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A Role for E2F6 in the Restriction of Male-Germ-Cell-Specific Gene Expression

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Summary

E2F transcription factors play a pivotal role in the regulation of cellular proliferation and can be subdivided into activating and repressing family members [1]. Like other E2Fs, E2F6 binds to E2F consensus sites, but in contrast to E2F1-5, it lacks an Rb binding domain and functions as an Rb-independent transcriptional repressor [2–5]. Instead, E2F6 has been shown to complex with Polycomb (PcG) group proteins [6, 7], which have a well-established role in gene silencing. Here, we show that E2F6 plays an unexpected and essential role in the tissue specificity of gene expression. E2F6-deficient mice ubiquitously express the α-tubulin 3 and 7 genes, which are expressed strictly testis-specifically in control mice. Like an additional E2F6 target gene, Tex12, that we identified, tubulin 3 and 7 are normally expressed in male germ cells only. The promoters of the α-tubulin and Tex12 genes share a perfectly conserved E2F site, which E2F6 binds to. Mechanistically, E2F6-mediated repression involves CpG hypermethylation locking target promoters in an inactive state. Thus, E2F6 is essential for the long-term somatic silencing of certain male-germ-cell-specific genes, but it is dispensable for cell-cycle regulation.

Results and Discussion

We used a gene-targeting strategy to mutate the mouse E2F6 gene in embryonic stem (ES) cells. Translation termination codons were introduced in all three open reading frames (ORFs) after codon 58, and the genomic sequences encoding the DNA binding domain were replaced with a neomycin resistance gene (Figure 1A). Correctly targeted clones were identified by Southern blotting (Figures 1B and 1C) and used to generate E2F6 mutant mouse strains. E2F6$^{-/-}$ animals were viable and fertile, were born at the expected Mendelian frequency, and were of similar size and weight to their wild-type litter mates (data not shown). E2F6$^{-/-}$ mice were used to isolate mouse embryonic fibroblasts (MEF). Western-blot analysis with two independent anti-E2F6 antibodies identified a major immunoreactive band, undetectable in extracts derived from E2F6$^{-/-}$ animals, of the expected size in extracts from wild-type MEFs and from mouse embryos (Figure 1D). We conclude that we have created a null allele of E2F6.

To identify target genes that strictly depend on E2F6 for their expression, we compared the expression profiles of wild-type (wt) and E2F6$^{-/-}$ proliferating and quiescent MEFs by microarray analysis employing Affymetrix gene chips. This identified a number of genes that were misexpressed in $^{-/-}$ MEFs. Here, we concentrate on the analysis of two genes, α-tubulin 3 (TUBA3) and α-tubulin 7 (TUBA7), that display similar DNA sequences and encode identical proteins [8]. TUBA3 and TUBA7 expression was virtually undetectable both in proliferating and in quiescent control fibroblasts, and both genes were strongly expressed in E2F6$^{-/-}$ MEFs (see Table S1 in the Supplemental Data available with this article online). To validate these data obtained by microarray analysis, we analyzed mRNAs derived from MEFs of E2F6$^{-/-}$ and wild-type (wt) animals by RNase protection assay. TUBA3 and TUBA7 mRNAs were undetectable in wt MEFs and strongly upregulated in proliferating and G_0 E2F6$^{-/-}$ MEFs (Figure 2A). α-tubulins are highly expressed in many cell types, and subtype-specific antibodies do not exist, which impedes a specific analysis of TUBA3 and TUBA7 proteins. Immunofluorescence analysis with a pan-α-tubulin antibody revealed neither quantitative nor qualitative differences in the microtubuli structure of E2F6$^{-/-}$ versus control MEFs (data not shown).

