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High Incidence of Thymic Epithelial Tumors in E2F2 Transgenic Mice*

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In virtually all human tumors, genetic and epigenetic alterations have been found which affect the *INK4/-CYCLIN D/RB* pathway, which regulates cell cycle entry and exit in normal cells. E2F transcription factors are important downstream components of this pathway, which act by controlling the expression of genes involved in DNA replication and cell cycle progression. To determine whether E2F2 deregulation promotes proliferation and tumorigenesis *in vivo*, we generated E2F2 transgenic mice, in which the Eμ and murine *pim1* promoter (pp) direct high expression of E2F2 in thymic epithelial cells. Eμ-pp-E2F2 mice start to develop cytokeratin- and ER-TR4-positive cortical thymomas from the age of 20 weeks, and within 1 year, nearly all mice succumb to gross thymic epithelial tumors. General thymic morphology is largely maintained, but T cell development is perturbed in thymomas, with proportionately less $CD4^+CD8^+$ double-positive thymocytes. In the first 3 months, E2F2 transgenic thymi exhibit only mild epithelial hyperplasia, and thereafter thymomas arise stochastically, probably following additional mutations. Interestingly, Eμ-pp-E2F1 mice do not display cortical thymomas. These data argue that E2F2 promotes unscheduled cell division and oncogenic transformation of thymic epithelial cells.

E2F transcription factors are key regulators of cell division and function by controlling the expression of genes that are critical for DNA synthesis, DNA repair, and mitosis (1–3). Six distinct genes encode seven different E2F proteins (E2F1, E2F2, E2F3a, E2F3b, and E2F4 to E2F6), which form heterodimers with DP1, or one of the four different splice variants (α, β, γ, and δ) of human DP2 (4) or mouse DP3 (5, 6). However, E2F7, which binds to consensus E2F DNA recognition sites and acts as a transcriptional repressor, lacks the residues necessary for DP dimerization (7, 8). E2F1, E2F2, and E2F3a are potent activators of E2F-responsive genes, but their transcriptional activity is inhibited by binding to the retinoblastoma protein (pRb).[1] pRb is functionally inactivated at the G_1-S transition by cyclin D-cyclin dependent kinase 4 (Cdk4)/Cdk6 and cyclin E-Cdk2-mediated phosphorylation (9, 10), thus enabling E2Fs to activate their target genes. Besides promoting cell division, E2F1, and to a lesser extent E2F2 and E2F3a, also induce programmed cell death through both p53-dependent and p53-independent mechanisms.

Studies performed on E2F gene-targeted mice have demonstrated both unique and redundant functions of the different E2Fs (11). *E2f1*$^{-/-}$ and *E2f2*$^{-/-}$ mice are fully viable, and each mutant shows distinct cell lineage-specific phenotypes: *E2f1*$^{-/-}$ thymocytes are defective in T cell receptor-mediated apoptosis (12–14), and T lymphocytes proliferate less upon antigen stimulation in the absence of E2F1 (15, 16). In contrast, E2F2-deficient thymocytes show normal apoptosis characteristics, whereas *E2f2*$^{-/-}$ splenic T cells display accelerated G_1-S phase progression (15, 16). On the other hand, erythropoiesis and pre-B cell differentiation are defective in *E2f2*$^{-/-}$ mice, whereas there is increased B cell maturation in *E2f1*$^{-/-}$ animals (17). E2F3 deficiency dramatically impairs cell proliferation of mouse embryonic fibroblasts and results in reduced neonatal viability (18). E2F1 and E2F2 share redundant roles with E2F3 during embryonic development because deficiency for either E2F1 or E2F2 exacerbates the lethal phenotype observed in *E2f3*$^{-/-}$ embryos (19, 20). Although *E2f1*$^{-/-}$; *E2f2*$^{-/-}$ mouse embryonic fibroblasts proliferate efficiently, the combined loss of E2F1, E2F2, and E2F3 completely abolishes entry into S phase and further cell division (20).

E2f1$^{+/-}$ as well as *E2f1*$^{-/-}$ mice develop a similar spectrum of tumor types after a latency of 16 months, including lymphomas, hemangiosarcomas, and histiocytic sarcomas, arguing that *E2f1* acts as a tumor-suppressor gene (21). The tumor-inhibitory function of E2F1 is illustrated further by the finding that overexpression of E2F1 inhibits glioma tumor growth (22), induces apoptosis in breast and ovarian carcinoma cell lines (23), and increases chemosensitivity of melanoma and pancreatic carcinoma cells (24, 25). Dominant negative mutants of E2F/DP inhibit apoptosis and induce tumor growth of a non-malignant breast epithelial cell line (26). In addition, E2F1 suppresses tumorigenesis in mice expressing a c-Myc transgene under control of an epithelial-specific keratin (K5) promoter (27). *E2f2*$^{-/-}$ mice display aberrant peripheral immune tolerance and acquire autoimmune disease (15), but on an *E2f1*$^{+/-}$ background, *E2f2*$^{-/-}$ mice largely succumb to additional hematological malignancies (16), suggesting that E2F2 may also act as a tumor suppressor. Importantly, loss of E2F3

