induce transcription of a reporter gene. Instead, it seems to repress E2F-dependent transcription in a dominant negative manner and this repression is dependent on an intact marked box domain. E2F6 levels are low during G0 and start to rise during the G0/G1 transition with maximum expression in mid-G1 (Dahme et al., 2002).

Initial studies showed that overexpression of E2F6 delays exit from S phase resulting in an accumulation of cells in S phase (Cartwright et al., 1998) whereas E2F6 microinjection into serum-arrested cells prevents S phase entry upon serum re-addition (Gaubatz et al., 1998). In agreement with the latter experiment, E2F6 has been shown to bind and repress the G1/S-, but not the G2/M-regulated, E2F target genes during S phase (Giangrande et al., 2004). The implication is that E2F6 would repress the G1/S target genes at the end of S phase while allowing E2F-mediated transcription of G2/M target genes. In this model, E2F6 is proposed to stimulate S phase progression. Yet another contradiction comes from the finding that E2F6 preferentially occupies certain E2F target promoters in G0 cells (Ogawa et al., 2002). This discrepancy could be due to differences in cell types since Giangrande et al. (2004) used T98G cells whereas Ogawa et al. (2002) used BJ-1 cells. Significantly, Ogawa et al. (2002) did not detect E2F4 and p130 bound to promoters during G0 in opposition to a report that in T98G cells, E2F4 and p130 occupy target promoters in G0 and early G1 phases (Takahashi et al., 2000).

In an attempt to understand the biological significance of E2F6, a yeast two-hybrid screen has identified RYBP (Ring1 and YY1 binding protein) as an E2F6 interactor (Trimarchi et al., 2001). RYBP is a member of the mammalian Polycomb Group (PcG) complex (which will be discussed in more detail in the second part of this
introduction). In fact, E2F6 associates with a number of the mammalian PcG proteins including Ring1A, MEL-18, Mph1 and Bmi-1, as determined by co-immunoprecipitation assays. At least the interaction with RYBP seems to be through direct contacts with the marked box domain, which is responsible for the E2F6-mediated repression. More recently, another E2F6-containing complex has been purified from cultured cells and mass spectrometry analysis has identified Mga and Max, chromatin modifiers such as histone methyltransferases, heterochromatin protein 1 gamma (HP1γ) and several PcG proteins such as RING1, RING2, MBLR, h-I(3)mbt-like protein and YAF2 (Ogawa et al., 2002). Finally, another yeast two-hybrid screen identified another PcG protein, EPC1, as an E2F6 interactor (Attwooll et al., 2005). The complex containing E2F6 and EPC1 also contains DP1, the PcG protein EZH2 and Sin3B. Interestingly, EZH2 is required for cellular proliferation, suggesting a role for E2F6 in the regulation of cell cycle progression. Although there is controversy over the identity of the PcG proteins that interact with E2F6, it is most likely that the E2F6-mediated repression is achieved by recruitment of repressive complexes such as the PcG complexes.

A suggested alternative mechanism for the E2F6-mediated repression is methylation of histone H3 at lysine 9 through the recruitment of a euchromatic-specific histone methyltransferase to DNA (Ogawa et al., 2002). This methylated lysine 9 is known to recruit the heterochromatic protein 1 (HP1) resulting in formation of a repressive heterochromatic structure. In contrast to this model, E2F6-regulated promoters do not seem to contain histone H3 methylated lysine 9 (Oberley et al., 2003). Instead, E2F1 is recruited to the target promoters in the absence of E2F6 indicating that
E2F6 is able to repress transcription simply by preventing the activating E2Fs from binding to DNA.

To identify E2F6 target genes, Oberley et al. (2003) have used chromatin immunoprecipitation followed by CpG island microarrays in cancer cells. The results from this screen were confirmed as they were up-regulated when E2F6 was knocked-down using RNA interference. Many of these genes encoded proteins that are involved in tumor suppression and maintenance of chromatin structure such as brca1, ctip, art27, hp1α and rbap48 (Oberley et al., 2003).

Despite in vitro evidence that suggest roles for E2F6 in cell cycle control as well as in mouse development, the biological significance of E2F6 remains unknown. E2f6 mutant mice may significantly broaden our understanding of E2F6 and its function.
Part II: The Polycomb group complex

The Polycomb group (PcG) proteins were first identified in *Drosophila* as repressors of the *homeotic* (*Hox*) genes that are responsible for the segmentation patterning of the embryo. In early fly development, the expression patterns of the *Hox* genes are established by the segmentation genes. About four hours into embryogenesis, the transcriptional state of *Hox* genes is no longer controlled by these genes but by the trithorax group (TrxG) and the PcG complexes. These complexes maintain the active or inactive state of *Hox* genes transcription. Specifically, the TrxG complex maintains the active state of those *Hox* genes that were initially on, whereas the PcG group maintains the inactive state of those genes that were initially off.

Many mammalian PcG proteins have been identified based on homology to members of the *Drosophila* PcG genes or by yeast two-hybrid screens (See Table 1). In mammals, there are many more PcG proteins suggesting a higher level of complexity and redundancy than in the fly. However, both in mammals and flies, there are at least two distinct polycomb complexes, commonly known as PRC1 and PRC2 (Table 1). The PRC2 complex functions early in development and initiates polycomb-mediated repression. The PRC1 complex functions later in development maintains the repression at later stages of development. However, there is mounting evidence suggesting that there may be many different "early" and "late" PcG complexes with different protein compositions.
1. The homeotic genes and their role in segmentation

Segmentation was defined by Bateson in 1894 as a repetition of pattern elements along the major anterior-posterior (A/P) axis of the body. He also described homeosis as the phenomenon in which one segment is transformed toward the identity of another (Bateson, 1894). The process of segmentation is best understood in the fruit fly, *Drosophila melanogaster*. The basic segmentation of Drosophila is established through the subdivision of the embryo body into increasingly specified body regions by the sequential action of a hierarchy of regulatory genes (reviewed in (Akam, 1987).

Initially, the egg polarity is specified by maternally active coordinate genes; then, zygotically active segmentation genes determine the patterning of the embryo (Nusslein-Volhard et al., 1980; Nusslein-Volhard and Wieschaus, 1980). There are three groups of segmentation genes: the gap, pair-rule and segment polarity genes. Mutations in these genes result, respectively, in gaps in the array of segments, deletion of alternate segments and defects in the sequence of pattern elements within segments. Finally, the homeotic (*Hox*) genes determine the unique identity of equivalent segmental units (Anderson et al., 1985; Anderson and Nusslein-Volhard, 1984; Lewis, 1978; Nusslein-Volhard et al., 1985; Nusslein-Volhard et al., 1980; Nusslein-Volhard and Wieschaus, 1980; Wieschaus et al., 1984) and their mutation usually leads to homeotic transformations in which one segment is changed into another. However, it is important to note that the *Hox* genes have also been implicated in the processes of morphogenesis and organogenesis (Hombria and Lovegrove, 2003) as well as in cell cycle control (Del Bene and Wittbrodt, 2005).

During development, *Hox* genes are expressed in specific zones on the A/P axis with very sharp anterior boundaries and less well-defined posterior boundaries. This
expression pattern is established in the syncytial blastoderm about two hours into embryogenesis and is maintained throughout the course of development. The gap and pair-rule proteins are responsible for specifying the initial \textit{Hox} genes expression boundaries. Specifically, gap proteins are repressors whereas pair-rule proteins are activators of transcription and the correct \textit{Hox} gene expression results from the coordinated actions of these two classes of transcription factors (Muller and Bienz, 1992; Qian et al., 1993; Shimell et al., 1994; Zhang and Bienz, 1992).

The first two homeotic mutants isolated were \textit{bithorax} and \textit{antennapedia}. The \textit{bithorax} mutant has a transformation of the halteres into an extra pair of wings (Lewis, 1978; Lewis, 1998) whereas the \textit{antennapedia} mutant transforms the antenna on the head of the fly into an extra pair of thoracic legs (Kaufman et al., 1990; Plaza et al., 2001). Since then, massive genetic screens designed to identify lethal mutations that alter the pattern of the larval cuticle led to the discovery of many more of these regulatory genes in \textit{Drosophila} (Nusslein-Volhard et al., 1985; Nusslein-Volhard and Wieschaus, 1980). Subsequently, multiple \textit{Hox} genes were found in all bilateral organisms. Mutational studies in mice revealed homeotic transformations of the axial skeleton confirming that vertebrate \textit{Hox} genes control the anterior-posterior patterning in a similar manner to that of their invertebrate counterparts. Loss of function mutations of \textit{Hox} genes in both flies and mice lead to anterior transformations whereas gain of function mutations result in posterior ones. This ability of a more posterior \textit{Hox} gene to impose its function on more anterior genes is called posterior prevalence.

Each of the \textit{Hox} gene products contains a 60-amino acid homeodomain (HD) that is conserved across the species (reviewed by (Scott et al., 1989). The structure of the HD
has been determined by nuclear magnetic resonance and crystallographic studies. These domains are related to the helix-turn-helix motif of prokaryotic DNA-binding proteins. The Hox proteins are monomeric transcription factors that positively or negatively regulate the expression of multiple target genes. *In vitro* experiments have shown that they recognize the DNA sequences 5’-TAAT-3’ (Laughon, 1991). Since this consensus sequence is very short, modulators and cofactors are thought to assist Hox proteins in assembling specific activation and repression complexes on the regulatory elements of Hox target genes (Kornberg, 1993; Mann and Chan, 1996). Although many Hox target genes have been described, only a few have been shown to be directly regulated by the Hox genes (Biggin and McGinnis, 1997; Liang and Biggin, 1998). It has been proposed that the Hox proteins function by controlling the expression of other genes called “realizators” that are directly involved in controlling cell proliferation, survival, shape changes and rearrangements (Garcia-Bellido, 1975). We now know that in many cases, the downstream targets of Hox proteins are actually networks that control the expression of the realizators.

Interestingly, the Hox genes are arranged in clusters where the position and order of orthologs are conserved in different species and are collinear with the order of the anterior boundaries of expression and the timing of activation during development (Zakany and Duboule, 1999a; Zakany and Duboule, 1999b). While Drosophila contains only one cluster of Hox genes, mammals have four Hox genes clusters (HOXA, HOXB, HOXC and HOXD).
2. Identification of the Polycomb Group complex

Mutations in the *Hox* genes lead to transformation of one body segment into another along the A/P axis. Similar phenotypes were observed in mutants of genes that lie outside of the *Hox* gene clusters. Initially the *extra sex combs* (*esc*) mutant was described by Slifer in 1940 and later, Lewis found the *polycomb* (*Pc*) mutant in 1947. Both of these mutations lead to additional sex combs on the second and third pairs of legs in males, instead of only on the first leg, where they usually belong. To date, 18 such genes have been identified and the group has been termed the Polycomb Group (PcG) (Paro, 1990; Ringrose and Paro, 2004). The posterior transformations observed in the PcG mutants are due to inappropriate derepression of *Hox* gene expression, equivalent to a gain-of-function mutation in *Hox* genes (Kennison, 1995; Kennison, 2004). Years later, the Trithorax Group (TrxG) was identified as regulators of *Hox* genes that function in opposition to the PcG proteins (reviewed by (Ringrose and Paro, 2004). Consistently, mutations in the 17 genes identified so far suppress the extra sex comb phenotype.

For most of the *Drosophila* PcG genes, multiple mammalian counterparts have been isolated (See Table 1) and they also appear to play an important role in the maintenance of *Hox* gene repression (Satijn and Otte, 1999; van Lohuizen, 1999). While there seems to be a higher degree of complexity in mammals, the proteins are fairly well conserved. In fact, many mammalian proteins are able to partially rescue the phenotypes caused by mutations on their *Drosophila* orthologs. These include the mouse Pc ortholog, M33 (Muller, 1995), the mouse polyhomeotic ortholog and the human pleiohomeotic ortholog, YY1 (Jooss et al., 1995).
Mutant mice for many PcG proteins display axial skeleton transformations and deregulation of Hox gene expression like the Drosophila mutants. Unlike the fly genes, the mammalian PcG genes are expressed differentially in different tissues and cell types (Alkema et al., 1997b; Lessard et al., 1998; Pearce et al., 1992; Raaphorst et al., 2001; Raaphorst et al., 2000; Sewalt et al., 1998; van Lohuizen et al., 1998). This probably explains the mutant-specific phenotypes, which include cerebellar defects in the Bmil-deficient mice, male-to-female sex reversal in M33 mutant mice, smooth muscle atrophy in Mel18 mutant mice and neural crest defects in Mph-deficient mice (Akasaka et al., 1996; Akasaka et al., 1997; Core et al., 1997; Katoh-Fukui et al., 1998; Raaphorst et al., 2001; Takihara et al., 1997; Tokimasa et al., 2001).

However, although the transformations in mutant mice are highly reminiscent of those found in flies, they are relatively mild compared to the drastic malformations observed in the Drosophila mutants. This may reflect a higher level of functional redundancy between the mammalian PcG proteins. The idea of redundancy in Hox gene repression was first suggested by Jürgens (1985) based on the phenotypes of double and triple mutants flies for the Polycomb Group genes. If the PcG proteins formed one functional complex, it would be expected that loss of one protein in the complex should completely abolish the function of the complex. Therefore the loss of additional proteins should not make a difference (Peterson and Herskowitz, 1992). However, in all cases examined, double and triple mutants for Polycomb Group genes have more severe phenotypes than the single mutants (Cheng et al., 1994; Moazed and O'Farrell, 1992; Peterson and Herskowitz, 1992). Subsequently, the analysis of double mutants in mice have also revealed a high degree of redundancy (Akasaka et al., 2001; Bel et al., 1998).
3. The composition of PcG complexes

The PcG proteins act in large multiprotein complexes that modify chromatin to maintain the repressed states of their target genes (Alkema et al., 1997a; Franke et al., 1992; Gunster et al., 1997). At least two distinct multiprotein Polycomb Group complexes have been purified (Table 1) (Lund and van Lohuizen, 2004). First, the Polycomb Repressive Complex 2 (PRC2) is a 600 kDa complex that was purified from Drosophila embryos and Hela cells and is involved in the initiation of silencing. It contains ESC, Enhancer of zeste (E(z)), Suppressor of zeste 12 (Su(z)12) and a small number of additional proteins depending on the purification protocol used (Table 1). These include histone deacetylases and histone methylases (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2004; Muller et al., 2002; Tie et al., 2001; van der Vlag and Otte, 1999). Consistent with their role in early development, deletion of PRC2 genes in mice result in early embryonic lethality during gastrulation (O'Carroll et al., 2001; Schumacher et al., 1998). Second, the Polycomb Repressive Complex 1 (PRC1) is a 1-2 MDa complex that was purified from Drosophila Schneider cells and human Hela cells and is implicated in the stable maintenance of gene repression. The core components of this complex are Polycomb, Polyhomeotic, Posterior Sex Combs and RING (Table 1) (Francis and Kingston, 2001; Lavigne et al., 2004). Mutant mice for most PRC1 genes survive until birth due to partial functional redundancy (Akasaka et al., 2001; Core et al., 1997; Takihara et al., 1997; van der Lugt et al., 1994b). An exception is Rnfl/Ring1b-deficiency, which leads to an early lethal phenotype similar to that of PRC2-deficient mice (Voncken et al., 2003). Although studies demonstrate that PRC1 functions later in
Table 1. Polycomb Group Complexes in flies, humans and mice. Adapted from (Valk-Lingbeek et al., 2004).