Expression of the TUBA3 and TUBA7 genes was originally identified in testis, and these proteins represent the only tubulin subtypes exclusively expressed in the male gonads [9]. Despite a high degree of conservation between different tubulin subtypes, specific needs for particular tubulin subtypes in particular cell types appear to exist [10]. Probes that detect eight less-restricted or ubiquitously expressed tubulin subtypes were also present on the microarray; these genes, however, were expressed at similar levels in control and E2F6$^{-/-}$ MEFs (Table S1). This suggested that E2F6 may be responsible for specifically silencing TUBA3 and TUBA7 gene expression in organs other than testis, thereby enabling a tissue-specific expression pattern for these genes. To test this further, we prepared RNAs from various organs derived from both wt and E2F6$^{-/-}$ mice and analyzed the expression of the TUBA3 and

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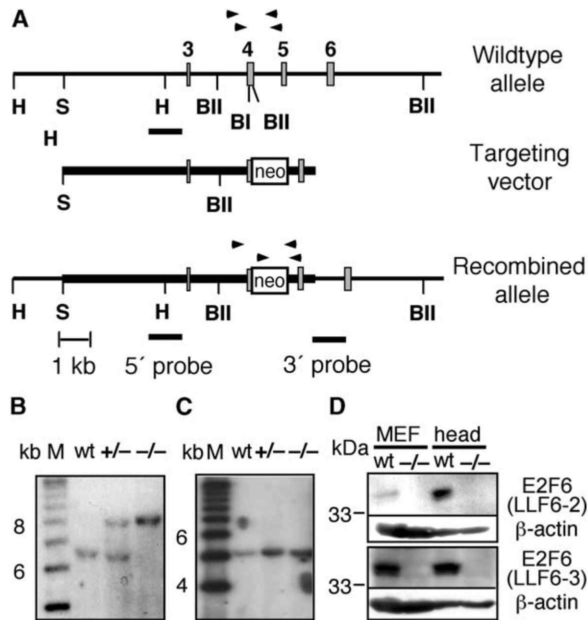

Figure 1. Targeted Disruption of the Murine *E2F6* Gene

(A) Schematic of the partial wild-type *E2F6* allele, the targeting vector used, and the recombined *E2F6* allele. Exons 3–6 are indicated as filled boxes, and intron sequences as lines. Exon 4 encodes the DNA binding domain and was replaced by the neomycin resistance cassette (neo). The 5′ arm of the targeting vector is a 7.5 kb SmaI/Bst1107I restriction fragment. The 3′ arm was generated by PCR (H, HpaI; S, SmaI; BI, Bst1107I; and BII, BglII). The positions of oligonucleotide primers used for the genotyping of animals are indicated by arrowheads.

(B) Homologous recombination in ES cells was controlled by Southern analysis of BglII-digested genomic DNA with the indicated 3′ probe.

(C) Positive clones were validated by hybridization of HpaI-digested DNA with the 5′ probe to control for single-copy integration of the long arm.

(D) To confirm the absence of the E2F6 protein in animals, we performed Western-blot analysis of extracts from E2F6$^{-/-}$ and wt MEFs and embryo heads with two independent antibodies (kindly provided by J. Lees, Massachusetts Institute of Technology) recognizing different epitopes of E2F6.

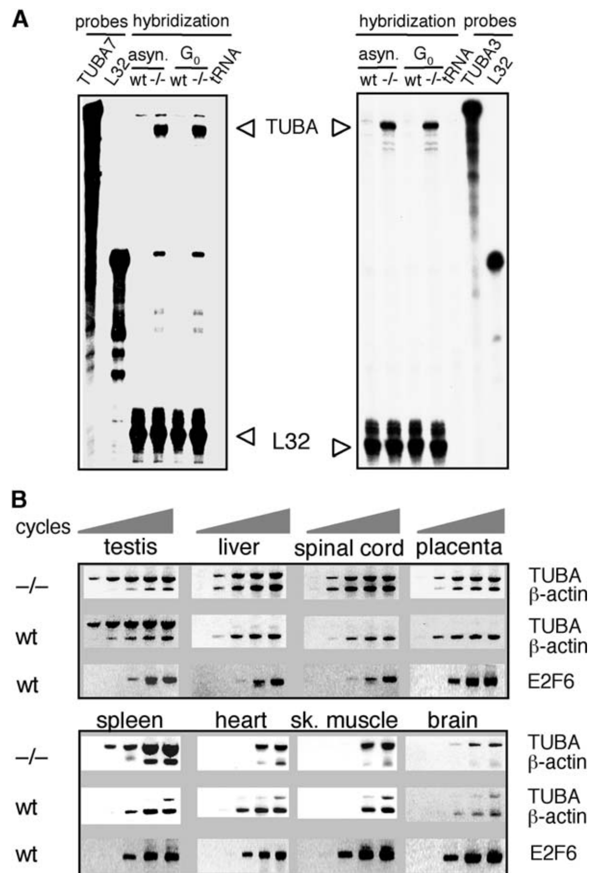

Figure 2. E2F6 Is Required for the Tissue-Specific Expression of the TUBA3/7 Genes

(A) RNase protection assays (RPA) for TUBA7 (left) and TUBA3 (right) expression in MEFs. Experiments were performed with extracts from both asynchronously (asyn.) or growth-arrested (G_0) fibroblasts. L32 was included as an internal control, and tRNA as a negative control. Undigested probes as indicated. A representative example of several experiments with independent preparations of MEFs is shown.

