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Homeotic transformations of the axial skeleton that accompany a targeted deletion of *E2f6*

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E2F transcription factors play an important role in regulating mammalian cell proliferation. E2F6, the most recently identified E2F family member, is a transcriptional repressor. In an effort to ascertain the *in vivo* biological function of E2F6, we have generated an *E2f6* mutant mouse strain. Mice lacking E2F6 are viable and healthy. Surprisingly, *E2f6*–/– embryonic fibroblasts proliferate normally. However, *E2f6*–/– animals display overt homeotic transformations of the axial skeleton that are strikingly similar to the skeletal transformations observed in polycomb mutant mice. This observation is compatible with the recent finding that endogenous E2F6 and one or more mammalian polycomb proteins are components of the same multiprotein complex. The accumulated evidence suggests that, during development, E2F6 participates in the recruitment of polycomb proteins to specific target promoters.

INTRODUCTION

E2F family members have been intensively studied for their ability to control cellular proliferation. E2F-responsive elements have been identified in genes that play key roles in cell cycle progression, DNA replication and apoptosis (Trimarchi and Lees, 2002). E2F forms a heterodimeric complex containing an E2F and a DP subunit. In mammals, six E2F proteins (E2F1–E2F6) and two DP proteins (DP-1 and DP-2) have been identified. E2F1–E2F5 function as transcriptional activators. Their transcriptional activity is negatively regulated by binding to pRB, the product of the retinoblastoma tumor suppressor protein, and to two related 'pocket proteins', p107 and p130 (Dyson, 1998).

Except for a closely related DNA-binding and dimerization domain, E2F6, the most recently identified E2F family member, is only weakly homologous to the other E2F proteins. It does not bind to pocket proteins, lacks a transactivation domain and is a transcriptional repressor (Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998; Trimarchi *et al.*, 1998). Ectopic overproduction of E2F6 can result in S-phase accumulation and, in quiescent cells, it can delay re-entry into the cell cycle (Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998). Recently, a novel complex that contains E2F6, polycomb proteins and chromatin modifiers was shown to occupy target promoters in G_0 (Ogawa *et al.*, 2002). Thus, it was suggested that one function of E2F6 is to inactivate E2F-dependent genes in quiescent cells.

In another study, consistent with a role for E2F6 in mediating transcriptional silencing, E2F6 was found to associate with a number of mammalian polycomb group (Pc-G) proteins, including Bmi-1, Mel-18, Mph1 and Ring1 (Trimarchi *et al.*, 2001). Pc-G proteins form large multimeric complexes needed to maintain stable transcriptional repression of *Hox* genes (Gould, 1997; Schumacher and Magnuson, 1997). *Hox* genes encode a family of regulatory proteins that are expressed along the antero-posterior axis in the vertebrate embryo (Cillo *et al.*, 2001). According to the 'Hox code' model, the particular combination of *Hox* genes expressed in an individual embryonic segment determines the identity of individual vertebrae (Kessel and Gruss, 1991). In Pc-G mutant mice, the expression boundaries of certain *Hox* genes are shifted anteriorly, resulting in posterior transformations of the axial skeleton. In addition to their role in *Hox* gene regulation, Pc-G proteins also display other activities. For example, Bmi-1 is a critical regulator of proliferation, senescence and apoptosis (Jacobs *et al.*, 1999).

Since most polycomb proteins cannot bind directly to DNA, it is not well understood how they are targeted to specific promoters (Pirrotta, 1999). Because E2F6 exists in a complex with certain polycomb proteins, one wonders whether E2F6 functions to recruit Pc-G complexes to target promoters. If so,

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one might speculate that E2F6 contributes to the specific biological functions of certain Pc-G proteins, e.g. the regulation of developmental patterning, proliferation and/or cellular senescence. However, to date, there have been no biological data to support this model.

We have begun to probe the function of E2F6 *in vivo* by introducing a null mutation into the *E2f6* gene in murine embryonic stem (ES) cells. Mice unable to synthesize the E2F6 transcription factor have now been generated and analyzed. These animals are viable and healthy. However, they appear to be defective in spermatogenesis, and they display homeotic transformations of the axial skeleton. The latter are strikingly similar to the skeletal transformations observed in polycomb knockout mice. Our observations support a model that foresees E2F6 mediating the recruitment of polycomb complexes to specific target promoters during development.