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<th>Human proteins</th>
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</table>

deviation than PRC2, the mode of action of these complexes is currently under debate and will be discussed later in this chapter.

Importantly, there is strong evidence that the function and composition of these core complexes in vivo is modulated in different tissues (Otte and Kwaks, 2003) and at different target genes (Rastelli et al., 1993; Strutt and Paro, 1997). Furthermore, many other proteins have been reported to associate with PcG proteins (Mulholland et al., 2003; Sewalt et al., 2002; Trimarchi et al., 2001; van der Vlag and Otte, 1999) suggesting that
an enormous functional diversity can be achieved by multiple combinations of core and auxiliary factors.

4. The Polycomb Group target genes

In *Drosophila*, the PcG proteins have been found to bind to more than 100 sites in polytenic chromosomes from larval salivary gland (Rastelli et al., 1993; Zink and Paro, 1989). The binding specificity has been proposed to be achieved through the cis-regulatory PcG response elements (PREs) that enable both the PcG and TrxG proteins to bind and maintain the status of transcriptional activity over many cell generations. The PREs are large sequences (100-300 bp) and tend to be very divergent. PREs act in groups of two or more elements that are modulated by tissue-specific enhancers and boundary elements (Barges et al., 2000; Mihaly et al., 1997; Pirrotta et al., 1995; Simon et al., 1993). Interestingly, only five proteins have been shown to have a sequence-specific DNA-binding activity (GAGA, PSQ, Zeste, PHO and PHO-like) (Simon et al., 1993). In mammals, much less is known about the PcG proteins binding to DNA. However, although no PREs have been identified, some targets are known (Hanson et al., 1999). For example, the deregulation of *Hoxc4, Hoxc5, Hoxc6, Hoxc8* and *Hoxc9* in the *Bmi1*−/− mice indicate that these are controlled by the Bmi1-containing PcG complex (van der Lugt et al., 1996).

In addition to the *Hox* genes, it appears that the PcG and TrxG proteins regulate the expression of a variety of genes such as the segmentation genes (Kassis, 1994; Maurange and Paro, 2002; McKeon et al., 1994; Pelegri and Lehmann, 1994) and genes involved in patterning of the imaginal discs which will later give rise to the adult
structures (Ringrose et al., 2003). The mammalian PcG proteins have also been shown to regulate genes implicated in cellular proliferation and tumorigenesis such as p16\textsuperscript{INK4A} and p19\textsuperscript{ARF} (Jacobs and van Lohuizen, 2002).

5. The role of PcG complexes in cellular memory

The specialized state of cells is maintained throughout many rounds of cell division that are necessary for body growth during Drosophila development. In fact, imaginal discs that are transplanted into a different larvae form the appropriate structures and appendages for which they were initially specialized. This maintenance of cellular memory is a dynamic process that is accomplished by the concerted functions of the PcG and TrxG proteins in the regulation of their many target genes.

First, these proteins must find the target gene while the initial determining transcription factors (i.e., segmentation genes) are still present. Indeed, PC and TRX have been found to bind the Bithorax Complex (BX-C) well before the segmentation genes disappear (Orlando et al., 1998). Furthermore, recent data indicates that the segmentation gene hunchback recruits the PcG complex to repressed target genes through the dMi2 deacetylase complex (Kehle et al., 1998; Zhang and Bieker, 1998).

Second, the PcG and TrxG proteins must assess the activity of the promoter. Transgenic studies suggest that PREs act as silencers by default (Sengupta et al., 2004) and that the TrxG proteins directly oppose this repression (Klymenko and Muller, 2004). This suggests that the active state of a promoter may lead to the recruitment of TrxG proteins through an unknown mechanism.
Third, the PcG and TrxG complexes must preserve the repressed or active state of (Kuzmichev et al., 2002) transcription of their target genes, respectively. It has been shown that PcG proteins do not exclude transcription factors from silenced promoters as it was previously thought (Saurin et al., 2001). Several alternative mechanisms of repression were proposed including the sumoylation and subsequent activation of transcriptional corepressors (Kagey et al., 2003), modulation of the polymerase activity (Dellino et al., 2004; Wang et al., 2004), histone methylation (Lachner et al., 2003) and deacetylation (Czermin et al., 2002; Huang et al., 2004; Kuzmichev et al., 2002; Poux et al., 2001; Tie et al., 2001; van der Vlag and Otte, 1999). In contrast, the activation mechanism seems to involve histone methylation (Beisel et al., 2002; Strutt et al., 1997) and acetylation (Cavalli and Paro, 1999).

Finally, after each cell division, the same transcriptional state must be maintained. In both flies and mammals, the PcG proteins dissociate from DNA during mitosis (Buchenau et al., 1998; Miyagishima et al., 2003; Voncken et al., 1999) whereas it is not known how the TrxG proteins behave during mitosis yet. Histone methylation is a very stable modification and therefore could account for the cellular memory after mitosis (Lachner et al., 2003). However, it has also been suggested that the PREs themselves are important for remembering the transcriptional state of the genes they regulate (Busturia et al., 1997; Sengupta et al., 2004).

6. The role of PcG complexes in cell cycle control

Although the PcG complex is well known for its role in segmentation through the regulation of Hox gene expression, it has become increasingly clear that this group of
negative regulators of transcription are also involved in cell cycle control. Most of the evidence in support of this idea comes from the study of Bmi1.

*Bmi1* was originally identified in a screen for genes that could cooperate with Eμ-Myc in lymphomagenesis (Haupt et al., 1991; van Lohuizen et al., 1991). Retroviral insertional mutagenesis was performed with the Moloney murine leukemia virus on Eμ-Myc transgenic mice, which were screened for a decrease in the latency period of pre-B-cell lymphoma formation. A frequent site of integration (35-47%) was near the *Bmi1* (B-cell-specific Moloney murine leukemia virus insertion site 1) gene, which resulted in its overexpression. Bmi1 is a 324 amino acid protein with high degree of homology to the *Drosophila* Psc and Su(z)2, two members of the PRC1 complex (Brunk et al., 1991; van Lohuizen et al., 1991). The predicted amino acid sequence reveals several structural motifs. The N-terminal portion of the protein contains a cysteine-rich zinc finger motif called the RING finger, which was later found to be required for Bmi1’s oncogenic capacity (Cohen et al., 1996). Although it has not been possible to demonstrate that Bmi1 binds directly to DNA, it contains a helix-turn-helix-turn-helix-turn (HTHTHT) motif in the center of the protein, which is a potential DNA-binding domain. Bmi1 also has two putative nuclear localization signals, KRRR and KRMK, but only the latter seems to be required for its nuclear localization (Cohen et al., 1996; Haupt et al., 1991). Finally, this protein contains a PEST domain, which is rich in proline, glutamic acid, serine and threonine and is associated with rapid intracellular protein degradation (Rogers et al., 1986).

Consistent with the retroviral mutagenesis studies, transgenic mice that overexpress both Bmi1 and Myc accelerate the onset of both B- and T-cell lymphomas supporting that
Bmi1 is an oncogene (Haupt et al., 1993). Further evidence of Bmi1's oncogenic properties comes from reports that Bmi1 can transform Rat1a cells by itself (Cohen et al., 1996) and MEFs in cooperation with Ras (Jacobs et al., 1999b). Furthermore, Bmi1-deficient MEFs have a reduced proliferation rate and senesce prematurely (Jacobs et al., 1999a). These oncogenic effects of Bmi1 appear to be mediated by its ability to repress the tumor suppressors p16\(^{INK4A}\) and p19\(^{ARF}\), the products of two alternative reading frames of the Ink4a/Arf locus. Indeed, Bmi1\(^{-/-}\) MEFs as well as lymphocytes have increased expression of both p16\(^{INK4A}\) and p19\(^{ARF}\). Conversely, Bmi1 overexpression results in decrease of p16\(^{INK4A}\) and p19\(^{ARF}\) protein levels (Itahana et al., 2003; Jacobs et al., 1999a). Deletion of the Ink4a/Arf locus partially rescues the Bmi1 mutant phenotypes (Jacobs et al., 1999a). However, it has not been possible to determine whether the repression of the Ink4a/Arf locus is direct or not.

In the past couple of years, the role of Bmi1 in cell cycle control has been extended to include the regulation of stem cell self-renewal and proliferation of hematopoietic and neural stem cells (HSCs and NSCs) (Lessard and Sauvageau, 2003; Leung et al., 2004; Molofsky et al., 2003; Park et al., 2003). The participation of Bmi1 in the proliferation of these cells also involves the repression of the Ink4a/Arf locus suggesting that this is a general function of Bmi1 in different cell types and tissues.

It has been recently shown that Bmi1 is required for the self-renewal of peripheral and central nervous system stem cells (Molofsky et al., 2003). In fact, loss of Bmi1 leads to the postnatal depletion of NSCs in the cerebellum. As a result, Bmi1 mutant mice have a smaller cerebellum due to severe loss of both the molecular and granular layer neurons (van der Lugt et al., 1994a). A substantial rescue of the cerebellar size is observed in
The partial rescue by loss of the Ink4a/Arf locus is in agreement with the upregulation of both p16^{INK4A} and p19^{ARF} in NSCs lacking Bmi1 (Molofsky et al., 2003; Park et al., 2003). However, it appears that the contributions of p16^{INK4A} and p19^{ARF} may be different in different cell types and tissues (Bruggeman et al., 2005; Molofsky et al., 2005).

Sonic hedgehog (Shh) secreted by Purkinje cells guides cerebellum development by driving a postnatal wave of proliferation of the cerebellar granule neuronal progenitors (CGNPs) in the external granular layer (EGL), which subsequently become post-mitotic, migrate inward and differentiate into cerebellar granule neurons (Goldowitz and Hamre, 1998). Bmi1 mutant CGNPs have been shown to have an impaired proliferative response upon Shh stimulation (Leung et al., 2004). Based on these results, it has been proposed that Shh binds the Patched receptor in the CGNPs leading to inhibition of smoothened and activation of the Gli transcription factors. The Gli transcription factors induce the expression of Bmi1, which in turn leads to the down-regulation of the Ink4a/Arf locus ensuring cell proliferation can continue (Leung et al., 2004).

Bmi1 also regulates the proliferative activity of normal hematopoietic stem and progenitor cells (Lessard and Sauvageau, 2003; Park et al., 2003). Consistent with this role, the expression of PcG genes during human hematopoietic cell differentiation is highly regulated (Jacobs and van Lohuizen, 2002); Bmi1 is specifically expressed in CD34+ immature progenitor cells, but is absent in differentiated cells (Lessard et al., 1998). Furthermore, the number of HSCs in postnatal Bmi1 mutant mice is markedly reduced with no detectable self-renewal of adult HSCs. As a consequence, mice deficient for Bmi1 suffer from various defects in the hematopoietic system such as hypoplasia in
spleen and thymus, reduction in overall T cell numbers, defects in B cell development and an impaired proliferative response of lymphoid precursors to IL-7 (van der Lugt et al., 1994b). These defects are also partially rescued by Ink4a/Arf loss as indicated by restored lymphocyte counts (Jacobs et al., 1999b).

In light of the transforming properties of Bmi1 from in vitro studies as well as its capacity to cooperate with Myc in lymphomagenesis in vivo, it is not surprising that Bmi1 has been found to be overexpressed in several human cancers. These include mantle cell lymphoma, colorectal carcinoma, liver carcinomas, non-small cell lung cancer, pediatric brain tumors and medulloblastomas (Bea et al., 2001; Hemmati et al., 2003; Kim et al., 2004a; Kim et al., 2004b; Leung et al., 2004; Neo et al., 2004; Vonlanthen et al., 2001). Significantly, the finding that Bmi1 is involved in stem cell self-renewal and proliferation raises the question of whether any of these tumors are of stem cell origin.