(B) E2F6 deficiency results in the derepression of tissue-specific TUBA3 and TUBA7 gene expression. RNA was prepared from the indicated organs (sk. muscle = skeletal muscle) of both E2F6-deficient (–/–) and wt animals. Combined analysis of TUBA3 and TUBA7 transcripts was performed by semiquantitative RT-PCR. The triangles indicate increasing numbers of rounds of PCR amplification (cycles). All primer pairs were selected so that sense and antisense primers anneal to sequences on adjacent exons to control for the amplification of genomic DNA. A β-actin internal control PCR was included in all samples. Expression of E2F6 mRNA as indicated.

TUBA7 genes (Figure 2B). In control animals, TUBA3 and TUBA7 expression was readily detected in testis (top left) but absent or only marginally detectable in other organs, such as the liver, spinal cord, placenta, spleen, heart, skeletal muscle, and brain. At the same time, all of these tissues, including testis, concomitantly expressed E2F6. In E2F6$^{-/-}$ mice, TUBA3 and TUBA7 expression had lost its tissue specificity, and high levels of transcripts were detected in all organs analyzed. In testis, the expression levels of TUBA3 and TUBA7 were similar in control and E2F6$^{-/-}$ animals (in relation to the β-actin control). This might indicate that the expression of TUBA3 and TUBA7 in normal testis is achieved by preventing the E2F6-mediated repression. We conclude from this set of experiments that E2F6 is essential for the repression of the TUBA3 and TUBA7 genes in nontesticular tissues, implying a role for E2F6 in establishing tissue-specific patterns of gene expression.

A recent study found the TUBA3/7 genes not to be expressed in somatic cells of the testis but rather in male germ cells, namely the spermatogonia that constitute the self-renewing, mitotic germ cells of the testis [11]. The same study also described several other spermatogonia-expressed genes that were not represented on the Affymetrix microarray used here. Out of this set of genes, we found the Tex12 gene (for testis-expressed 12) to be also derepressed in E2F6$^{-/-}$ MEFs (Figure 3A). In contrast, the expression of several other spermatogonia-expressed genes was absent from both

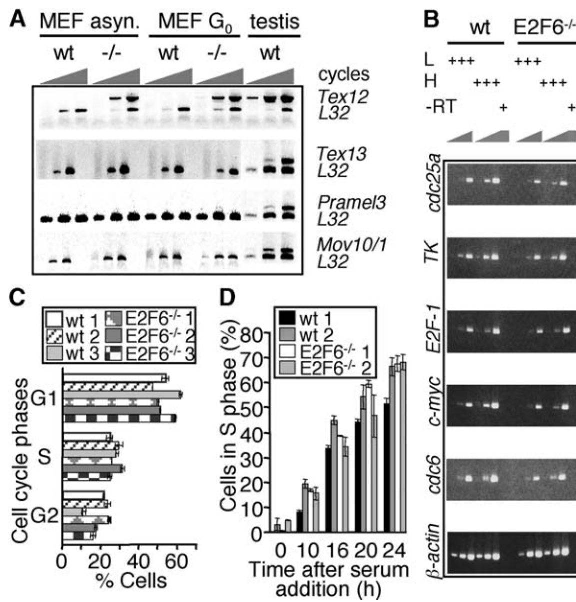

Figure 3. E2F6 Is Required for the Somatic Repression of Germ-Cell-Specific Gene Expression but Is Dispensable for the Regulation of Classical E2F Response Genes

(A) The indicated germ-cell-specific genes were analyzed by semiquantitative RT-PCR for their expression in asynchronously (asyn.) growing and G_0-arrested wt and E2F6$^{-/-}$ MEFs or in testis.