RESULTS

E2F6 is not required for mouse viability

We inactivated the *E2f6* gene in mice using a targeting strategy that replaced exons 3–5 of *E2f6* with a neomycin resistance gene. This deletes the dimerization and DNA-binding domains of E2F6 and is, thus, expected to result in a null mutation (Figure 1). Interbreeding of heterozygous mutant animals yielded *E2f6* null (*E2f6–/–*) animals, from which E2F6 transcripts and protein were absent.

E2f6 null animals were born with a normal Mendelian frequency, showed no obvious external defects and appeared to be healthy for >12 months (not shown). The average growth of *E2f6+/+* and *E2f6–/–* animals over time from birth to 1 year of age was indistinguishable (not shown). Female and male *E2f6* nullizygous animals were fertile and gave rise to healthy offspring. The appearance and size of their internal organs were indistinguishable from those of wild-type (wt) animals. Furthermore, there were no significant differences in the abundance of the various classes of circulating hematopoietic cells between *E2f6–/–* and wt animals (not shown).

A detailed histological analysis of the organs of several 2- to 3-month-old male *E2f6–/–* animals revealed no abnormalities except in the testes (Figure 2; data not shown). The testes of these animals showed a paucity of mature spermatocytes and unusually abundant Leydig cells. In addition, there was an incomplete filling of the ductus epididymidis with spermatocytes (Figure 2). Taken together, these data suggest a defect in spermatocyte development in *E2f6–/–* animals. Notably, these abnormalities failed to affect fertility. Interestingly, in *E2f1–/–* animals, there was also an increase in interstitial Leydig cells and an incomplete filling of tubuli with spermatocytes, and they, too, are fertile (Yamasaki *et al.*, 1996). Presumably, the extent of the defect is insufficient to affect fertility. In this regard, others have shown that rodent spermatogenesis must be reduced by at least 90% to affect fertility (Russell *et al.*, 1990).

Homeotic transformations of the axial skeleton in *E2f6–/–* mice

Mice deficient in certain Pc-G genes display homeotic transformations of the axial skeleton. For example, posterior

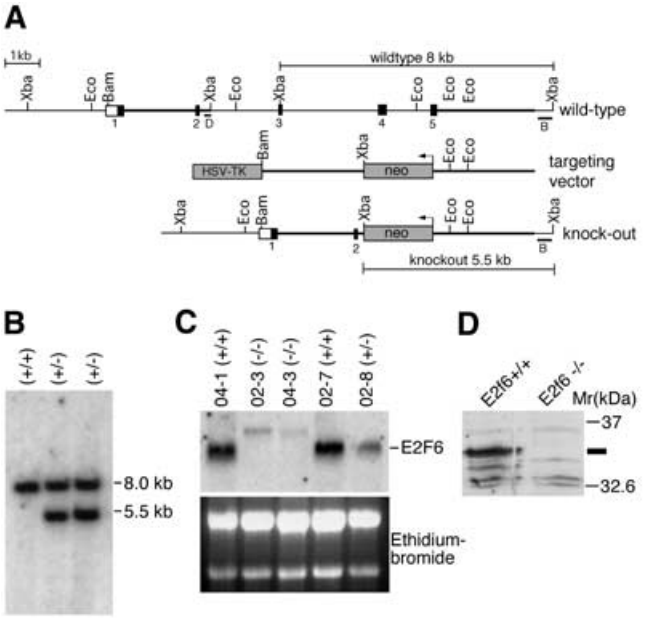

Fig. 1. Targeting strategy and germline transmission. (**A**) Top: genomic organization of the *E2f6* gene. Middle: targeting vector. Bottom: targeted allele. Exons are shown by boxes and introns by lines. The open box represents the non-coding region of exon 1. (**B**) Southern blot of genomic DNA isolated from different ES cell lines digested with *Xba*I and hybridized with probe B [see (A)]. Correct targeting of the left flank was confirmed by hybridizing *Eco*RI-digested genomic ES cell DNA with probe D (not shown). Genotypes are indicated. (**C**) Northern blot analysis to confirm the absence of E2F6 mRNA in MEFs derived from *E2f6–/–* animals. Genotypes are indicated. (**D**) Western blot analysis of lysates from *E2f6+/+* and *E2f6–/–* MEFs to confirm the absence of the E2F6 protein in animals.