In summary, Bmi1 is involved in the control of proliferation of several cell types, at least in part, through the control of p16\(^{\text{INK4A}}\) and p19\(^{\text{ARF}}\) expression. Importantly, other PcG proteins have also been implicated in cell cycle control (Gil et al., 2005). In fact, M33, Mel18 and Cbx7 have been shown to regulate the Ink4a/Arf locus expression (Core et al., 2004; Gil et al., 2004; Jacobs et al., 1999a). However, the mechanism by which Bmi1 (and other PcG proteins) is recruited to the Ink4a/Arf locus promoters remain to be elucidated. Interestingly, the p19\(^{\text{ARF}}\) promoter contains E2F binding sites (Bates et al., 1998) and E2F6 has been found in association with members of the mammalian PcG complex including Bmi1 (Trimarchi et al., 2001). These findings raise the possibility that E2F6 recruits Bmi1 to the p19\(^{\text{ARF}}\) promoter for its PcG-mediated repression.
E2F6 is a unique member of the E2F family of transcription factors that cannot be regulated by the pocket proteins. The aim of this study is to understand the biological significance of E2F6 with respect to its significant homology to the E2F transcription factors and its potential role in the PcG-mediated transcriptional repression. The study of mutant mice and MEFs has been critical for defining the role of the individual E2Fs in cell cycle progression and tumorigenesis. Furthermore, mice and MEFs deficient for mammalian PcG genes have also been essential in confirming the biological significance of PcG proteins in development as well as in uncovering novel roles in the control of cell cycle and tumorigenesis. Thus, to gain insight into the role that E2F6 plays in vivo, E2f6-deficient mice were generated and characterized in a wild-type (Chapter 2) or Bmil mutant (Chapter 3) background.
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Chapter Two

*E2f6* deletion leads to posterior transformations of the axial skeleton

María Courel & Jacqueline A. Lees

The author contributed all text and figures. All mouse work and cell culture experiments were done in the laboratory of Jacqueline A. Lees.
ABSTRACT

E2F6 is a member of the E2F family of transcription factors that are involved in the control of the cell cycle. Unlike other E2Fs, E2F6 does not contain a transactivation domain and is not regulated by pocket protein binding. This E2F has been shown to be a repressor of transcription that seems to act through the recruitment of the Polycomb Group complex. This complex is responsible for the maintenance of Hox gene expression patterns during development and thus ensures the correct anterior-posterior segmentation of the embryo. One of the most studied members of the PcG complex is Bmi1, which also has a role in cell cycle control through its negative regulation of p16\textsuperscript{INKA} and p19\textsuperscript{ARF} expression. In order to determine the biological function of E2F6, we have generated and characterized E2f6\textsuperscript{-/-} mice and mouse embryonic fibroblasts (MEFs). The mutant mice are viable and survive into adulthood with no difference in lifespan compared to their littermate controls. Furthermore, the E2f6 null MEFs are indistinguishable from wild-type MEFs in asynchronous proliferation, cell cycle re-entry from quiescence, senescence and E2F target genes expression levels. Instead, we found that the loss of E2F6 results in posterior axial skeleton transformations that are reminiscent of the Bmi1-deficient mice defects. These findings suggest that E2F6 does not play a major role in cell cycle control or that its function can be compensated by the action of other factors. However, our data indicates that E2F6 participates in the segmentation of the embryo during development providing proof that it is a \textit{bona fide} Polycomb Group protein.
INTRODUCTION

The E2F transcription factors are a family of key regulators of cell proliferation and differentiation (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). They function by controlling the expression of genes that are necessary for S phase entry and progression. Deregulation of these E2F target genes leads to inappropriate proliferation, transformation and apoptosis. Recently, members of the E2F family have also been implicated in the control of genes that are important for DNA repair and recombination, mitosis and development (Cam and Dynlacht, 2003; DeGregori, 2002; Muller et al., 2001; Stevaux and Dyson, 2002). The E2F family consists of 10 genes that can be subdivided into two families, the E2Fs (1-8) and the DPs (1, 2). Functional E2F complexes are formed when one E2F subunit and one DP subunit heterodimerize. Although both subunits are required for E2F activity, the functional specificity is conferred by the E2F moiety. The E2F proteins can be further subdivided into three groups based on structural and functional similarities.

The first group is comprised of E2F1, E2F2 and E2F3a, which are strong transcriptional activators that are able to drive quiescent cells into the cell cycle through activation of E2F target genes (DeGregori et al., 1997; Kowalik et al., 1995; Lukas et al., 1996; Verona et al., 1997). These proteins are not detected during quiescence but rather their levels start to rise during G1 and reach peak levels just before the G1/S transition. This E2F activity is exclusively regulated by the retinoblastoma protein (pRB) and functions mainly by activation of genes that are responsible for cell cycle progression.

E2F4 and E2F5 are poor transcriptional activators and are unable to induce quiescent cells to enter S phase (Lukas et al., 1996; Muller et al., 1997; Verona et al.,
In fact, these proteins are thought to actively repress E2F target genes through their ability to bind all pocket proteins and recruit histone deacetylases to the promoters (Brehm and Kouzarides, 1999; Dyson, 1998). E2F3b does not share the same level of structural similarity observed between E2F4 and E2F5 (He et al., 2000; Leone et al., 2000) and appear to bind exclusively to pRB (Leone et al., 2000). Despite this, E2F3b has been grouped with E2F4 and E2F5 due to its ability to repress target genes and because its expression pattern through the cell cycle is reminiscent of the repressive E2Fs (Aslanian et al., 2004; He et al., 2000; Leone et al., 2000).

The last group consists of the most novel members of the E2F family, E2F6, E2F7 and E2F8 (de Bruin et al., 2003; Logan et al., 2004; Maiti et al., 2005). These proteins lack the characteristic transactivation and pocket protein domains found in the rest of the E2F family members and are therefore unable to activate transcription and are not regulated by pocket protein binding. Instead, they have been shown to repress the E2F-mediated transcription of target genes. However, there are differences between the proteins of this group. E2F7 and E2F8 contain two DNA binding domains, lack a DP dimerization domain and are able to bind DNA in the absence of DP. In contrast, E2F6 contains both DNA binding and dimerization domains and requires DP for effective interaction with DNA (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998).

The mouse E2f6 gene produces two mRNAs through alternative splicing of exon 2 that give rise to two proteins (Kherrouche et al., 2001). E2F6a lacks this exon whereas E2F6b is truncated at the N-terminal end of the protein and translation begins with exon 2 (Dahme et al., 2002). E2F6 is widely expressed during embryogenesis and is present in
most, if not all, adult murine tissues (Dahme et al., 2002; Kherrouche et al., 2001). In MEFs, E2F6 expression rapidly increases during the G0/G1 transition reaching its highest level in mid-G1 and remains relatively constant thereafter (Dahme et al., 2002; Kherrouche et al., 2001). This is consistent with the finding that in primary human T lymphocytes, E2F6 expression starts well before the S phase peak after PHA stimulation (J.M.Trimarchi and J.A.Lees, unpublished results). Several experiments indicate that E2F6 may have a role in cell cycle control. Overexpression of E2F6 in asynchronous Saos2 or NIH3T3 cells leads to a modest increase in the number of cells in G1 and microinjection of E2F6 into quiescent NIH3T3 cells leads to a 50-60% reduction in the number of cells that are able to enter S phase upon serum stimulation (Gaubatz et al., 1998). In contrast, transient expression of E2F6 in asynchronous U2OS results in a small increase in the number of cells in S phase (Cartwright et al., 1998). Moreover, the levels of the cell cycle inhibitor p19ARF are decreased when E2F6 is overexpressed in wild-type MEFs arguing in favor of a positive role for E2F6 in the regulation of the cell cycle (J.M.Trimarchi and J.A.Lees, unpublished results). Finally, E2F6 has been recently found at E2F target promoters that are activated during G1/S but does not associate with promoters of E2F target genes that are activated during G2/M (Giangrande et al., 2004). This finding implies a role for E2F6 in progression through the cell cycle by ensuring that the transcription of G1/S target genes is extinguished before the activation of G2/M target genes. Therefore, the role of E2F6 in cell cycle regulation remains to be clarified since most studies were done by overexpressing E2F6 and have yielded conflicting results.
E2F6 has been shown to function as a transcriptional repressor (Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998). At least when overexpressed, it can inhibit the transcriptional activity of the other E2F complexes. It appears that this repression does not occur via sequestration of DP molecules given that the repression is not relieved by an excess of DP (Gaubatz et al., 1998; Trimarchi et al., 1998).

In an attempt to elucidate the mechanism of the E2F6-mediated repression, we and others have identified proteins that interact with E2F6 (Attwooll et al., 2005; Ogawa et al., 2002; Trimarchi et al., 2001). Surprisingly, E2F6 has been found in several complexes containing proteins of the mammalian Polycomb Group (PcG). The PcG proteins, first identified in *Drosophila*, form large multimeric complexes that are responsible for the repression of the Hox genes, which determine the patterning of the developing embryo (Kennison, 1995; Simon, 1995). In mammals, there seem to be many more PcG proteins and at least two distinct PcG complexes (Otte and Kwaks, 2003). The Eed-containing PcG (PRC2) initiates polycomb-mediated repression, whereas the Bmi-1-containing PcG (PRC1) maintains the repression at later stages of development. Loss of proteins of the PcG-PRC2 complex in mice generally result in early embryonic lethality whereas PcG-PRC1 mutant mice display posterior transformations of the axial skeleton due to anterior shifts in Hox genes expression boundaries. Furthermore, several PcG proteins have been implicated in the control of the cell cycle (Jacobs and van Lohuizen, 2002). For example, Bmi1 regulates cell proliferation through its ability to repress the tumor suppressors p16<sup>INKA</sup>A and p19<sup>ARF</sup> (Jacobs et al., 1999).

A yeast two-hybrid screen has identified the PcG-PRC1 protein, RYBP, as a direct E2F6 interactor (Trimarchi et al., 2001). Further biochemical analysis revealed
that E2F6 is found in association with several Polycomb Group proteins including Ring1, Mel-18, Mph1 and the oncoprotein Bmi1. In another study, an E2F6-containing complex was purified from human HeLa cells (Ogawa et al., 2002). Mass spectrometry analysis showed that several proteins are present in this complex including DPI, Mga and Max, histone methyltransferases, HP1γ and several Polycomb Group proteins (RING1, RING2, MBLR, h-l(3)mbt-like protein and YAF2). The presence of E2F6, Max and HP1γ on E2F- and myc-target promoters during G0 but not at later stages of the cell cycle suggests that this complex is involved in stable repression required for quiescence (Ogawa et al., 2002). A third group has performed a yeast two-hybrid screen with the full length human E2F6 and has identified the PcG-PRC2 protein, EPC1 (Enhancer of Polycomb 1), as a direct interactor (Attwooll et al., 2004). This interaction mediates the association of E2F6 with Sin3B and EZH2 specifically in proliferating cells.

Significantly, only a few PcG proteins have been shown to bind DNA directly including Mel-18 and YY1 (Brown et al., 1998; Kanno et al., 1995). These cannot account for all the Polycomb DNA binding activity suggesting that other DNA binding factors may be responsible for targeting the PcG complexes to specific promoters. Since E2F6 associates with PcG proteins and is able to directly bind DNA, it is reasonable to speculate that E2F6 functions to recruit PcG complexes to target promoters and therefore contributes to their biological function.

To test this hypothesis and to address the role of E2F6 in cell cycle control, we have generated E2f6-deficient mice. If E2F6 were central to PcG function, we would expect E2f6−/− mice to display phenotypes similar to those described for other PcG mutants. Indeed, these mice display posterior axial skeleton transformations indicating
that E2F6 participates in the role of the PcG complex in segmentation of the developing embryo. However, the analysis of the cell cycle properties of E2f6−/− MEFs revealed no defects suggesting that E2F6 is dispensable for cell cycle control.

RESULTS

Generation of E2f6 mutant mice

In order to study the role of E2F6 in vivo, we generated E2f6-deficient mice. To do so, we have mapped and cloned a region of the E2f6 genomic locus that encompasses the translation initiation site through the stop codon (Fig. 1a). We constructed a vector that targets most of the E2f6 gene including the exons that encode the DNA binding, leucine zipper and marked box domains. This deletion should effectively abrogate E2F6's ability to bind DNA, dimerize with DP and interact with RYBP and should therefore constitute a null allele. Figure 1a shows the targeting vector construct, which contains two regions of homology to the E2f6 genomic sequences upstream of the DNA binding domain and downstream of the marked box domain. These homologous arms flank a neomycin resistance cassette for positive selection of the recombinant embryonic stem (ES) cells. The vector also includes a Herpes simplex virus tk gene for negative selection of random integration events. E2f6−/− ES cells were generated and used to produce two independent lines of E2f6−/− mice and mouse embryonic fibroblasts (MEFs). The ES cells were genotyped by Southern blot and the mice and MEFs were genotyped by PCR (Fig. 1b and c). As expected, no E2F6 was detected in E2f6−/− MEFs by Western blots (Fig. 1d).
Figure 1. Generation of E2f6−/− mice. 

a. Wild-type E2f6 genomic locus (top), targeting vector (middle) and targeted allele (bottom).

b. Southern blot of genomic DNA from ES cell lines digested with EcoRI and hybridized with the probe shown in a.

c. PCR genotyping of genomic DNA from ear punches.

d. Western blot analysis of MEFs lysates to confirm the absence of E2F6 protein. The asterisk denotes an unspecific band.
E2F6 is dispensable for mouse viability and survival

E2f6-deficient mice are viable and display no obvious morphological defects. These animals survive into adulthood with no difference in growth or fertility compared to their wild-type littermates (Table 1). E2f6+/+, E2f6+/- and E2f6-/- mice were compared for lifespan and cause of death. We observed no difference in the percentage of animals that were alive after 530 days (70%, 68% and 75%, respectively) (Figure 2a).

<table>
<thead>
<tr>
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<th>E2f6 +/+</th>
<th>E2f6 +/-</th>
<th>E2f6 -/-</th>
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<tr>
<td>Expected</td>
<td>58.25</td>
<td>116.5</td>
<td>58.25</td>
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<tr>
<td>Observed</td>
<td>60</td>
<td>103</td>
<td>70</td>
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**Table 1. E2f6-deficient mice are viable.** Heterozygous crosses were performed generating a total of 233 animals.

Histological analysis of the dead animals was performed to determine the cause of death and revealed a few interesting observations although their significance is still not clear (See Table 2). E2f6+/+ and E2f6-/- mice seem to have a modest increase in the incidence of certain tumor types insinuating the possibility that E2F6 may have a role in the formation of certain tumors. First, 35% of E2f6+/- mice and 20% of E2f6-/- mice died of blood tumors including lymphomas, hemangiosarcomas and histiocytic sarcomas. Second, 20% of E2f6+/- mice and 28% of E2f6-/- mice died of liver tumors. Third, 3 out of 5 E2f6-/- mice analyzed had developed odontomas, which normally arise as a consequence
Figure 2. Loss of E2F6 does not affect the lifespan of adult mice. a. Survival curves of E2f6+/+ (blue, n=35), E2f6+/- (green, n=50) and E2f6-/- (red, n=53) mice. b. Survival curves of E2f6+/+ (blue, n=14), E2f6+/- (green, n=13) and E2f6-/- (red, n=18) mice treated with 4 Grays of γ-irradiation at 6 days of age.
of defective osteoclast function during tooth eruption (Amling et al., 2000). Furthermore, 20% of $E2f6^{+/+}$ mice and 28% of $E2f6^{-/-}$ mice displayed testicular atrophy. Finally, two $E2f6^{+/+}$ mice and two $E2f6^{-/-}$ mice displayed glomerulonephritis whereas two $E2f6^{-/-}$ mice were found to have only one kidney. It is noteworthy that two other mice that were homozygous mutant for $E2f6$ generated from a cross to $Bmi1$-deficient mice also were found to have a single kidney.