(B) Expression of the indicated E2F target genes in asynchronously growing cells was monitored by semiquantitative RT-PCR analysis. Low (L) and high (H) concentrations of template cDNAs were used along with increasing numbers of rounds of PCR amplification (triangles) as indicated. Amplification of β-actin was used as a positive control. As a negative control, the reverse transcriptase was left out of the template reaction mix (-RT).

(C) Cell-cycle distribution of asynchronously growing MEFs. Results of three independently prepared MEF lines are shown. Experiments were performed at least three times. Columns indicate mean values with standard deviations shown (error bars).

(D) MEFs were synchronized by serum starvation, and their ability to reenter the cell cycle after serum readdition was analyzed by BrdU incorporation over time. Results of two independently MEF lines are shown. Experiments were performed at least three times. Columns indicate mean values with standard deviations shown (error bars).

E2F6$^{-/-}$ and wt MEFs but well detectable in testis (Figure 3A; data not shown). The Tex12 gene encodes a protein of unknown function with an estimated size of 15 kDa and a 90% conservation between human and mouse. Thus, more specifically, E2F6 appears to silence a subset of germ-cell-specific genes in somatic cells.

E2F6 has previously been shown to physically associate with a number of canonical E2F-responsive genes such as Cdc6, E2F1, or TK in vivo [6, 12]. However, in this study none of these genes were found deregulated either in asynchronously proliferating or in serum-starved E2F6$^{-/-}$ MEFs (Figure 3B; data not shown). In addition, a further set of genes (including BRAC1, RbAP48, and HP1α) that had also been shown by chromatin immunoprecipitation (ChIP) to be occupied by E2F6 in vivo was similarly expressed in wt and E2F6$^{-/-}$ fibroblasts (data not shown). Consistent with these data, our microarray analysis also did not identify typical E2F target genes to be deregulated in E2F6-deficient MEFs. Therefore, our data suggest that E2F6 is not required for the regulation of an E2F-driven program of cell-cycle-dependent gene expression. This is further supported by the finding that the overall cell-cycle control of E2F6$^{-/-}$ MEFs is unperturbed when compared to wt MEFs (Figure 3C; data not shown). Also, both cell types reentered the cell cycle after serum starvation with similar kinetics (Figure 3D), which is consistent with a previous study [13]. In conclusion, E2F6 is not required for the correct expression of E2F-responsive cell-cycle genes.

The TUBA3 and TUBA7 promoters are well conserved and lack canonical TATA sequences (Figure 4A). As shown by RNase protection assay, the TUBA3 promoter contains two major transcription start sites at positions −103 and −71 in relation to the translation initiation codon (Figure 4A). This is consistent with the sequence of EST clones found in the National Center for Biotechnology Information (NCBI) databank. Both promoters contain conserved transcription factor binding sites such as CCAAT (C/EBP, NF-Y) and Myb elements that have been shown to serve as positive sites in the E2F-responsive cdc2 and cyclin B gene promoters [14]. The TUBA3 and TUBA7 promoters also contain a single E2F consensus binding site that is located in a conserved position at approximately 180 bp upstream of the major transcription start sites. This E2F site is identical in the mouse TUBA3 and TUBA7 genes, in the testis-specific human TUBA3/7 homolog, TUBA2 [15], and in two primate TUBA3/7 homologs that were identified in the NCBI databank by homology search (Figure S1). The Tex12 gene also contains this very E2F site (at position −98 in relation to the transcription start site), and, interestingly, this particular binding site perfectly reflects the high-affinity E2F6 binding element that we identified in a binding site selection assay with bacterially expressed E2F6 and DP1 proteins (Figure 4B). This high-affinity binding site is characterized by a core element that in all of the 28 distinct clones we identified in the selection assay displays the sequence TCCCGC followed by a C or G. Also, the core is typically flanked on one or both sides by a poly A or poly T stretch (Figure S2).

To determine whether the E2F consensus site is of functional significance for the E2F6-mediated regulation of the α-tubulin promoters, we first analyzed the ability of E2F6 to interact with this site in DNA bandshift assays. With extracts from HeLa cells, the TUBA7 E2F site was recognized by two endogenous protein complexes that contain heterodimers of DP1 and either E2F1, E2F3, or E2F4 ("free E2F"), as shown by "supershift" experiments (Figure 4C; data not shown). In contrast, no binding of endogenous E2F6 protein was detected by this approach, although recombinant HA-tagged E2F6 readily binds to the wt (Figure 4C, lane 2) but not to a mutant TUBA3 E2F recognition sequence (lane 3) and can be "supershifted" by an anti-HA antibody (lane 4). Similarly, overexpressed E2F4 binds to the wt but not the mutant probe and comigrates with the slowest of the "free E2F" complexes.