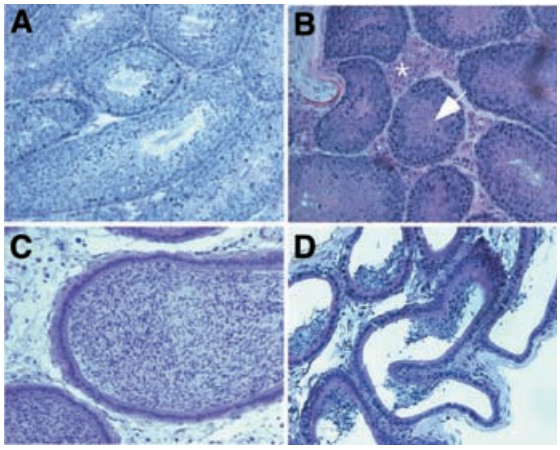

Fig. 2. Testicular abnormalities in *E2f6–/–* animals. Histological sections (hematoxylin and eosin) of testes (**A** and **B**) and ductus epididymidis (**C** and **D**) of wt (A and C) and *E2f6–/–* (B and D) animals. An increase of interstitial Leydig cells (asterisk) and of eosinophilic material (residual bodies) in the seminiferous tubuli (arrow) is evident in *E2f6–/–* testes (B). Incomplete filling of the ductus epididymidis with spermatocytes in *E2f6–/–* animals (D). In (C), a wt ductus epididymidis is shown at the same magnification as (D).

transformations of the axial skeleton have been observed in mice deficient for the Bmi-1 polycomb protein, a protein known to associate with E2F6 (van der Lugt *et al.*, 1994). Like Bmi-1 and