<table>
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<th>$E2f6^{+/+}$ (n=9)</th>
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<th>$E2f6^{-/-}$ (n=25)</th>
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<td>3</td>
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Table 2. Histological analysis of $E2f6^{+/+}$, $E2f6^{-/-}$ and $E2f6^{-/-}$ mice.

Mutation of genes that are required for resistance to oncogenic radiation leads to a reduction in the latency of tumor formation following $\gamma$-irradiation. To test the possibility that absence of E2F6 may stimulate tumor formation through sensitization to DNA damage, we performed a $\gamma$-irradiation experiment. For this purpose, 14 $E2f6^{+/+}$ mice, 13 $E2f6^{-/+}$ mice and 18 $E2f6^{-/-}$ mice were treated with 4 Grays of $\gamma$-irradiation at 6 days of age and monitored until moribund, then sacrificed and autopsied. We observed no significant difference in the lifespan or tumor progression between all the genotypes.
(Figure 2b; data not shown) implying that if E2F6 has a role in tumorigenesis it does not involve the DNA damage response. These results are not consistent with the increase in the frequencies of certain tumors in \(E2f6^{+/}\) and \(E2f6^{-/-}\) mice (Table 2). One possibility is that this increase is not significant due to the small number of wild-type animals analyzed. Another possibility may reflect the fact that the irradiated mice did not receive the correct dose of \(\gamma\)-irradiation due to problems in the calibration of the \(\gamma\)-irradiator. To definitively test the possibility that E2F6 behaves as a tumor suppressor we would have to perform loss of heterozygosity studies on tumors isolated from \(E2f6^{+/}\) mice.

**E2f6 null mice display posterior transformations of the axial skeleton**

Loss of Bmi1 in mice results in posterior transformations along the entire axial skeleton (van der Lugt et al., 1994). Since E2F6 has been found to associate with Bmi1 (Trimarchi et al., 2001), we wished to determine if \(E2f6\) mutant mice also displayed transformations of the axial skeleton. We analyzed \(E2f6^{+/}, E2f6^{+/+}, \) and \(E2f6^{-/-}\) mice at post-natal day 3 (P3) and embryonic day 18.5 (E18.5) by staining of the skeletons with Alcian blue and Alzarin red, which stain the cartilage in blue and the bone in red (Figure 3). \(E2f6^{+/+}\) and \(E2f6^{-/-}\) mice display two types of posterior transformations that had been previously described for the \(Bmi1\) mutants. First, the thoracic vertebra T13 is transformed into a lumbar vertebra L1 as evidenced by degeneration or the complete lack of ribs in 9% of \(E2f6^{+/+}\) and 67% of \(E2f6^{-/-}\) animals. Second, the lumbar vertebra L6 is transformed into the sacral vertebra S1 as evidenced by its association with the ilial bones in 35% of \(E2f6^{+/+}\) and 80% of \(E2f6^{-/-}\) mice (Table 3). These results suggest that \(E2f6\)
mutation results in dosage-dependent posterior transformations of the axial skeleton that are reminiscent of the Bmil-deficient mice transformations.

Figure 3. Posterior axial skeletal transformations of E2f6+/- mice. Ventral view of the thoraco-lumbo-sacral region of E2f6+/+ (left) and E2f6-/- (right) mice. The E2f6-/- animals have only 12 pairs of ribs compared to 13 in the wild-type control due to degeneration of the thirteenth ribs. Also, the lumbar vertebra L6 has formed sacro-iliac joints, which are typical of sacral vertebra S1.
Penetrance of skeletal abnormalities (%)

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Table 3. Skeletal transformation of E2f6++ and E2f6++ mice.

**E2F6 is not required for cell proliferation and senescence**

E2F6, a member of the E2F transcription family of cell cycle regulators, has been implicated in the regulation of the cell cycle by several findings. First, it is expressed in a cell cycle-dependent manner (Dahme et al., 2002; Kherrouche et al., 2001). Second, it is found at the promoters of cell cycle-regulated genes (Giangrande et al., 2004; Ogawa et al., 2002). Third, E2F6 overexpression leads to both a reduction or increase in the percentage of cells in S phase depending on the cell type and conditions (Cartwright et al., 1998; Gaubatz et al., 1998). Finally, E2F6 associates with Bmi1, which has also been implicated in cell cycle control through its negative regulation of the Ink4/Arf locus. Bmi1 mutant MEFs have an impaired proliferation and premature senescence, which appear to be due to the up-regulation of both p16INK4A and p19ARF.
Based on these findings, it is reasonable to believe that E2F6 may function in the control of cellular proliferation. In order to address this possibility, we generated $E2f6^{-/-}$ MEFs and performed several cell cycle assays. In asynchronous proliferation assays as well as on cell cycle re-entry experiments, $E2f6^{-/-}$ MEFs performed as well as their wild-type counterparts (Figure 4). No deregulation of E2F target genes (cyclin E, cyclin A, E2F1, PCNA, p107) or p16$^{INK4A}$ and p19$^{ARF}$ was observed (data not shown). Furthermore, $E2f6^{-/-}$ MEFs underwent normal senescence when successively passaged in a 3T3 protocol (Figure 5). These results indicate that E2F6 is not required for normal proliferation and senescence.
Figure 4. E2F6 is dispensable for normal proliferation of mouse embryonic fibroblasts. a. Proliferation of asynchronously growing E2f6+/+ (blue), E2f6+/− (green) and E2f6−/− (red) MEFs in medium with 10% serum. b. Thymidine incorporation of E2f6+/+ (blue), E2f6+/− (green) and E2f6−/− (red) MEFs after serum arrest and release. Curves are typical results obtained with different cell lines.
Figure 5. *E2f6*^/+^ MEFs undergo normal senescence. *E2f6*^+++^ (blue), *E2f6*^+/−^ (green) and *E2f6*^−−^ (red) MEFs were passaged according to a standard 3T3 protocol. Fold replication is the number of cells determined in each passage divided by the number of cells plated (3x10^5). Curves are typical results obtained with different cell lines.
DISCUSSION

The aim of this study was to determine the biological significance of E2F6. Since E2F6 has been shown to associate with several PcG proteins, we anticipated that loss of E2F6 would result in typical defects observed in the Polycomb mutants. Indeed, we have found that E2f6−/− mice display posterior axial skeletal transformations that are reminiscent of those found in Polycomb mutants. Moreover, E2f6−/− MEFs were not defective in proliferation or senescence as it might have been expected in light of the roles of some PcG and E2F proteins in cell cycle control.

Viability and lifespan of E2f6−/− mice

E2f6−/− mice are viable and display no difference in lifespan compared to their control littermates. Furthermore, we did not observe a significant difference in the response to γ-irradiation. We did find a modest increase in the incidence of certain tumors of liver and blood suggesting that E2F6 may function as a tumor suppressor. However, it is important to note that these tumors were observed in mutant mice that were over one year old and the number of wild-type animals analyzed at this age was much smaller. In addition, most of the overexpression studies as well as E2F6's ability to associate with the Bmi1 oncogene propose an oncogenic rather than a tumor suppressive role for E2F6. In agreement with this, E2F6 has recently been shown to repress the transcription of the tumor suppressor BRCA1 (Oberley et al., 2003). However, in order to test the possibility that E2F6 is a tumor suppressor, a loss of heterozygosity study should be performed on tumor samples from E2f6−/− mice.
Significantly, 3 out 5 mice analyzed had developed odontoma. These are benign tumors that arise in osteopetrotic mice as a consequence of defects in osteoclast function or differentiation (Amling et al., 2000; Ida-Yonemochi et al., 2002; Ida-Yonemochi and Saku, 2002; Oberley et al., 2003). A reduced osteoclast function during tooth development leads to morphological abnormalities that result in the formation of odontomas. The significance of this finding is not clear yet since we have not studied the effects of loss of E2F6 in osteoclast function and differentiation. It would therefore be of interest to isolate osteoclast precursor from peripheral blood mononuclear cells of E2f6 mice and assay their differentiation and function.

E2f6+/ and E2f6-/ mice display testicular atrophy with low penetrance. However, it is important to note that this is a common age-related defect and all the mice were at least 500 days old. A histological analysis of males at different ages would be necessary to determine if this atrophy develops earlier in the mutants. Significantly, while this work was in progress, another laboratory reported the generation and characterization of E2f6-/ mice and observed a reduction of spermatogenesis that did not affect the fertility of these mice (Storre et al., 2002).

A few E2f6 mice were found to have a single kidney while the mutants that had both kidneys did not exhibit any abnormalities. It is not clear if this observation reflects a defect in kidney development or degeneration. In order to address this, a timed analysis should be performed. However, the low penetrance of this phenotype would require the generation of an enormous number of E2f6 animals. Significantly, the Hox11 paralog group (HoxA11, HoxC11 and HoxD11) are involved in the induction of ureter budding from the nephric duct (Wellik et al., 2002).
Proliferation and senescence of $E2f6^{-/-}$ MEFs

We have shown here that E2F6 is dispensable for proliferation and senescence in MEFs given that $E2f6^{-/-}$ MEFs are undistinguishable from their wild-type littermates. This data is supported by similar findings by Storre et al., 2002. Previous data suggested that E2F6 may repress the transcription of p19ARF (J.M.Trimarchi and J.A. Lees, unpublished results). However, we do not observe a deregulation of p19ARF expression in $E2f6^{-/-}$ MEFs. Together these findings suggest that E2F6 does not participate in the Bmi1-mediated repression of p19ARF.

Alternatively, other factors could compensate for the absence of E2F6. Interestingly, although initial studies indicated that E2F6 was the only E2F able to interact with RYBP, a subsequent report has suggested that E2F2 and E2F3 may also associate with this protein (Schlisio et al., 2002; Trimarchi et al., 2001). This would provide alternative proteins for targeting the PcG proteins to DNA in the absence of E2F6. In agreement with this hypothesis, E2F3b has been found at the p19ARF promoter and has been implicated in its repression (Aslanian et al., 2004). Moreover, recent findings suggest that E2F4 is able to compensate for the loss of E2F6 in MEFs (Giangrande et al., 2004). In this study, although E2F6 was found at the promoters of E2F target genes that are activated during G1/S, no deregulation was observed in $E2f6^{-/-}$ MEFs. However, when E2F4 levels were reduced using siRNAs, these genes were de-repressed. Further studies are needed to determine if similar compensation for E2F6 loss occurs at the p19ARF and PcG target genes.
Posterior transformations in $E2f6^{-/-}$ mice

Usually, the discovery of novel PcG proteins begins with the identification of a protein that associates with other PcG proteins followed by its characterization as a transcriptional repressor and its localization to Polycomb bodies on DNA. Nevertheless, the ultimate proof that a protein is a member of the PcG complex has come, without exception, from the observation of axial skeleton transformation in mutant mice. It has been previously shown that E2F6 interacts with several PcG proteins (Attwooll et al., 2004; Ogawa et al., 2002; Trimarchi et al., 2001) and that E2F6 may repress E2F-mediated transcription (Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998). Although E2F6 has been shown to be a nuclear protein, its expression seems to be diffuse instead of localized to Polycomb bodies (J.M.Trimarchi and J.A.Lees, unpublished results). Here we show that $E2f6^{-/-}$ mice display a subset of the posterior transformations of the axial skeletons observed in $Bmi1^{-/-}$ mice (van der Lugt et al., 1994) demonstrating that E2F6 is a bona fide PcG protein. Although there is a difference in the penetrance of these transformations, an independent analysis of $E2f6^{-/-}$ mice revealed identical posterior transformations of the axial skeleton (Storre et al., 2002).

However, the posterior transformations in the other PcG mutants are observed throughout the entire length of the axial skeleton whereas the ones observed in $E2f6^{-/-}$ mice are restricted to the more posterior region of the axial skeleton (Akasaka et al., 1996; Core et al., 1997; Takihara et al., 1997; van der Lugt et al., 1994). Although we have not examined the expression patterns of Hox genes in the E2f6-deficient mice, this finding implies that E2F6 contributes to the PcG regulation of a subset of target genes.
EXPERIMENTAL PROCEDURES

Generation and genotyping of \textit{E2f6}\textsuperscript{--} mice

The BAC clone b39J22 (Research Genetics) known to contain the \textit{E2f6} genomic locus (Peterfy et al., 1999) was mapped and cloned to obtain the sequences necessary for designing the targeting strategy (Figure 1a). The targeting vector described above was introduced into 129Sv J1 ES cells by electroporation and the cells were selected with G418 and Gancyclovir. 96 resistant clones were picked for genotyping. \textit{E2f6} +/- cells were detected by Southern blot using external 5’ and 3’ probes as well as a neomycin probe (Fig. 1a). Once the heterozygous clones were identified and verified to contain a diploid genome by karyotyping, they were injected into C57/BLACK6 3.5 d.p.c. blastocysts. The injected blastocysts were subsequently implanted into pseudo-pregnant females and the chimeric progeny were identified by coat color. Mice with a high contribution of agouti cells were mated to pure C57/BLACK6 mice. The agouti progeny of these mice were genotyped by PCR of DNA obtained from ear or tail pieces using the common primer 5’-ATCTCTGTCTGGTCTGATCC-3’, the wild-type \textit{E2f6}-specific primer 5-GATGCCATCCAAGACATTGG-3’, and the mutant targeting vector specific primer 5-GCCGCATAACTTCGTATAGC-3’ (Fig. 1c). The \textit{E2f6}++ mice were then interbred to produce \textit{E2f6}-- mice.