E2F6 is contained in large complexes [6]. DNA binding of endogenous E2F6 has not been demonstrated in

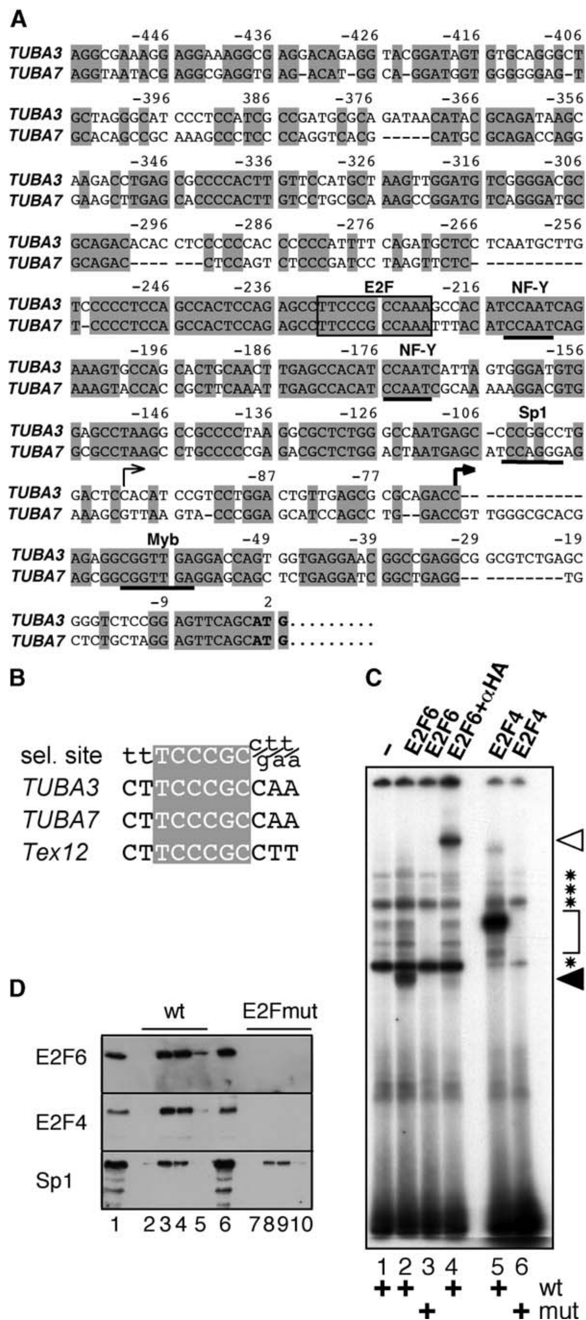

Figure 4. Testis-Specific E2F6 Target Genes Contain a Conserved E2F Site that Serves as a High-Affinity E2F6 Binding Site

(A) Promoter sequences of the TUBA7 and TUBA3 genes were retrieved from the NCBI databank (accession numbers NT_085812 and AC140324). Minor (arrow) and major (bold arrow) transcription start sites were determined by ribonuclease protections assay (RPA; not shown). Numbering is according to the TUBA3 sequence, with −1 being the first nucleotide upstream of the translation initiation codon (ATG). Conserved NF-Y, Sp1, and Myb binding sites are underlined, and the E2F site is boxed.

(B) The E2F site identified in a binding site selection assay as a high-affinity E2F6 binding element (sel. site) is aligned with the E2F sites from the TUBA3, TUBA7, and Tex12 promoters. Identical nucleotides among all sites are shaded (also see Figure S2 for details on the binding site selection assay).

(C) Gel mobility shift assays were performed with whole-cell extracts from HeLa cells transfected with control (lane 1), HA-tagged (HA) E2F6 (E2F6, lanes 3–4), or E2F4 (lanes 5 and 6) expression plasmids. The E2F binding site of the TUBA7 promoter was used as probe. In lanes 3 and 6, the E2F site was mutated (TTC CCGCCAAA to TTCTTAACAAA). "Free E2F" complexes are indicated by a bracket. The E2F6-specific complex (closed arrowhead), its "supershifted" form (open arrowhead), and nonspecific complexes (asterisks) are indicated.