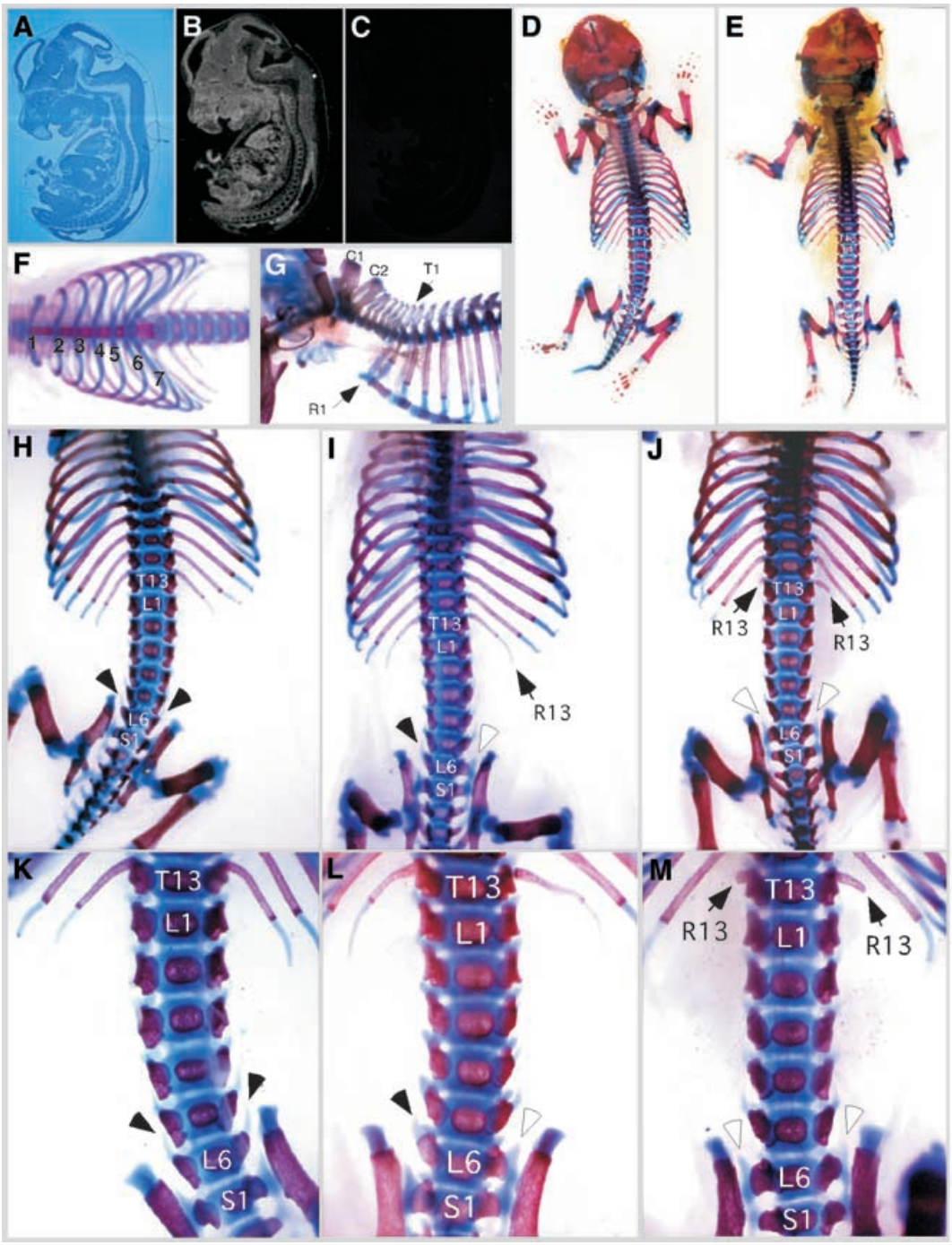

Fig. 3. Skeletal transformations of *E2f6–/–* animals. (**A–C**) *In situ* hybridization of E2F6 in 14.5-day-old mouse embryos: (A) Bright field; (B) E2F6 antisense probe; (C) E2F6 sense probe (control). (**D–M**) Alcian Blue (cartilage) and Alizarin Red (bone) staining of cleared newborn animals. Overview of the skeletons of wt (D) and *E2f6–/–* (E) skeletons. Ventral view of the thoracic region (F) and lateral view of the cervical region (G) of *E2f6–/–* animals. Note the normal number and attachment of the ribs, and normal size and appearance of the C1 and C2 vertebrae. Dorsal view of the thoraco-lumbo-sacral region of wt (H) and *E2f6–/–* (I and J) animals. In (J), the thirteenth ribs are degenerated (arrows). Moreover, the sixth lumbar vertebra (L6) has been transformed into the first sacral vertebra (S1) on one side (I and L) or two sides (J and M) (white arrowheads). Black arrowheads point to normal L6 vertebral elements. The skeletal transformations of *E2f6–/–* animals can also be seen in the ventral view of the lumbo-sacral region (L and M). The formations of sacro-iliac joints at L6 are evident in (L) and (M). Compare with the lumbo-sacral region of a wt animal (K).

other polycomb group genes, E2F6 is ubiquitously expressed at low levels in mouse embryos, as shown by *in situ* hybridization on sections from wt 14.5 d.p.c. embryos (Figure 3A–C). We were interested in the skeletal development of *E2f6–/–* animals and compared skeletal preparations of *E2f6–/–* embryos and newborn mice with those of wt animals (Figure 3D–M). These

Table I. Skeletal abnormalities in *E2f6–/–* animals

Phenotype		Penetrance of skeletal abnormalities (%)		
		E2f6+/+ (*n* = 16)	*E2f6+/–* (*n* = 5)	*E2f6–/–* (*n* = 16)
T13 > L1[a]	One side	0	0	19
	Both sides	0	0	6
L6 > S1	One side	0	0	44
	Both sides	0	40	31

[a]Demonstrated by rudimentary ribs.

analyses revealed abnormalities along the antero-posterior skeletal axis of the knockout mice. Specifically, we observed a transformation of the sixth lumbar vertebra (L6) to the first sacral vertebra (S1), indicated by abnormally located sacro-iliac joints at L6 (Figure 3I, J and L, M). There was also a transformation of the thirteenth thoracic vertebra (T13) to the first lumbar vertebra (L1), as demonstrated by degeneration of the ribs that are normally associated with T13 (Figure 3J and M). The aforementioned changes can be interpreted as posterior transformations, because the T13 and L6 vertebrae acquire morphological characteristics of adjacent posterior vertebrae. In contrast to *Bmi-1*-deficient mice, which reveal more widespread skeletal transformations involving the atlas and multiple ribs (van der Lugt *et al.*, 1994), the *E2f6–/–* transformations were restricted to the lumbar and sacral regions (Figure 3F and G). No other gross abnormalities of the skeletons were observed (Figure 3D and E). As indicated in Table I, in some *E2f6–/–* animals, there were asymmetric skeletal transformations, which presented on only one side of the body. Also, the penetrance of the transformations within litters was incomplete, similar to what was observed for many polycomb nullizygous mice, e.g. *bmi-1–/–* mice (van der Lugt *et al.*, 1994). Skeletal transformations were also noted in heterozygous *E2f6+/–* animals, albeit at a lower frequency (see Table I).