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[1] The abbreviations used are: pRb, retinoblastoma protein; Cdk, cyclin-dependent kinase; DP, double positive; Eμ, immunoglobulin heavy chain enhancer; HA, hemagglutinin; K5, keratin 5; LTR, long terminal repeat; pp, *pim1* promoter; TEC, thymic epithelial cell; DAB, 3,3′-diaminobenzidine tetrahydrochloride.

expression harbors no increased susceptibility for the development of tumors, both in wild type and *E2f1* mutant mice.

On the other hand, enforced expression of E2F1 in the liver results in hepatocellular adenomas as well as large cell dysplasia (28). Transgenic K5-E2F1 expression induces spontaneous tumors that originate from epithelial basal cells (29), accelerates formation of benign skin papillomas in collaboration with a v-Ha-*ras* transgene (30), and enhances skin tumorigenesis in $p53^{-/-}$ mice (31). Furthermore, E2F1 deficiency reduces thyroid and pituitary tumorigenesis in $Rb^{+/-}$ mice (32), delays the onset of Eμ-Myc-induced pre-B cell lymphomas (33), and enhances keratinocyte apoptosis after UVB exposure and γ-radiation (34). Interestingly, the absence of E2F1 reverts the UVB-induced apoptosis defect in primary fibroblasts and early onset thymic lymphomas seen in $p53^{-/-}$ mice, arguing that E2F1 acts functionally downstream of p53 (34).

These data demonstrate that *in vivo* E2F1 has both tumor-suppressive as well as tumor-promoting activity depending on cell lineage, stage of differentiation, status of the p53/ARF/MDM2 pathway, and expression level of pRb. However, there is much less information about the potential diverse functions of E2F2 in different cell lineages. E2F2 has an unexpected negative role in controlling antigen-stimulated T cell proliferation (15, 16), whereas in mouse embryonic fibroblasts endogenous E2F2 contributes to promote cell division (20), and in hematopoietic progenitor cells E2F2 is required for efficient S phase progression (17). In postmitotic lens fibers overexpression of E2F2 induces cell cycle entry and subsequent apoptosis (35). In this study, we provide evidence that E2F2 has a distinct function in promoting oncogenic transformation of nonlymphoid thymic epithelial cells (TECs).

EXPERIMENTAL PROCEDURES

E2F2 Transgenic Mice—For the generation of Eμ-pp-E2F2 mice, a 1.3-kb HA-tagged human E2F2 cDNA was inserted into the SacI-KpnI site of pJ3Ω. The NotI-HpaI fragment containing HA-E2F2, followed by splice donor and acceptor elements derived from SV40 small t antigen intervening sequences present in pJ3Ω, was cloned in EagI-HpaI site of Eμ-*pim1* promoter-Moloney murine leukemia virus LTR transgenic vector. The assembled transgene was liberated with HindIII from the vector backbone, microinjected into pronuclei of FVB zygotes, and transferred to (B6 $\times$ DBA)F_1 foster mice. Eμ-pp-E2F founder mice and subsequent transgenic progeny were identified with PCR analysis on genomic DNA isolated from tail biopsies with transgene specific primers TDK5′ 5′-CGGCCTTTGATGGCTTTG-3′ and EMU3′ 5′-AGGGTATGAGAGAGCCTC-3′.

Northern and Southern Blot Analyses—Total RNA was isolated from frozen sections of normal and hyperplastic thymi or isolated TECs and thymocytes using TRIzol (Invitrogen). TECs were isolated as described previously (36). Samples of 20 μg of total RNA were separated on a 1% paraformaldehyde-containing agarose gel, transferred to Protran nitrocellulose filter (Schleicher & Schuell), and hybridized to random priming labeled [α-^{32}P]dATP *U3LTR* or 0.8-kb β-actin cDNA probes. Genomic DNA (15 μg), isolated from frozen tissue samples, was digested with PvuII, separated on 0.7% 1 $\times$ TAE-agarose gel, blotted on Protran nitrocellulose filter, and hybridized to probe J15, a 900-bp ClaI-EcoRI fragment of Jβ2 locus, to check for T cell receptor rearrangements.

Western Blot Analysis—Total cell extracts were generated by lysis of frozen thymic tissues in ice-cold ELB buffer (250 mM NaCl, 0.1% Nonidet P-40, 50 mM HEPES pH 7.0, and 5 mM EDTA) supplemented with protease inhibitors (Complete, Roche Applied Science). Samples were cleared by centrifugation for 10 min at 14,000 rpm, lysates separated with SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Polyclonal antibodies against E2F2 (C20), cyclin A (C19), cyclin E (M20), and actin (C11) (Santa Cruz) were used for immunoblotting. Results were visualized by Enhanced Chemiluminescence (Amersham Biosciences).