(D) Aliquots of the same wild-type and mutated E2F oligonucleotides used in (B) were covalently coupled to a sepharose column. The column was loaded with extracts from HEK293 cells under binding conditions. After being washed (lanes 2 and 7), bound proteins were eluted with high-salt buffer (lanes 3–5 and 8–10) and analyzed by immunoblot with specific antibodies as indicated.

a band shift assay to date, possibly owing to the instability of such complexes under electrophoresis conditions. To overcome this limitation, we covalently coupled double-stranded DNA oligonucleotides containing the TUBA7 E2F binding site (or a mutant site) to sepharose and used this for affinity chromatography. The affinity-chromatography columns were loaded with whole-cell extracts (Figure 4D, lane1) and washed. Bound proteins were eluted with high salt and analyzed by Western blotting. Under these experimental conditions, an interaction of endogenous E2F6 with the wt (lanes 2–5) but not the mutant (lanes 7–10) TUBA7 E2F site can readily be observed. In addition, E2F4 also binds to the column in an E2F-site-specific manner, but the unrelated transcription factor Sp1 does not. Thus, both overexpressed and endogenous E2F6 can specifically bind to the TUBA3 E2F site.

Given that several E2F family members are able to bind to the TUBA3 E2F site in vitro, we next analyzed the occupancy of this site in vivo. Employing the genomic footprinting methodology [16], we show that at times when this promoter is active in E2F6$^{-/-}$ MEFs, the E2F site is protected, indicating the association of endogenous transacting factors with this DNA element (Figure 5A). Also, additional footprints protecting a CCAAT box and a predicted Sp1 binding site clearly indicate that the TUBA3 promoter (including the E2F site) is bound by sequence-specific factors in vivo. In contrast, none of these sites were occupied to a significant degree in wt MEFs, and, moreover, no other protected sequence elements could be detected. In addition, the hypersensitive site observed in E2F6$^{-/-}$ MEFs at position −148 was absent from the repressed promoter. These data indicate that in wt fibroblasts, when the TUBA3 gene is silent, the promoter is not occupied to an appreciable extent by transacting factors but that it "opens up" in E2F6$^{-/-}$ MEFs, leading to factor accessibility that in turn correlates with active transcription. Consistent with the lack of E2F site occupancy of the repressed TUBA3 promoter, we were unable to detect significant E2F6 (or E2F1) binding to the TUBA3 promoter in wt fibroblasts by ChIP (Figure 5B). However, we could readily detect binding of the transactivating factors E2F1 and Sp1 to the TUBA3 gene promoter in E2F6$^{-/-}$ MEFs (Figure 5B), which is again in close agreement with the in vivo footprinting pattern and the activity of the TUBA3 promoter in these cells.