**Histological and skeletal analysis**

Dead mice from our aging colonies were dissected and processed for histological analysis. Soft tissues were fixed in 10\% formalin and hard tissues were fixed in Bouin’s fixative. Paraffin sections were prepared and stained with hematoxylin and eosin. Skeletal analysis was performed on 3-day old mice. After removing the skin and viscera,
the skeletons were fixed in acetone and stained with cartilage-specific Alcian Blue and bone-specific Alzarin Red. Soft tissue was cleared with KOH.

**Mouse embryonic fibroblasts**

MEFs were prepared from 13.5 d.p.c embryos as previously described (Humbert et al., 2000) and genotyped by PCR of DNA obtained from yolk sacs. Proliferation curves were obtained by plating 2x10^4 MEFs in triplicate in 24-well plates. At the indicated time points, MEFs were trypsinized and counted. For cell cycle re-entry assays, 2x10^5 MEFs were plated in triplicate in 6-well plates. After 2 days of growth in media containing 10% serum, they were incubated in media containing 0.1% serum for 3-4 days. Re-entry into the cell cycle was induced by incubation in media containing 10% serum and at the indicated time points, 5 μCi of ^3^H-thymidine was added to the cells for 1 hour. Cells were then scraped from the plates and cell pellets were analyzed for ^3^H-thymidine incorporation using a scintillation counter. A 3T3 protocol was followed to monitor senescence. 3x10^5 MEFs were plated in duplicates in 6-cm plates and re-fed 2 days later. On the third day, they were trypsinized, counted and replated. The fold replication was determined by dividing the number of cells obtained at day 3 by 3x10^5. Western blots were performed as described previously (Moberg et al., 1996) with 50-100 μg of whole cell lysates.
ACKNOWLEDGEMENTS

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Chapter Three

E2F6 and Bmi1 synergize in axial patterning in a gene dosage-dependent manner

María Courel & Jacqueline A. Lees

The author contributed all text and figures. All mouse work and cell culture experiments were done in the laboratory of Jacqueline A. Lees.
ABSTRACT

E2F6 is a member of the E2F family of transcription factors that does not contain a transactivation domain and cannot be regulated by pocket protein binding. Instead, this protein appears to repress transcription through the recruitment of the Polycomb Group (PcG) complex. This complex participates in the anterior-posterior patterning of the developing embryo by stably repressing Hox gene expression. Consequently, genetic ablation of PcG proteins leads to posterior transformations of the axial skeletons.

Furthermore, other developmental defects are often observed in these mutants such as hematopoietic, cerebellar and smooth muscle defects. Finally, the PcG complex has been implicated in cell cycle control since at least one of its members, the oncoprotein Bmi1, appears to repress the transcription of p16\textsuperscript{INK4A} and p19\textsuperscript{ARF}. Consistent with E2F6’s association with PcG proteins, E2F6 loss results in posterior axial skeleton transformations that are reminiscent of the Bmi1-deficient mice defects. However, none of the other commonly observed Polycomb mutant phenotypes have been observed in the \textit{E2f6}\textsuperscript{-} mice suggesting that E2F6 only participates in a subset of PcG functions. To investigate the possibility that E2F6 may be involved in other aspects of PcG activity, we have generated \textit{E2f6}\textsuperscript{-};\textit{Bmi1} compound mutant mice and MEFs. Our results confirm the gene dosage effect of E2F6 on axial skeletal patterning and suggest that E2F6 may possibly synergize with Bmi1 in mice viability and survival as well as cell proliferation and senescence.
INTRODUCTION

E2F6 is a recently identified member of the E2F family of transcription factors that has significant homology to other family members within the DNA binding, dimerization and marked box domains (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998). However, it is truncated at the C-terminus and therefore lacks transactivation and pocket protein binding domains. Consistent with its structure, E2F6 can bind DNA as a heterodimer with DP, is not able to activate transcription of target genes and is not regulated by pocket protein binding.

E2F6 is widely expressed during embryogenesis and is present in all adult mouse and human tissues examined (Cartwright et al., 1998; Dahme et al., 2002; Kherrouche et al., 2001; Trimarchi et al., 1998). The expression of this protein in MEFs rapidly increases during the G0/G1 transition reaching its highest level in mid-G1 and remaining relatively constant thereafter (Dahme et al., 2002; Kherrouche et al., 2001). In agreement with this, in primary human T lymphocytes, E2F6 expression starts well before the S phase peak after PHA stimulation (J.M.Trimarchi and J.A.Lees, unpublished results).

The role of E2F6 in the cell cycle remains elusive although several pieces of data suggest that it may stimulate cell cycle progression. First, transient transfection of E2F6 in asynchronous U2OS cells leads to a modest increase in the number of cells in S phase (Cartwright et al., 1998). Second, overexpression of E2F6 in MEFs results in the downregulation of the cell cycle inhibitor p19ARF (J.M.Trimarchi and J.A.Lees, unpublished results). Finally, E2F6 has been proposed to allow cell cycle progression through S phase by repressing the transcription of E2F target genes that are activated during the G1/S transition (Giangrande et al., 2004). Thus, E2F6 would ensure that these
G1/S-specific genes are no longer expressed before the next phase of the cell cycle begins. In contrast, a reduction in the number of S phase cells was reported when E2F6 was microinjected into quiescent NIH3T3 cells before re-stimulation to enter the cell cycle by serum addition (Gaubatz et al., 1998). These differences may simply reflect distinct functions of E2F6 during different phases of the cell cycle, may be the result of overexpression of E2F6 at artificially high levels, or may be due to cell type specificity. However, the analysis of E2f6−/− MEFs has revealed no cell cycle defects arguing against a role for E2F6 in the cell cycle (Chapter 2; Storre et al., 2002). An alternative explanation is that E2F6’s function in the cell cycle can be compensated by other factors. In fact, Giangrande et al (2004) have reported that E2F4 can compensate for E2F6 loss in the regulation of the G1/S genes repression during S phase. Another candidate is E2F3b, which has recently been implicated in the repression of p19ARF (Aslanian et al., 2004).

E2F6 has been shown to act as a dominant-negative transcriptional repressor by binding to E2F-target promoters and blocking the access of other activating E2Fs (Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998). Overexpression of E2F6 can counteract the E2F1-mediated transcriptional activation of a reporter gene even in the presence of non-limiting concentrations of DP. More recently, however, it has been suggested that the E2F6-mediated repression may be the result of active recruitment of repressive complexes to E2F target genes (Attwooll et al., 2004; Ogawa et al., 2002; Trimarchi et al., 2001). Three independent studies have reported that E2F6 is present in complexes that contain members of the mammalian Polycomb Group (PcG) as well as chromatin remodeling enzymes.
The PcG proteins were first identified in *Drosophila* as a group of proteins that are necessary for the maintenance of stable repression of *Hox* genes during development (Kennison, 1995; Simon, 1995). Subsequently, many mammalian orthologs have been discovered revealing a similar function despite the higher level of complexity (Gould, 1997). The correct spatial and temporal expression of *Hox* genes during development is essential for the normal anterior-posterior segmentation of the embryo. To achieve the repression of these genes, the PcG proteins form large multimeric complexes that are thought to act by changing the local chromatin structure (Pirrotta, 1997).

In mammals, the PcG proteins form part of at least two different complexes (Otte and Kwaks, 2003). The PRC2 complex contains Eed, Enx1/Ezh2 and Enx2/Ezh1 and is responsible for initiating the Polycomb-mediated repression (Hobert et al., 1996; Laible et al., 1997; Schumacher et al., 1998; Sewalt et al., 1998; van Lohuizen et al., 1998). Deletions of these genes in mice result in early embryonic lethality underscoring the importance of their products during development (O'Carroll et al., 2001; Pasini et al., 2004). The PRC1 complex contains Bmi1, Mel-18, M33, Ring1a and RYBP among others (Gunster et al., 1997; Hemenway et al., 1998; Satijn et al., 1997; Schoorlemmer et al., 1997; Tagawa et al., 1990; van Lohuizen et al., 1991). This complex maintains the repression established by PRC2 at later stages of development. In contrast to PRC2 mutants, loss of most PRC1 proteins leads to anterior shifts in the *Hox* genes expression boundaries and the corresponding posterior transformations of the axial skeleton (Akasaka et al., 1996; Akasaka et al., 1997; del Mar Lorente et al., 2000; van der Lugt et al., 1994). These relatively mild defects observed in PRC1 mutants may reflect the functional redundancy between PcG proteins. In fact, in flies which do not have as many
PcG proteins, loss-of-function mutations of these genes lead to widespread abnormalities that are not restricted to a few segmental shifts as in mice.

Furthermore, several PcG proteins have been implicated in hematopoiesis (Jacobs and van Lohuizen, 2002). First, the expression of PcG genes is highly regulated in hematopoietic differentiation (Fukuyama et al., 2000; Lessard et al., 1998; Lessard et al., 1999; Peytavi et al., 1999; Raaphorst et al., 2001; Raaphorst et al., 2000). Second, all PcG mutant mice display defects in different hematopoietic compartments and have severely reduced body weights and sizes (Akasaka et al., 1996; Core et al., 1997; Katoh-Fukui et al., 1998; Kimura et al., 2001; Lessard et al., 1998; Takihara et al., 1997; van der Lugt et al., 1994). Finally, at least the hematopoietic defects observed in $Bmi1^{-/-}$ mice have been attributed to a deficiency in the self-renewal capacity of the hematopoietic stem cells (Lessard and Sauvageau, 2003; Park et al., 2003).

Apart from the common defects in axial patterning and hematopoiesis observed in all PRC1-PcG mutants, specific defects were also reported including decreased cerebellar cellularity in $Bmi1^{-/-}$ mice, smooth muscle atrophy in $Mel-18^{-/-}$ mice, male-to-female sex reversal in $M33^{-/-}$ mice and neural crest defects in $Mph1^{-/-}$ mice (Akasaka et al., 1996; Core et al., 1997; Katoh-Fukui et al., 1998; Takihara et al., 1997; van der Lugt et al., 1994). Interestingly, it has also been proposed that the cerebellar proliferation defects of $Bmi1^{-/-}$ mice are due to decreased proliferation of neural stem cells (Leung et al., 2004; Molofsky et al., 2003; Zencak et al., 2005) suggesting a general role for Bmi1 in the self-renewal of adult stem cells.

The proliferation defects observed in vivo are consistent with a role for the PcG proteins in the control of the cell cycle. Indeed, $Bmi1^{-/-}$, $Mel-18^{-/-}$ and $M33^{-/-}$ MEFs are
impaired in proliferation (Core et al., 1997; Jacobs et al., 1999a). The tumorigenic properties of Bmi1 have made it a major focus of interest in the field. The Bmi1 gene was originally identified as an oncogene that could cooperate with Myc in the formation of B-cell lymphomas in mice (Haupt et al., 1991; van Lohuizen et al., 1991). Subsequently, Bmi1 was shown to transform rodent fibroblasts in vitro (Cohen et al., 1996). In harmony with its oncogenic capacity, loss of Bmi1 in MEFs resulted in impaired proliferation and premature senescence due to an up-regulation of p16\textsuperscript{INK4A} and p19\textsuperscript{ARF} (Jacobs et al., 1999a). In fact, loss of the Ink4a/Arf locus at least partially rescues the proliferation defects in MEFs and hematopoietic and neural stem cells (Bruggeman et al., 2005; Jacobs et al., 1999a; Jacobs et al., 1999b; Park et al., 2003).

We and others have previously shown that E2F6 is a bona fide member of the mammalian PRC1-Polycomb complex (Chapter 2; Storre et al., 2002). The finding that E2F6 was found in association with several PcG proteins, led to the hypothesis that E2F6 may act by recruiting these proteins to, at a minimum, a subset of their target promoters (Attwooll et al., 2004; Ogawa et al., 2002; Trimarchi et al., 2001). The investigation of E2f6-deficient mice is consistent with this model. E2f6\textsuperscript{-/-} mice display some of the same axial skeletal transformations observed in Bmi1\textsuperscript{-/-} mice suggesting that E2F6 only participates in the repression of a subset of Hox genes (Chapter 2; Storre et al., 2002). Unlike Bmi1\textsuperscript{-/-}, E2f6\textsuperscript{-/-} mice do not exhibit hematopoietic or other developmental defects. Finally, E2f6\textsuperscript{-/-} MEFs present normal proliferation and senescence properties. These findings suggest that the function of E2F6 is restricted to the correct patterning of the thoraco-lumbo-sacral region of the axial skeleton. Other possibilities are that E2F6 loss
is compensated by other factors as discussed above or that E2F6 has a minor role in these processes resulting in subtle defects that have not been detected.

Compound mutants of PRC1-PcG proteins have been generated in flies and mice resulting in dramatic synergistic effects (Adler et al., 1991; Akasaka et al., 2001; Bel et al., 1998; Kwon et al., 2003). The transformations and mortality frequencies were more severe in the double mutants suggesting a strong gene dosage effect. In an attempt to uncover further unknown roles for E2F6 in development and proliferation and investigate the extent of cooperation between E2F6 and Bmi1, we have generated mice and MEFs that are deficient for both E2f6 and Bmi1 genes. The most striking observation is a significant increase in the penetrance of posterior axial skeleton transformations in the compound mutant mice compared to the single knockout littermates. Furthermore, although more experiments are required, our initial observations suggest synergistic effects between E2F6 and Bmi1 in mouse viability as well as in cell proliferation and senescence.