Proliferation of *E2f6* mutant and wt fibroblasts

In addition to their role in *Hox* gene regulation during development, polycomb proteins are also involved in the control of cell cycle proliferation and senescence. The proliferation defects of *bmi-1–/–* cells result, at least in part, from the upregulation of the $p16^{INK4a}$ and $p19^{ARF}$ tumor suppressor genes (Jacobs *et al.*, 1999), suggesting that transcriptional repression of these genes is normally mediated by Bmi-1-containing polycomb complexes. Since E2F proteins have also been implicated in the regulation of $p19^{ARF}$ expression (DeGregori *et al.*, 1997; Bates *et al.*, 1998), it was speculated that E2F6 could mediate the polycomb-dependent repression of the *INK4a* locus (Trimarchi and Lees, 2002). Moreover, E2F6, when overproduced, can restrain exit from quiescence (G_0) in mouse embryonic fibroblasts (MEFs) (Gaubatz *et al.*, 1998), and, in human cells, a complex that contains E2F6 and chromatin modifiers was recently shown to occupy target promoters in G_0 (Ogawa *et al.*, 2002).

To directly assess the role of endogenous E2F6 in cell proliferation, we cultured MEFs from *E2f6* null and littermate wt embryos. Surprisingly, proliferation of both sets of MEFs was indistinguishable