The finding that the TUBA3 promoter is obviously not

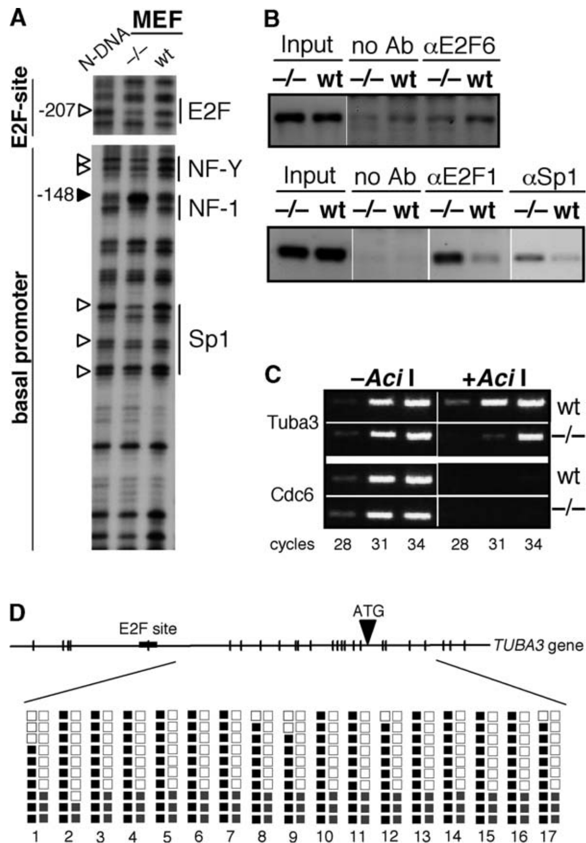

Figure 5. TUBA3 Promoter Occupancy and Protein Interaction In Vivo

(A) Genomic footprinting of the TUBA3 promoter in wt and E2F6$^{-/-}$ MEFs. E2F6$^{-/-}$ and wt MEFs were treated with dimethylsulfate (DMS) to analyze the occupancy of transcription factor binding sites in vivo. Modified genomic or naked (N-DNA) DNA was subjected to piperidine cleavage that induces strand brakes at DMS-methylated guanosine residues. The sense strand of the TUBA3 promoter was analyzed by ligation-mediated PCR (LMPCR) starting from –67 in relation to the ATG start codon. Transcription factor binding sites for E2F, NF-Y (C/EBP), NF-1, and Sp1 as well as protected (open arrowheads) and hypermethylated guanosine residues (filled arrowheads) are indicated.

(B) In vivo binding of transcription factors to the mouse TUBA3 promoter. Extracts were prepared from formaldehyde-treated E2F6$^{-/-}$ and wt MEFs and, after sonification, crosslinked chromatin was precipitated with antibodies directed against E2F1, E2F6, and Sp1 or in the absence of antibodies (no Ab). After de-crosslinking, TUBA3 promoter fragments were either amplified from the precipitated material or from the pre-precipitation input material (input).

(C) Restriction enzyme/PCR analysis of the methylation state of the TUBA3 promoter in E2F6$^{-/-}$ and wt MEFs. The methylation status of the TUBA3 and Cdc6 genes was analyzed by restriction enzyme PCR. Genomic DNA from E2F6$^{-/-}$ and wt MEFs was incubated with the methylation-sensitive restriction endonuclease AciI, and then promoter sequences were PCR amplified. The amplified TUBA3 and Cdc6 promoter fragments each contain two AciI recognition sites.

(D) E2F6-dependent switch in TUBA3 promoter methylation. The individual CpG methylation status of the TUBA3 promoter in E2F6$^{-/-}$ and wt MEFs was analyzed by bisulphite sequencing. Sodium-bisulphite-modified DNAs were PCR amplified, and TUBA3 promoter fragments covering sequences from –175 to +121 in relation to the ATG start codon were subcloned. The TUBA3 promoter is schematically shown in the top panel with the E2F site and the ATG start codon indicated. Small vertical lines represent CpG dinucleotides, of which 17 are located in the indicated amplified promoter fragment. The methylation state of each of these CpG dinucleotides was determined in ten independent clones by direct sequencing. Methylated CpGs of the TUBA3 promoter fragments are shown as black (from wt MEFs) or gray (from E2F6$^{-/-}$ MEFs) squares. Open squares indicate nonmethylated CpG dinucleotides.

occupied by transacting factors in E2F6$^{-/-}$ fibroblasts suggested to us that in wt fibroblasts, the repressed TUBA3 gene might be DNA methylated and hence in an inaccessible state for specific DNA binding proteins. Consistent with this notion, the TUBA3 promoter contains a predicted (EMBL-EBI EMBOS CpGPlot) CpG island spanning the proximal promoter and the first exon. A single CpG dinucleotide is also located within the E2F site. In order to directly analyze the state of DNA methylation of the TUBA3 gene, we first performed a restriction digest analysis of genomic DNAs with the methylation-sensitive endonuclease AciI followed by PCR amplification of a promoter fragment encompassing nucleotides –370 to +9 in relation to the ATG start codon. This region contains two AciI recognition sites, one of them overlapping the E2F binding element. In the absence of the AciI digestion (–AciI), the TUBA3 and a Cdc6 control promoter could readily be amplified by PCR (Figure 5C). However, AciI digestion prevented amplification of the Cdc6 promoter, indicating its nonmethylated state in both E2F6$^{-/-}$ and wt MEFs. In contrast, AciI treatment (+AciI) did not hamper amplification of the TUBA3 promoter from wt MEFs, suggesting that this promoter (including the E2F binding site) is methylated in wt fibroblasts. Because DNA methylation of E2F sites has been shown to interfere with E2F DNA binding [17], these data also help to rationalize the lack of E2F site occupancy of the repressed TUBA3 promoter described above. Importantly, in E2F6$^{-/-}$ MEFs, methylation of the TUBA3 promoter was found to be severely reduced, as indicated by an approximately 8–10-fold decrease in the efficiency of promoter amplification (Figure 5C). The majority of CpG dinucleotides of the amplified TUBA3 promoter region were methylated in wt MEFs. Strikingly, in E2F6$^{-/-}$ MEFs, the situation was reversed, with the majority of CpGs being nonmethylated (Figure 5D). This result is not only consistent with the above data but also with the state of TUBA3 promoter activity in these cells. Together, these data show that E2F6 is essentially required for the epigenetic imprint of the repressed TUBA3 gene and that this imprint is lost from the TUBA3 promoter in E2F6$^{-/-}$ MEFs in which this gene is highly expressed.