RESULTS

Viability and survival of compound mutants

To further characterize E2F6 function, we have generated E2f6<sup>-/-</sup>;Bmi1<sup>-/-</sup> mice from double heterozygous crosses. We had previously shown that E2f6 deletion alone does not affect the viability and survival of mice (Chapter 2) while it has been reported that Bmi1 deficiency leads to a reduction in the survival of newborn mice (van der Lught et al., 1994). Although Bmi1<sup>-/-</sup> mice are born at the expected frequency, only approximately 50% survive into adulthood. Those that do survive are significantly smaller than their
wild-type littermates and display poor health that result in early lethality (3 to 20 weeks). In agreement with the published data, only 51% of Bmi1−/− mice survived to weaning at 3 weeks of age in our crosses (Table 1). E2f6+/−;Bmi1−/− mice were underrepresented at both weaning age and E18.5 (38% and 46% of expected numbers, respectively). Like the

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<th></th>
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<td>7</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
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<td>122</td>
<td>53</td>
<td>46</td>
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</table>

Table 1. Offspring from intercrosses of double heterozygous mice. a. Results obtained from 250 animals genotyped at 3 weeks of age. B. Results obtained from 105 animals genotyped at E18.5.
Bmi1−/− mice, the compound mutants eventually became severely anemic and had to be sacrificed. Interestingly, the frequency of E2f6−/−;Bmi1−/− mice is higher than that of both Bmi1−/− and E2f6−/−;Bmi1−/− mice at weaning age but not at E18.5. This does not fit our hypothesis of gene dosage effects but rather suggests an alternative unknown mechanism whereby removing only one allele of E2f6, but not both, would confer a survival advantage after birth. However, it is important to note that the total number of animals analyzed is relatively small (n=250) resulting in low statistical power that could explain these results.

The E2f6−/−;Bmi1−/− mice, as the Bmi1−/− mice, were significantly smaller than their wild-type littermates as evidenced by their body weight at 2-3 months of age (Figure 1). Yet, as discussed before, the number of animals analyzed is not enough to establish whether there is a significant difference in weight between these mice.

**Neurological abnormalities of compound mutants**

The Bmi1-deficient mice develop ataxia at the age of 2-4 weeks as a result of decreased cell density in the different layers of the cerebellum. Our results show that E2f6−/−;Bmi1−/− mice also develop ataxia. We examined animals from our double heterozygous crosses that survived to 2 months of age. Unlike Bmi1+/+ mice, 60% of Bmi1−/−, 65% of E2f6+/−;Bmi1−/− and 70% of E2f6−/−;Bmi1−/− mice developed ataxia by 2 months of age. We are currently analyzing histological sections of E2f6−/−;Bmi1−/− brains to assess the state of the cerebellum defect compared to Bmi1−/− brains. Although it is difficult to quantify the level of ataxia in the different genotypes, we could attempt to
Figure 1. Weight distribution of 2-3 month old animals. a. Females and b. males of all genotypes were weighed at 2-3 months of age. Blue dots represent animals that are wild-type for Bmil; green dots, Bmil heterozygous animals; and red/orange dots, Bmil knock-out animals (all irrespective of E2f6 genotype).
measure the severity of this phenotype by determining the age of onset of the ataxia or by performing a Rotarod test where mice are tested for their ability to stay on a rotating rod.

Interestingly, we have observed that the ataxia development is correlated with the body weight of the animals. Specifically, animals that had lower body weights consistently developed ataxia whereas bigger mice did not (Figure 2). It is unclear if any of these defects is a consequence of the other or if they merely indicate that some mutants are more affected in all phenotypes.

![Graph showing ataxia and weight correlation for 2-3 month-old females and males.](image)

**Figure 2. Ataxia and weight correlation for 2-3 month-old females and males.**

Shown in red are the weights of animals that displayed the ataxic phenotype whereas blue represents the weights of animals that did not develop ataxia. The animals included in this chart are mice that are heterozygous or homozygous mutant for *Bmi1*. 
Axial skeletal transformations of \( E2f6^{-/-};Bmi1^{-/-} \) mice

\( Bmi1^{-/-} \) mice display several morphological abnormalities along the anteroposterior axis (van der Lugt et al., 1994). These changes indicate posterior transformations of vertebra identity and consist of (1) an extra piece of bone rostral to the cervical vertebra C1, (2) a C1 to C2 conversion, (3) a C7 to T1 (thoracic vertebra) conversion evidenced by the presence of ribs at C7 which then fuse on the ventral side with the ribs associated with T1, (4) a T7 to T8 conversion resulting in only six instead of seven vertebrosternal ribs, (5) a T13 to L1 (lumbar vertebra) conversion shown by the absence or degeneration of ribs at T13 and (6) a L6 to S1 (sacral vertebra) conversion evidenced by the joints between L6 and the iliac bones. As expected, we also observed all of these transformations, which are illustrated in Figure 3. \( E2f6 \)-deficient mice exhibit the T13 to L1 and L6 to S1 conversions with a penetrance of 67% and 80%, respectively (Chapter 2; Storre et al., 2002). Data from both \( Bmi1^{-/-} \) and \( E2f6^{-/-} \) mice indicate that the severity of the defect is dependent on the gene dosage.

We have examined the axial skeletons of all genotypes generated from a double heterozygous cross and noted a higher penetrance of all except one of the \( Bmi1^{-/-} \) defects in mice doubly-deficient for \( E2f6 \) and \( Bmi1 \) (Table 2). Specifically, our results showed no effect on the penetrance of the T7 to T8 conversion by additional deletion of \( E2f6 \). For the remaining transformations, deletion of only one allele of \( E2f6 \) was enough to increase the penetrance of each abnormality. Further deletion of the remaining \( E2f6 \) allele led to an even higher penetrance, at least in the \( Bmi1^{+/+} \) background, suggesting a gene dosage effect. In the \( Bmi1^{-/-} \) background, deletion of both copies of \( E2f6 \) appears to result in a lower penetrance of the certain transformations. However, only two animals
Figure 3. Posterior transformations of the axial skeletons of Bmil-deficient mice. 

**a.** Lateral view of the cervical and upper thoracic region of wild-type (left) and Bmil<sup>−/+</sup> (right) skeletons. 

**b.** Ventral view of the thoraco-lumbo-sacral region of wild-type (left) and Bmil<sup>−/+</sup> (right) skeletons. See text for explanation.
were analyzed for this genotype and the remaining data is consistent with a gene dosage effect. Still, more animals should be analyzed to confirm this observation. Significantly, the conversions of C1 to C2 and C7 to T1 were detected in all of the $E_{2f6}^+;Bmi1^-$ and $E_{2f6}^+;Bmi1^-$ mice analyzed. In contrast, the T13 to L1 and L6 to S1 conversions were observed in 100% of $E_{2f6}^+;Bmi1^-$ mice and only in 50% of $E_{2f6}^+;Bmi1^-$ mice analyzed. These results suggest that E2F6 and Bmi1 act synergistically in the anteroposterior segmentation of the axial skeleton.

<table>
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<th>WT</th>
<th>HET</th>
<th>KO</th>
<th>WT</th>
<th>HET</th>
<th>KO</th>
<th>WT</th>
<th>HET</th>
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<tr>
<td>E2f6 BT Bmi1</td>
<td>WT</td>
<td>HET</td>
<td>KO</td>
<td>WT</td>
<td>HET</td>
<td>KO</td>
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<td>0</td>
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<td>0</td>
<td>60</td>
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<tr>
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<td>31</td>
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<td>50</td>
<td>60</td>
<td>100</td>
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</tr>
<tr>
<td>T7 to T8</td>
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<td>0</td>
<td>0</td>
<td>4</td>
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Table 2. Penetrance of axial skeletal transformations.

Cell cycle properties of double mutant MEFs

$Bmi1^-$ MEFs exhibit impaired proliferation and premature senescence due to an up-regulation of $p16^{INK4A}$ and $p19^{ARF}$ protein levels (Jacobs et al., 1999a). In contrast,
E2f6-/- MEFs display no defects in either proliferation or senescence (Chapter 2; Storre et al., 2002). Since E2F6 and Bmi1 share a common and synergistic role in axial patterning, we wished to determine if these proteins could also cooperate in cell cycle control. To do so, we have compared wild-type, Bmi1-/-, E2f6-/- and E2f6-/-;Bmi1-/- MEFs in several cell cycle assays.

Our analyses of Bmi1-/- MEFs revealed the same defects that had been previously reported (Figures 4 and 6). In addition, we found that serum deprived Bmi1-/- MEFs were impaired in their ability to re-enter the cell cycle following the re-addition of serum (Figure 5). The asynchronous proliferation rate of three out of four E2f6-/-;Bmi1-/- cell lines were even lower than that of Bmi1-/- MEFs (Figure 4). However, only one out of four double mutant cell lines displayed a stronger impairment in S phase re-entry than the Bmi1 single mutant (Figure 5). Finally, in a standard 3T3 protocol, two out of four E2f6-/-;Bmi1-/- cell lines exhibited an earlier senescence than observed in the Bmi1-/- MEFs (Figure 6). These results suggest that E2f6 deletion affects the cell cycle defects of Bmi1 mutant MEFs. However, more cell lines need to be examined to corroborate these findings.

To further investigate these observations, we have collected RNA samples from the experiments described above for real-time PCR analysis of target gene expression. These experiments are currently underway. Specifically, we wish to determine whether an increase in p16INK4A and p19ARF levels correlates with the stronger impairment in proliferation and cell cycle re-entry as well as the earlier senescence in the double mutant MEFs. If so, this would argue in favor of a synergism between E2F6 and Bmi1 in the repression of the Ink4a/Arf locus. However, if no changes are observed in the levels of
p16\textsuperscript{INK4A} and p19\textsuperscript{ARF} between $Bmi1^{-/-}$ and $E2f6^{-/-};Bmi1^{-/-}$ MEFs, this would suggest that E2F6 modulates the cell cycle in the $Bmi1$-deficient background through a different mechanism. Alternatively, E2F6 and Bmi1 could be cooperating without synergizing in the repression of the $Ink4a/Arf$ locus. In fact, this result would be expected if these proteins were present in one complex that regulates the expression of p16\textsuperscript{INK4A} and p19\textsuperscript{ARF}.
Figure 4. Proliferation of asynchronously growing MEFs. a. and b. Two separate experiments using different cell lines of the following genotypes: wild-type (blue), $E2f6^{-/-}$ (green), $Bmi1^{-/-}$ (green) and $E2f6^{-/-};Bmi1^{-/-}$ (red).
Figure 5. Cell cycle re-entry of MEFs. a. and b. Two separate experiments using different cell lines of the following genotypes: wild-type (blue), $E2f6^{-/-}$ (green), $Bmi1^{-/-}$ (green) and $E2f6^{-/-} ; Bmi1^{-/-}$ (red).
Figure 6. Senescence properties of MEFs. a. and b. Two separate experiments using different cell lines of the following genotypes: wild-type (blue), $E2f6^+$ (green), $Bmi1^+$ (orange) and $E2f6^+;Bmi1^+$ (red).
DISCUSSION

E2F6 has been previously shown to play a role in PcG-mediated axial patterning during murine development (Chapter 2; Storre et al., 2002). Although data from overexpression as well as promoter occupancy studies implicated E2F6 in the regulation of the cell cycle (Cartwright et al., 1998; Gaubatz et al., 1998; Giangrande et al., 2004; Ogawa et al., 2002), the examination of the proliferation and senescence properties of E2f6-deficient MEFs suggests otherwise (Chapter 2; Storre et al., 2002).

Bmi1 deficiency leads to posterior axial skeletal transformations, reduced newborn survival, ataxic gait and hematopoietic defects in the mouse (van der Lugt et al., 1994). Furthermore, the Bmi1- MEFs exhibit impaired proliferation and premature senescence due to up-regulation of p16INK4A and p19ARF (Jacobs et al., 1999a). This suggests that Bmi1 normally represses the transcription of these cell cycle inhibitors. However, very few PcG proteins are able to directly bind DNA, including Bmi1 (Brown et al., 1998; Kanno et al., 1995). Since E2F6 is a transcription factor that associates with DNA at E2F sites (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998) and is associated with Bmi1, it is possible that Bmi1 is recruited to E2F sites in the p19ARF promoter (Bates et al., 1998) through the action of E2F6.

The purpose of this study was to test whether E2F6 functions in cooperation with Bmi1 in cell cycle control as well as in axial skeletal patterning. To do so, we have generated E2f6;Bmi1 compound mutants. In both flies and mammals, the deletion of two PcG proteins leads to stronger axial skeletal transformations suggesting a synergistic effect (Akasaka et al., 2001; Bel et al., 1998). Indeed, we observe a higher penetrance of these defects as the E2f6 dosage decreases in the Bmi1-deficient background. Moreover,
our preliminary analyses of the double mutant cells indicate that E2F6 may have a role in PcG function that is not restricted to axial patterning.

**Viability and lifespan of compound mutant mice**

The $E2f6^+/Bmi1^+$ mice arise at lower frequencies and seem to be smaller than $Bmi1$ single mutants although the analysis of a larger number of mutants would be necessary to reach statistical significance. Similarly, the $M33^+/Bmi1^+$ and $Mel-18^+/Bmi1^+$ mice also arise at lower frequencies than the $Bmi1^+/+$ mice (Akasaka et al., 2001; Bel et al., 1998). $Bmi1^-/+$ mice are known to be born at expected frequencies and die perinatally (van der Lugt et al., 1994). Our data is consistent with a similar trend although we did not find 100% of expected $E2f6^+/Bmi1^+$ embryos at E18.5. This could mean that the $E2f6^+/Bmi1^+$ mice die before birth suggesting an even stronger phenotype. It would be helpful to look at earlier time points in development to determine if, in fact, the $E2f6^+/Bmi1^+$ embryos have reduced viability. Alternatively, our numbers may be too small and if we increase them, we might discover that $E2f6^+/Bmi1^+$ mice are born at the expected Mendelian frequency as well.

We have not observed a significant difference in the lifespan of the $E2f6^+/Bmi1^+$ and $Bmi1^+$ mice that reach adulthood, indicating that loss of E2F6 does not alter the survival potential of the $Bmi1^+/+$ animals. Furthermore, the double mutant animals exhibit poor health, have lower body weights and develop an ataxic gait. Eventually, they become severely anemic and need to euthanized. Histological analysis of these animals reveals no other defects except for decreased hematopoiesis in the bone marrow and atrophic spleen.
Neurological abnormalities of $E2f6^{\text{lt}};Bmi1^{\text{lt}}$ mice

The $Bmi1^{\text{lt}}$ mice have been described to develop ataxia between 2-4 weeks of age due to a decreased cellularity of all the layers in the cerebellum, which is the major motor coordination center (van der Lugt et al., 1994). The ataxic phenotype appears to be more penetrant in $E2f6^{\text{lt}};Bmi1^{\text{lt}}$ mice. At the moment, we have not been able to determine if the severity of this phenotype is also increased. We are in the process of analyzing histological sections of the cerebellum to determine if the $E2f6^{\text{lt}};Bmi1^{\text{lt}}$ mice display a more severe defect. In addition, we plan to determine the age of onset of the ataxic gait in an attempt to quantify the severity of the defect.