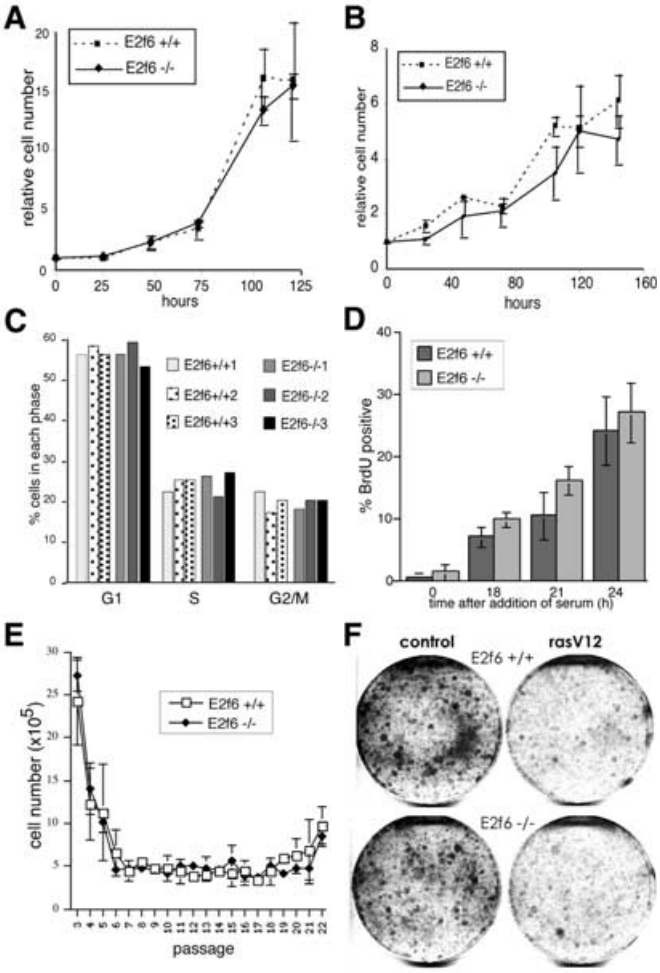

Fig. 4. Properties of *E2f6* null fibroblasts. (**A**) Proliferation of wt and *E2f6–/–* MEFs in medium with 10% FCS. Growth values represent the average of three independently isolated wt MEF cultures and three different *E2f6–/–* MEF cultures isolated from littermate embryos. Error bars represent the standard deviation. (**B**) Growth of *E2f6–/–* and *E2f6+/+* MEFs under low serum conditions [for details see (A)]. (**C**) Cell cycle distribution of independently isolated asynchronously growing *E2f6–/–* MEF lines. (**D**) Serum starvation and re-entry into the cell cycle of *E2f6+/+* and *E2f6–/–* MEFs was analyzed by BrdU incorporation. (**E**) Wild-type and *E2f6–/–* MEFs were passaged according to a standard 3T3 protocol. Cell numbers were determined at each passage. (**F**) Premature senescence of *E2f6+/+* and *E2f6–/–* MEFs: *E2f6+/+* and *E2f6–/–* MEFs were infected with either a control retrovirus or a RasV12-expressing retrovirus together with a puromycin resistance gene. Dishes were stained with Crystal Violet following 10 days of selection with puromycin.

both in normal and low serum-containing medium (Figure 4A and B), and, by FACS analysis, neither cell strain revealed a significant cell cycle abnormality (Figure 4C). Moreover, the nullizygous cells entered and exited G_0 normally (Figure 4D). We conclude that E2F6 is neither required for growth arrest following serum removal nor for timely re-entry into the cell cycle from G_0.

Senescence of *E2f6–/–* and wt fibroblasts

Bmi-1 knockout fibroblasts undergo premature senescence stimulated by the upregulation of $p16^{INK4a}$ and $p19^{ARF}$ (Jacobs *et al.*,

1999). To determine whether *E2f6* nullizygous MEFs have a similar phenotype, we analyzed their longevity in culture. Like wt MEFs, proliferation of the mutant cells began to slow after passage 5 and, eventually, most cells senesced. Moreover, spontaneous immortalization was observed after passage 20, as was the case for the wt cells. This demonstrates that loss of E2F6 is not sufficient to result in premature senescence (Figure 4E). In addition, retrovirus-mediated expression of oncogenic Ras (RasV12) resulted in senescence of both wt and *E2f6–/–* MEFs (Figure 4F), demonstrating that E2F6 is also not required for the RasV12-induced premature senescence process.

DISCUSSION

We have generated E2F6-deficient mice to establish the role of E2F6 in cellular proliferation and mouse development. Surprisingly, we found that E2F6 is dispensable for proliferation and quiescence of MEFs. This was unexpected as it was recently shown that an E2F6 complex that contains chromatin modifiers occupies promoters of cell cycle genes in G_0 cells (Ogawa *et al.*, 2002). Although our findings seem to contradict the conclusion that E2F6 is important for quiescence, there are several possible explanations for the seeming discrepancy between these two studies. First, it is likely that E2F6 is normally involved in quiescence, but that in its absence its function is provided by at least one other protein. In this regard, the E2F6 complex purified by Ogawa *et al.* (2000) contains several distinct DNA-binding activities in addition to E2F6. Thus, in the absence of E2F6, the complex might be targeted to certain cell cycle promoters through its Mga/Max DNA-binding subunit. Therefore, it will be critical to determine whether the complex is able to bind to cell cycle promoters in *E2f6–/–* cells and, if so, how it is targeted to the relevant promoters. Secondly, it is possible that in the absence of E2F6, E2F–pocket protein complexes compensate for its function in G_0. For example, previous experiments have shown that E2F4–p130 complexes occupy promoters of cell cycle genes in quiescence (Takahashi *et al.*, 2000; Wells *et al.*, 2000). Thus, in future, it will be important to characterize E2F–pocket protein complexes in E2F6-deficient cells in detail. Finally, effects on target gene expression in *E2f6–/–* cells may be too subtle to abolish entry into or exit from quiescence. It will be interesting to carefully analyze expression of cell cycle genes in E2F6-deficient cells.