Downregulation of TUBA3-like genes by E2F6 is evidently distinct in many ways from the E2F6-mediated repression of Cdc6-like genes. First, TUBA3 (and also TUBA7 and Tex12) is completely silenced by E2F6, whereas downregulation of Cdc6-like cell-cycle genes by E2F6 in S phase is "leaky" [12]. Second, TUBA3-like target-gene repression essentially depends on E2F6, whereas the loss of E2F6 can be compensated for by E2F4 in the repression of the Cdc6 gene [12]. In fact, we found none of the typical cell-cycle-dependent E2F target genes analyzed here to be deregulated in E2F6$^{-/-}$ MEFs. Third, in contrast to Cdc6, which shows contin-

ued promoter occupancy with transacting factors in the repressed state [18], the silenced TUBA3 promoter is characterized by a severely reduced accessibility for transacting factors, resulting in a "locked promoter state." Fourth, again in contrast to Cdc6 promoter regulation, in which we did not find evidence for DNA methylation, TUBA3 is heavily DNA methylated in the repressed state. Thus, there seem to exist at least two classes of E2F6 target genes, one class reflecting classical cell-cycle-regulated genes, such as Cdc6, that do not strictly depend on E2F6, and another class so far comprising the male germ-cell-specific α-tubulin and Tex12 genes that all have a cell-type-restricted expression pattern and that are permanently switched off with the help of E2F6. Silencing of the latter class of genes involves DNA methylation resulting in a locked promoter state with a severely reduced accessibility for transacting factors. Interestingly, DNA-methylation-based restriction of gene expression has also been shown for the testis-specific follicle-stimulating hormone receptor gene in males [19] and for the endothelial-cell-restricted expression of the endothelial nitric-oxide synthase gene [20], suggesting that DNA methylation might be a common mechanism for cell-type-dependent long-term silencing of specific genes.

The existence of two classes of E2F6 target genes is reminiscent of findings described for the repressive E2F (dE2F2) in *Drosophila*. Although dE2F2 does associate with numerous cell-cycle-regulated E2F target genes in vivo, it is not strictly required for their regulation. Instead, dE2F2 has important and nonredundant functions in the repression of gender-, developmental-, and/or differentiation-specific genes [21]. Mouse TUBA3 and 7 as well as Tex12 likely constitute examples of such E2F target genes in mammals, and further genes are likely to be identified. Consistent with this view, E2F6-deficient mice were reported to have a developmental defect displaying a mild homeotic transformation [13]. Therefore, the long-term silencing of gender- and developmental-specific genes appears to be a conserved function of repressive E2Fs from *Drosophila* to mammals. In contrast to dE2F2, repression by E2F6 is Rb-independent and can involve instead the recruitment of PcG repressor complexes [22]. Whether PcG proteins are also involved in the repression of testis-specific E2F6 target genes remains to be seen. However, a switch in the recruitment of repressor complexes may be advantageous for uncoupling the control of those types of E2F response genes that need to be silenced in a long-term manner from the cell-cycle-controlled activity of Rb-dependent repressor complexes.

Supplemental Data

Detailed Experimental Procedures, as well as two supplemental figures and a supplemental table, are available at http://www.current-biology.com/cgi/content/full/15/11/1051/DC1/.

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