Posterior transformations of $E2f6;Bmi1$ double mutant mice

$Bmi1^{\text{lt}}$ mice exhibit six different transformations along the entire anterior-posterior axis (van der Lugt et al., 1994). In contrast, $E2f6^{\text{lt}}$ mice display defects restricted to the thoraco-lumbo-sacral region indicating that E2F6 controls the expression of no more than a subset of $Hox$ genes. In this study, we have shown that reducing the E2F6 dosage in the Bmi1-deficient background leads to a progressively higher penetrance of five out of the six transformations observed in $Bmi1^{\text{lt}}$ mice. This finding strongly supports a model in which E2F6, like M33 and Mel-18, synergizes with Bmi1 in a dose-dependent manner in axial patterning. However, unlike the $M33^{\text{lt}};Bmi1^{\text{lt}}$ and $Mel-18^{\text{lt}};Bmi1^{\text{lt}}$ studies, we have not detected any novel transformations in the $E2f6^{\text{lt}};Bmi1^{\text{lt}}$ mice. This result further supports the hypothesis that E2F6 plays a limited role in the PcG-mediated anteroposterior segmentation during embryogenesis. Since E2F6 is
found in association with several PcG proteins, it would be interesting to determine whether this protein synergizes with other members of the PcG complex.

**Proliferation and senescence of E2f6⁻;Bmi1⁺ MEFs**

E2F6 appears to be dispensable for normal proliferation and senescence given that E2f6⁻ MEFs are indistinguishable from their wild-type littermates (Chapter 2; Storre et al., 2002). Although previous data suggested that E2F6 may repress the transcription of p19ARF (J.M.Trimarchi and J.A. Lees, unpublished results), we do not observe a deregulation of this protein in E2f6⁻ MEFs by western blot analysis. Together, these findings suggest that E2F6 does not participate in the Bmi1-mediated repression of p19ARF. Alternatively, other factors could compensate for the absence of E2F6. Our working model indicates that E2F6 participates in Polycomb function through its ability to target PcG complexes to appropriate target genes. Thus, a factor that replaces E2F6 in the recruitment of PcG proteins to target genes has to have similar DNA binding properties making other E2Fs the most likely candidates.

Indeed, E2F4 has been shown to associate with G1/S gene promoters during S phase only in the absence of E2F6 (Giangrande et al., 2004). In agreement with this finding, these genes were deregulated exclusively in E2f6⁻ MEFs in which E2F4 had been knocked-down by RNAi. Moreover, E2F3b has been found at the p19ARF promoter and has been implicated in its transcriptional inhibition (Aslanian et al., 2004). We had originally reported that E2F6 was the only E2F capable of RYBP association (Trimarchi et al., 2001). However, another study indicated that E2F2 and E2F3, but not the other E2Fs including E2F6, associate with this PcG protein (Schlisio et al., 2002). These
discrepancies may be due to subtle differences in the experimental protocols or could reflect the well-established diverse compositions of the PcG complexes in different cell types and conditions. Indeed, we have now been able to detect RYBP association with E2F1-6 (P.J.Iaquinta and J.A.Lees, unpublished results) in agreement with the knowledge that it is the well-conserved Marked box domain of E2F that mediates the direct interaction with RYBP (Trimarchi et al., 2001).

We analyzed four different E2f6-/-;Bmi1-/- MEF cell lines for their properties in asynchronous proliferation, cell cycle re-entry and senescence. First, three double mutant cell lines displayed a stronger proliferation impairment than the Bmi1 single mutant MEFs. Second, only one E2f6-/-;Bmi1-/- MEF cell line exhibited a more severe cell cycle re-entry defect. Third, two compound mutant MEF cell lines underwent senescence even earlier than the Bmi1 single mutant. Although more cell lines need to be analyzed, these results support that E2F6 participates in regulating the cell cycle in cooperation with Bmi1. It is a distinct possibility that these proteins work together in the repression of the Ink4a/Arf locus. Therefore, we are in the process of examining the expression levels of p16INK4 and p19ARF by real-time PCR in the E2f6-/-;Bmi1-/- MEFs.

The results described in this chapter indicate that E2F6 and Bmi1 act synergistically in the anteroposterior patterning of the developing embryo. Furthermore, they are consistent with our model that E2F6 recruits the PcG complex to a subset of target genes. Moreover, although more experiments are needed, our work raises the possibility that E2F6 may have a role in the control of proliferation and tumorigenesis.
EXPERIMENTAL PROCEDURES

Histological and skeletal analysis

Dead mice from our aging colonies were dissected and processed for histological analysis. Soft tissues were fixed in 10% formalin and hard tissues were fixed in Bouin’s fixative. Paraffin sections were prepared and stained with hematoxylin and eosin. Skeletal analysis was performed on 3-day old mice. After removing the skin and viscera, the skeletons were fixed in acetone and stained with cartilage-specific Alcian Blue and bone-specific Alizarin Red. Soft tissue was cleared with KOH.

Mouse embryonic fibroblasts

MEFs were prepared from 13.5 d.p.c embryos as previously described (Humbert et al., 2000) and genotyped by PCR of DNA obtained from yolk sacs. Proliferation curves were obtained by plating 2x10⁴ MEFs in triplicate in 24-well plates. At the indicated time points, MEFs were trypsinized and counted. For cell cycle re-entry assays, 2x10⁵ MEFs were plated in triplicate in 6-well plates. After 2 days of growth in media containing 10% serum, they were incubated in media containing 0.1% serum for 3-4 days. Re-entry into the cell cycle was induced by incubation in media containing 10% serum and at the indicated time points, 5 μCi of ³H-thymidine was added to the cells for 1 hour. Cells were then scraped from the plates and cell pellets were analyzed for ³H-thymidine incorporation using a scintillation counter. A 3T3 protocol was followed to monitor senescence. 3x10⁵ MEFs were plated in duplicates in 6-cm plates and re-fed 2 days later. On the third day, they were trypsinized, counted and replated. The fold replication was determined by dividing the number of cells obtained at day 3 by 3x10⁵.
Western blots were performed as described previously (Moberg et al., 1996) with 50-100 μg of whole cell lysates.
ACKNOWLEDGEMENTS

We are grateful to Marteen van Lohuizen for the generous gift of the Bmi1⁻/⁻ mice. We also thank Alicia Caron and Roderick Bronson for the generation and analysis of histological sections and Mark García for help with colony maintenance and dissections. We would also like to thank the members of the Lees lab for reagents and helpful discussions.

REFERENCES


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Chapter Four

Conclusions
Despite the *in vitro* evidence that suggests a role for E2F6 in cell cycle control as well as its interaction with the PcG complex, the biological significance of this protein had not been analyzed *in vivo*. This work unequivocally shows that E2F6 is a *bona fide* member of the PcG complex that functions in the anteroposterior segmentation of the developing embryo. Furthermore, our results allude to a broader role for E2F6 in PcG that extends to control of cell proliferation. In chapter two, we have established that loss of E2F6 results in posterior transformations of the axial skeleton that are reminiscent of defects seen in other PcG mutants providing genetic evidence of a PcG function for E2F6. However, our study of *E2f6*-deficient MEFs did not reveal a clear role for E2F6 in cell cycle control. In chapter three, we further examined the axial skeletal transformations of *E2f6*<sup>+/−</sup>;<*Bmi1*<sup>−/−</sup> mice and found a clear gene dosage effect. In addition, our preliminary evidence suggests that E2F6 may participate in the control of cell proliferation in MEFs and cerebellar neurons by Bmi1. Although it would be expected that the *E2f6*<sup>−/−</sup> mice and MEFs would also display these proliferative defects albeit with decreased severity, we cannot rule out the possibility that the E2F6 plays a minor role in the cell cycle and therefore, ablation of this gene alone has no effect. If that was the case, it is possible that in the presence of another mutation, the effects of *E2f6*-deficiency become apparent. Taken together, our data show that E2F6 is an important effector of PcG function.

**E2F6 in axial patterning**

The identification of PcG proteins as E2F6 interactors led to the hypothesis that E2F6 may play a role in the anterior-posterior patterning of the developing embryo.
(Attwooll et al., 2004; Ogawa et al., 2002; Trimarchi et al., 2001). The transcriptional repressive property of E2F6 is compatible with a role in the PcG complex, which negatively regulates Hox genes expression (Gaubatz et al., 1998; Kennison, 1995; Morkel et al., 1997; Trimarchi et al., 1998). However, in order to prove that a protein is a *bona fide* member of the PcG complex, it is essential to demonstrate that it is a functional component of this complex. In order to do this, we and others have generated E2f6-deficient mice and analyzed their axial skeletons (Chapter 2; Storre et al., 2002). These mice display two types of posterior transformations of the axial skeleton with dose-dependent penetrance. First, the thoracic vertebra T13 is transformed into the lumbar vertebra L1 as evidenced by the absence or degeneration of the ribs normally associated with T13. Second, the lumbar vertebra L6 is transformed into the sacral vertebra S1 as indicated by the presence of sacral-iliac joints. These identical defects are present among several transformations along the entire axial skeleton of in Bmil- mice suggesting the E2F6 and Bmi1 may function together at least in some regions (van der Lugt et al., 1994). Significantly, similar results have been reported by an independent study (Storre et al., 2002).

The T13-to-L1 transformation is found in 9% of E2f6+/− mice and in 67% of E2f6−/− mice whereas the L6-to-S1 transformation is found in 35% of E2f6+/− mice and in 80% of E2f6−/− mice. These observations indicate a clear gene dosage effect and imply that E2F6 loss has a more profound effect in the more posterior regions of the axial skeleton. It is possible that E2F6 has a more prominent role in the repression of those Hox genes that specify the identities of the more posterior vertebra. The analysis of the expression patterns of Hox genes during embryogenesis in E2f6−/− mice would provide
further insight into the role of E2F6 in axial patterning. Of particular interest would be the Hox10 and Hox9 paralog groups, which are known to have their anterior expression boundaries at the lumbosacral and thoracic region respectively (Burke et al., 1995). Another good candidate would be Hoxc8 since its deletion, leads to anterior transformation of the lumbar vertebra L1 into a thoracic vertebra T13 (Le Mouellic et al., 1992). Interestingly, the expression of Hoxc8 is among the affected ones in Bmi1+/− mice suggesting that lack of Hoxc8 activity leads to anteriorization of L1 to T13, whereas ectopic expression of Hoxc8 results in the posteriorization of T13 to L1 (van der Lugt et al., 1996). Based on these observations, it would be reasonable to speculate that E2F6 only participates in the repression of specific Hox genes that determine the identity of the more caudal structures.

The fact that the transformations observed in the E2f6−/− mice are also found in the Bmi1+/− animals implies that these proteins may act in synergism in the thoracic/lumbar and lumbar/sacral transitions. In agreement with this model, we have shown in chapter three that the posterior transformations observed in the Bmi1 mutants are found with a higher penetrance as the E2f6 gene dosage decreases. Specifically, we have shown that five out of six defects are enhanced in the compound mutants. For example, the penetrance of the C7-to-T1 transformation went up from 31% in Bmi1+/− to 50% in E2f6−/−;Bmi1+/− and from 60% in Bmi1−/− to 100% in E2f6−/−;Bmi1−/−. These results strongly support a synergistic and dose-dependent genetic interaction between E2F6 and Bmi1.
E2F6 in viability and survival

E2f6-deficient mice are viable, survive into adulthood and display no gross morphological defects, growth abnormalities or fertility problems. Moreover, their lifespan is not significantly different from that of their littermate controls even when treated with γ-irradiation at 6 days of age. In contrast, whereas E2f6+/−;Bmi1−/− mice are underrepresented at E18.5 and 3 weeks of age, the Bmi1−/− animals are underrepresented only at 3 weeks of age. This result argues in favor of a synergistic relationship between E2F6 and Bmi1 in viability and survival. However, it is important to note that our numbers are still relatively small and may not reflect statistically significant results (250 animals were genotyped at 3 weeks of age whereas 105 embryos were analyzed at E18.5). We are currently attempting to increase the total number of mice examined.

In chapter two, we have analyzed by histology all the deceased mice from our aging colonies in an attempt to determine the cause of death of the E2f6−/− mice. The examination of these animals revealed a few interesting observations although further studies are required to determine their significance.

Among the causes of death of the E2f6+/− and E2f6−/− aging mice, liver tumors and several blood tumors were the most prominent suggesting a potential tumor suppressor role for E2F6. However, the tumors observed in E2f6 mutant mice may be merely the result of old age since the mice that developed these tumors were over one year of age. In fact, based on the biochemical and cell cycle data, it is more likely that E2F6 behaves as an oncogene through the repression of important negative regulators of the cell cycle. For example, it has recently been shown that E2F6 is found at the promoter of the tumor suppressor BRCA1 gene (Oberley et al., 2003). Furthermore, the association and
synergism of E2F6 with the Bmi1 oncogene suggests that it may function in an analogous manner by repressing the Ink4a/Arf locus (Jacobs et al., 1999). The discovery of E2F target sites in the p19ARF promoter supports this hypothesis and led to the proposition that E2F6 is responsible for Bmi1 recruitment to p19ARF promoters (Bates et al., 1998). If this were the case, we would expect p19ARF levels to be similarly up-regulated in Bmi1−/−, E2f6−/− and E2f6−/−;Bmi1−/− MEFs since removal of one or the other should be enough to abrogate the repression of this gene. However, although overexpression of E2F6 in MEFs leads to down-regulation of p19ARF, E2f6−/− MEFs do not exhibit increased levels of this cell cycle inhibitor (J.M.Trimarchi and J.A.Lees, unpublished results; Chapter 2). We are currently in the process of determining the p19ARF levels in E2f6−/−;Bmi1−/− MEFs. Additionally, overexpression studies would address the role of E2F6 in oncogenesis. First, if E2F6 functions in a similar manner to Bmi1, overexpression of E2F6 and other known oncogenes should result in transformation of primary cells (Cohen et al., 1996; Jacobs et al., 1999). Second, if Bmi1 requires E2F6 to bind the p19ARF promoter, it would be expected that overexpression of Bmi1 would not be able to transform E2f6−/− MEFs. Third, transgenic mice that overexpress E2f6 should cooperate with other oncogenes in tumor formation (Alkema et al., 1997; Haupt et al., 1991; Haupt et al., 1993; van Lohuizen et al., 1991b). Finally, E2f6−/−;Eμ-Bmi1 mice should not cooperate with Eμ-myc mice in lymphomagenesis.