Although E2F6 is not essential for quiescence of mouse fibroblasts, we found that it is critical for developmental patterning. *E2f6–/–* mice are born with posterior homeotic transformations of the axial skeleton. The skeletal transformations in *E2f6–/–* mice are strikingly similar to those observed in mice deficient in the Bmi-1, Mel-18, Rae28 and M33 polycomb proteins (van der Lugt *et al.*, 1994; Akasaka *et al.*, 1996; Core *et al.*, 1997; Takihara *et al.*, 1997). However, while these polycomb knockout animals present with transformations along the complete antero-posterior axis of the skeleton, homeotic transformations in *E2f6–/–* mice are restricted to the lumbar and sacral regions of the vertebral column. Posterior transformations can arise when *Hox* genes are mis-expressed in an anterior position where they are normally transcriptionally repressed by polycomb proteins. Therefore, together with the previous observations that E2F6 is a component of the polycomb complex (Trimarchi *et al.*, 2001; Ogawa *et al.*, 2002), our findings suggest that E2F6 is normally required for the polycomb-dependent transcriptional repression of a subset of *Hox* genes. However, we can not exclude a more indirect role for E2F6 in the regulation of Hox function. Further work will be necessary to define the role of E2F6 in the regulation of the spatial and temporal expression of selected *Hox* genes.

In conclusion, our data demonstrate that the function of E2F6 is distinct from that of other E2F family members. We propose that E2F6 acts as a sequence-specific DNA-binding subunit of certain Pc-G complexes that mediate *Hox* gene regulation during development.

METHODS

Gene targeting and generation of *E2f6* mutant animals. An *Xba*I–*Hin*dIII restriction fragment from mouse E2F6 cDNA was used to screen a Lambda Fix II library derived from 129Sv genomic DNA. *E2f6* exons were identified by Southern blotting and sequence analysis. A 3 kb *Bam*HI–*Xba*I fragment containing exon 1 and intron 1 sequences and a 3 kb *Hpa*I–*Not*I fragment containing intron 4 sequences were inserted into the targeting vector, pPNT, which was electroporated into 129/Sv J1 ES cells. Cells were selected in medium containing 300 μg/ml G418 and 2 μM gancyclovir. Two correctly targeted clones were obtained from 150 selected ES cell clones. They were injected into C57BL/6 and Balb/c blastocysts to generate chimeric animals that passed the mutant *E2f6* allele to their offspring, as determined by PCR on DNA isolated from tail biopsies. Sequences of primers used for PCR genotyping of E2f6 animals are available upon request.

Primary fibroblasts. MEFs were isolated from 13.5 d.p.c. embryos. Growth was analyzed by plating MEFs in triplicate in 24-well plates. At the indicated time points, cells were fixed and stained with Crystal Violet. The dye was extracted with 10% acetic acid and the optical density was determined. Proliferation of three independently isolated wt and three *E2f6* null fibroblast lines was compared in media containing 10 and 2% serum. For serum-starvation/release experiments, MEFs were growth arrested by incubation in medium containing 0.2% fetal bovine serum for 60 h. Cells were then stimulated with 10% fetal bovine serum and pulsed with 10 μM bromodeoxyuridine (BrdU). BrdU incorporation was analyzed by immunostaining. For retroviral expression, MEFs were infected with a RasV12-expressing retrovirus or with a control virus. Two days after infection, MEFs were selected with 1.5 μg/ml puromycin. After 10 days, cells were fixed and stained with Crystal Violet. For immunoblotting, MEFs were lysed, and aliquots of these lysates (500 μg) were separated by SDS–gel electrophoresis and transferred to Immobilon-P. E2F6 was detected with affinity-purified polyclonal antiserum (C10) raised in rabbits against the keyhole limpet hemocyanin-coupled peptide C-10 (CPEKEDEPPQ).

Histology, skeletal preparations and *in situ* hybridization. Spleen, thymus, pancreas, stomach, duodenum, colon, kidney, lung, heart, skeletal muscle, cerebellum and testis were harvested from 3-month-old male animals and fixed in 60% ethanol, 30% chloroform, 10% acetic acid. Paraffin sections (6 μm) were prepared and stained with hematoxylin and eosin. Skeletal analysis was performed with E2f6 mice that were bred for 3–4 generations to the C57/Bl6 strain. E16.5 embryos and newborn pups were stained with cartilage-specific Alcian Blue and bone-specific Alizarin Red. Soft tissue was cleared with alkali. For

in situ hybridization, 14.5-day-old embryos were embedded in Tissue-Tek, frozen on the surface of a liquid nitrogen pool. Sectioning, post-fixation, preparation of a single-stranded DNA probe and hybridization were performed as described previously (Millauer *et al.*, 1993). Slides were coated with Kodak NTB2 film emulsion and exposed for ~7 days.

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