Another interesting finding is that three out of five mice analyzed had developed odontoma. These benign tumors of the tooth germs arise in osteopetrotic mice as a consequence of defects in osteoclast function or differentiation (Amling et al., 2000; Ida-Yonemochi et al., 2002; Ida-Yonemochi and Saku, 2002; Tiffee et al., 1999). Defects in
bone remodeling during tooth development results in morphological abnormalities that lead to the formation of odontomas. Significantly, Hoxc8 represses the transcription of two proteins produced by osteoblasts, Osteoprotegnerin (OPG) and Osteopontin (OPN). OPG is a secreted decoy receptor that binds to osteoclast differentiation factor and inhibits osteoclast maturation (Wan et al., 2001) whereas OPN facilitates bone resorption by decreasing bone mineralization (Lei et al., 2005). It is reasonable to speculate that in the absence of the appropriate PcG proteins, in this case E2F6, deregulated expression of Hoxc8 inappropriately inhibits OPG and OPN expression resulting in an impaired bone resorption. Therefore, it would be very interesting to study the function and differentiation of osteoclasts isolated from E2f6−/− mice as well as to determine the expression levels of OPG and OPN in E2f6 mutant osteoblasts.

A low percentage of E2f6-deficient mice exhibited testicular atrophy, a common age-related defect. Although the mice that displayed testicular atrophy were at least 500 days old, it is interesting to note that another group reported a reduction of spermatogenesis in E2f6−/− mice (Storre et al., 2002). Surprisingly, our colony of E2f6−/− mice exhibits no fertility problems.

Finally, we have noticed that two E2f6−/− mice as well as one E2f6−/−;Bmi1−/− mouse and one E2f6−/−;Bmi1+/− mouse have only a single kidney. It is not clear if this is a result of failure to develop a second kidney or of the degeneration of an existing kidney. To address this question, a timed analysis of mice of different ages should be performed but given the low penetrance of this phenotype this would require the generation of a large number of animals. The phenotype in the kidney establishes another connection between E2F6 and the PcG complex. In fact, the Hox genes have been implicated in kidney
development. The Hox11 paralog group (HoxA11, HoxC11 and HoxD11) are involved in the induction of ureter budding from the nephric duct (Wellik et al., 2002). In addition, mutant mice for Hoxa10 and Hoxd10 display altered kidney placement and size (Lin and Carpenter, 2003). In fact, the expression of 37 Hox genes was analyzed in the developing kidney at E12.5, E13.5 and E17.5 (Patterson and Potter, 2003; Patterson and Potter, 2004). Multiple Hox genes were found to be expressed in kidney and the colinearity of expression boundaries and the location of the genes in the clusters was conserved. It appears that these Hox genes regulate the expression of renal morphogens. Therefore, it would be interesting to study the expression levels of these Hox genes in E2f6−/− kidneys.

**E2F6 in the control of cell proliferation**

Several observations indicate that E2F6 may function in the cell cycle control. First, E2F6 is a member of the E2F transcription factor family, which have been known to play an important role in the control of cell proliferation (Trimarchi and Lees, 2002). Second, the cell cycle-dependent expression of E2F6 probably reflects a differential requirement for E2F6 function at different stages of the cell cycle (Dahme et al., 2002; Kherrouche et al., 2001; J.M.Trimarchi and J.A.Lees, unpublished results). Third, overexpression studies have implicated E2F6 in the control of S phase entry (Cartwright et al., 1998; Gaubatz et al., 1998). Fourth, E2F6 overexpression in MEFs results in down-regulation of p19ARF, an important cell cycle inhibitor. Finally, E2F6 has been found at the promoters of E2F target genes that are activated during G1/S (Giangrande et al., 2004).
In contrast to these observations, $E2f6^{-/-}$ MEFs exhibit no proliferation, senescence or target gene expression abnormalities arguing against a role for E2F6 in cell cycle control (Chapter 2; Storre et al., 2002). However, it is possible that E2F6 may participate in the control of cell proliferation yet the $E2f6^{-/-}$ MEFs display no phenotypes. In fact, $E2f1^{-/-}$ and $E2f4^{-/-}$ MEFs also present normal cell cycle properties despite their known role in cell cycle regulation (Humbert et al., 2000a; Humbert et al., 2000b).

One possibility is that compensation by other E2F family members may occur. In support of this model, E2F4 appears to compensate E2f6 deficiency in the regulation of G1/S genes repression during S phase (Giangrande et al., 2004). E2F4 was found at the promoters of G1/S-activated genes during S phase in MEFs lacking E2F6 whereas in wild-type MEFs E2F4 is found in association with DNA exclusively during G0 (Takahashi et al., 2000). Another potential candidate for E2F6 loss compensation is E2F3b, which has recently been implicated in the regulation of the p19ARF gene expression (Aslanian et al., 2004). This could explain why p19ARF is not up-regulated in $E2f6^{-/-}$ MEFs. Interestingly, like E2F6, E2F3 has also been shown to associate with the PcG protein RYBP suggesting that it may participate in the recruitment of the PcG complex to target gene promoters (Schlisio et al., 2002). It would therefore be interesting to determine the cell cycle properties of MEFs doubly deficient for $E2f6$ and $E2f4$ or $E2f6$ and $E2f3b$.

Another explanation for the lack of detectable phenotypes in $E2f6^{-/-}$ MEFs is that E2F6 plays a minor role in this cell type and therefore its loss does not have an effect. However, it is possible that by combining the $E2f6$ and $Bmil$ mutations, the $Bmil^{-/-}$ MEFs phenotypes are exacerbated. This would be reminiscent of the effects that E2F6 loss in
the Bmil mutant background has on axial patterning. In this case, while the analysis of E2f6 / mice has indicated that E2F6 participates in the establishment of the correct identities of only the more posterior vertebrae, when E2f6/Bmil compound mutant mice were analyzed, the role of E2F6 in axial patterning was extended to the entire axial skeleton. This suggests that certain roles of E2F6 cannot be uncovered by deletion of E2f6 alone. Similarly, we could speculate that the analysis of E2f6 /;Bmil / MEFs could reveal a function for E2F6 in the cell cycle. Our preliminary analysis of four such MEF lines suggests that the cell cycle defects observed in Bmil / MEFs are exacerbated by deletion of E2f6. We are currently in the process of studying the expression levels of several E2F-target genes including p19ARF to further understand how E2F6 participates in the control of cell proliferation.

The role of Bmi1 in the cell cycle through the regulation of 16NK4A and p19ARF levels appears to be the cause for the cerebellar and hematopoietic defects found in Bmil / mice. In recent years, it has become apparent that Bmi1 is required for the self-renewal and proliferation of both neural and hematopoietic stem cells (NSCs and HSCs) (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003; Zencak et al., 2005). It has also been shown that Bmi1 plays a crucial role in the maintenance and expansion of immature granule cell precursors (Leung et al., 2004).

In the cerebellum, the proliferative defects in both NSCs and the more committed cerebellar precursor cells lead to a progressive loss of cerebellar neurons in Bmil mutant mice that results in the development of ataxia (Leung et al., 2004; Molofsky et al., 2003; van der Lugt et al., 1994). In addition, as a consequence of the HSCs and progenitor cells self-renewal defects, Bmil / mice exhibit several hematopoietic abnormalities which
affect the more immature compartments more severely (Lessard and Sauvageau, 2003; Park et al., 2003; van der Lugt et al., 1994). Significantly, Ink4a/Arf locus deletion partially rescues both the cerebellar and hematopoietic defects of Bmi1−/− mice (Jacobs et al., 1999). It is important to note that while the proliferative defects of NSCs and cerebellar precursors could explain the cerebellar defects and ataxic phenotype of Bmi1−/− mice, other possibilities are also worth investigating. It has been shown that the mutation of certain Hox genes lead to problems in motor neurons which could also lead to motor coordination defects (Lin and Carpenter, 2003).

The E2f6;Bmi1 compound mutant mice develop ataxic gait with a modestly higher penetrance than the Bmi1 mutant mice (70% versus 60%). However, we have not quantified yet the level of ataxia. This could be done in at least three ways. First, we could measure the average time each mutant is able to stay on a rotating rod (Rotarod test). Second, we could determine the latency period for the onset of the ataxia for each mutant. Third, we could determine the extent of the cerebellum defect by histological analysis. We are currently pursuing the last two options.

We are also in the process of examining the hematopoietic defects of E2f6−/−;Bmi1−/− mice. Like the Bmi1−/− mice, the double mutants that survive into adulthood are significantly smaller than their wild-type littermates and exhibit poor health throughout their lives. Eventually, they become very anemic and need to be euthanized. We are performing FACS analysis on the peripheral blood, bone marrow, spleen and thymus of these animals to determine if the hematopoietic defects observed in the Bmi1−/− mice are exacerbated by additional loss of E2F6.
It would be interesting to study the self-renewal capacity of $E2f6^{+/-};Bmi1^{+/-}$ HSCs and NSCs. However, it is important to note that the defects observed in $Bmi1^{+/-}$ mice are so profound that it might prove hard to detect a significant exacerbation of such a strong phenotype.

**Mode of action of E2F6**

Our working model proposes that E2F6 is responsible for recruiting PcG complexes to a subset of target genes. Only a few PcG proteins have been shown to bind DNA in a sequence-specific manner suggesting that the recruitment could be mediated by additional factors (Brown et al., 1998; Dejardin et al., 2005; Mohd-Sarip et al., 2002; Mulholland et al., 2003; Poux et al., 2001; Srinivasan and Atchison, 2004). However, most of this work has been performed in *Drosophila* where the discovery of PcG responsive elements (PREs) has allowed the study of PcG-DNA binding. PREs are large and complex regulatory elements that are able to repress reporter genes in a PcG-dependent manner (Chan et al., 1994; Christen and Bienz, 1994; Simon et al., 1993).

To confirm our model, it would be important to establish that E2F6 1, regulates the expression of some PcG target genes; 2, directly binds the promoters of those genes; and 3, is required for PcG recruitment to DNA and repression.

First, it would be interesting to examine the *Hox* gene expression patterns during the development of $E2f6^{+/-}$ embryos. A few good candidates are those *Hox* genes known to be deregulated in $Bmi1^{+/-}$ mice. These include *Hoxa4, Hoxc4, Hoxa5, Hoxc5, Hoxb6, Hoxc6, Hoxc8* and *Hoxc9* (Brunk et al., 1991; van der Lught et al., 1996; van der Lught et al., 1994; van Lohuizen et al., 1991a; van Lohuizen et al., 1991b). Other good candidates
are the Hox paralog families Hox10 and Hox11, which regulate the identity of the vertebrae in the lumbar and sacral regions of the axial skeleton which are affected in the E2f6−/− mice (Burke et al., 1995). In addition, although we do not observe an upregulation of p19ARF in E2f6−/− MEFs, it would be interesting to analyze the p19ARF levels in E2f6−/−;Bmi1−/− MEFs. A more unbiased approach to identify E2F6 target genes would be to do microarrays using E2f6−/− MEFs. In fact, such an analysis has been recently reported and this study shows that E2F6 represses the transcription of α-tubulin 3 and α-tubulin 7 (TUBA3 and TUBA7) in all organs except in the male gonads (Pohlers et al., 2005). Significantly, no deregulation of E2F target genes was observed in E2f6−/− MEFs in agreement with previous data (chapter 2; Giangrande et al., 2004).

Second, we would like to determine if E2F6 is found at Hox genes as well as the p19ARF promoters. So far, we have not been able to consistently detect E2F6 at any of the promoters tested and the lack of a known E2F6 target gene to use as a positive control has made it impossible to interpret these results (P.J. Iaquinta and J.A. Lees, unpublished results). Recent studies have shown that E2F6 binds and regulates the expression of certain tumor suppressor as well as G1/S-activated E2F target genes (Giangrande et al., 2004; Oberley et al., 2003). Therefore, it would now be possible to use these known E2F6 target genes as positive controls. Due to the little information available on Hox gene regulatory elements in mammals, it is impossible to examine E2F6’s binding to these promoters at the present time.

Finally, it is important to establish E2F6’s role in the recruitment of the PcG proteins to target genes. If we could detect Bmi1 and E2F6 at the p19ARF promoter, we could test this in a very simple manner as we should not detect Bmi1 at this promoter in
E2f6−/− MEFs. An alternative in vitro approach has been used in Drosophila (Mulholland et al., 2003). A recruitment assay was developed using the promoter sequences of the Ubx Hox gene to demonstrate that the GAGA factor, which binds to several GAGA sequences in the Ubx promoter, can recruit the PcG complex to DNA. This DNA was assembled into chromatin and pre-incubated with either buffer alone or GAGA factor prior to the addition of the purified PRC1 core complex. Subsequent Western blot analysis revealed an increase in the PRC1 binding in the presence of GAGA factor. A similar experiment could be envisaged by using E2F binding sites to recruit E2F6 to DNA. One caveat to this experiment is that we would need to purify the mammalian PRC1 complex and demonstrate that it can associate with E2F6.

Taken as a whole, our studies have demonstrated a clear role for E2F6 in PcG function in the anterior-posterior patterning of the developing embryo. The part that E2F6 plays in cell cycle control remains unresolved although preliminary evidence supports its participation in the proliferation control by Bmi1. Therefore, this work has uncovered at least one biological function of E2F6 and, at the same time, has opened the field to a number of interesting questions.
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