Histology and Immunohistochemistry—For histopathological examination, thymi were removed, fixed with 10% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. For immunohistology of frozen sections, tissues were immersed in optimal cutting temperature compound and snap frozen in liquid nitrogen. 5 μM frozen sections were acetone fixed and stained with mouse monoclonal pan-cytokeratin (Sigma), rabbit polyclonal E2F2 (Santa Cruz), or rat monoclonal (ER-TR4, ER-TR5, and ER-TR7) (37) antibodies. Primary antibodies were visualized with fluorescein isothiocyanate-conjugated secondary antibodies. For paraffin-embedded sections tissues were processed using a standard protocol for DAB immunostaining, incorporating an antigen retrieval step, and using a 1:250 dilution of the mouse monoclonal HA.11 antibody (Covance). DAB-stained sections were counterstained with hematoxylin.

Flow Cytometry—Single cell suspensions were freshly prepared from wild-type thymus or hyperplastic thymi, and 10^6 cells were resuspended in 2% fetal calf serum, 5 mM HEPES in phosphate-buffered saline. Cells were stained with fluorescein isothiocyanate-conjugated CD4 (RM4-5) and phycoerythrin-conjugated CD8α (53-6.7) (BD Pharmingen), washed and analyzed on FACScan (BD Biosciences) with CellQuest software package.

RESULTS

Generation of Eμ-pp-E2F Transgenic Mice—To compare their ability to promote cell proliferation and regulate apoptosis when overexpressed *in vivo*, E2F1 and E2F2 transgenic mice were generated. For this purpose, human E2F1 and E2F2 cDNAs were subcloned in a vector containing a duplicated version of the immunoglobulin heavy chain enhancer (μ) inserted into the murine *pim1* promoter (pp) region, and the Moloney murine leukemia virus LTR, which encodes a polyadenylation signal (Fig. 1*A*). The Eμ-pp transgenic promoter has been shown to direct ubiquitous expression during embryonic development (38) and confers high expression to different hematopoietic cell lineages in adult mice (39, 40).

Four founders containing the Eμ-pp-E2F1 (TEF1-5, -21, -43, and -62) or Eμ-pp-E2F2 (TEF2-6, -33, -35, and -37) transgene were identified by PCR analysis on genomic DNA, and each of the founders was transmitted the E2F transgene to their progeny, except founder TEF2-6. To analyze expression of the different Eμ-pp-E2F transgenes in the thymus and compare transgene-driven *E2F* mRNA levels, Northern blot hybridization was performed using the Moloney murine leukemia virus *U3LTR* probe. Both Eμ-pp-E2F1 (TEF1-5) and Eμ-pp-E2F2 (TEF2-35 and TEF2-37) mice showed significant *E2F1* and *E2F2* transgenic mRNA levels in thymus (Fig. 1*B*). Immunoblotting confirmed protein expression of transgene-derived HA-tagged E2F1 and E2F2 (data not shown). Western blotting for cyclin E and cyclin A protein levels on thymic cell extracts indicated that transgene-driven E2F2 expression, but not E2F1, resulted in enhanced levels of cyclin E (Fig. 1*C*). Both cyclin E1 (*Ccn1*) and cyclin E2 (*Ccn2*) gene transcriptions are controlled by the pRb/E2F pathway (3), suggesting that overexpression of E2F2 in murine thymus activates cyclin E expression.

Eμ-pp-E2F1 and Eμ-pp-E2F2 founder lines were mated with FVB mice to generate larger cohorts of E2F transgenic mice. Interestingly, we observed a remarkable difference in phenotypes between E2F1 and E2F2 transgenic mice. Eμ-pp-E2F1 mice displayed a strong delay in endochondral ossification with concomitant dwarfism and a relatively high perinatal lethality among Eμ-pp-E2F animals (60% of transgenic offspring) (39). Thus with great difficulty, only one Eμ-pp-E2F1 line (TEF1-5) could be maintained. In contrast, progeny of all three Eμ-pp-E2F2 founder lines (TEF2-33, -35, and -37) were born alive with normal Mendelian ratios and showed no evidence of skeletal abnormalities. Therefore, subsequent functional studies were largely restricted to E2F2 transgenic mice.

Eμ-pp-E2F2 Transgene Is Highly Expressed in TECs—We decided to examine in more detail the expression pattern of the Eμ-pp-E2F2 transgene in the two major cell types present in thymus, thymocytes and TECs. Thymic epithelium forms a structurally and functionally important component of the thymus microenvironment and is required for selection of the

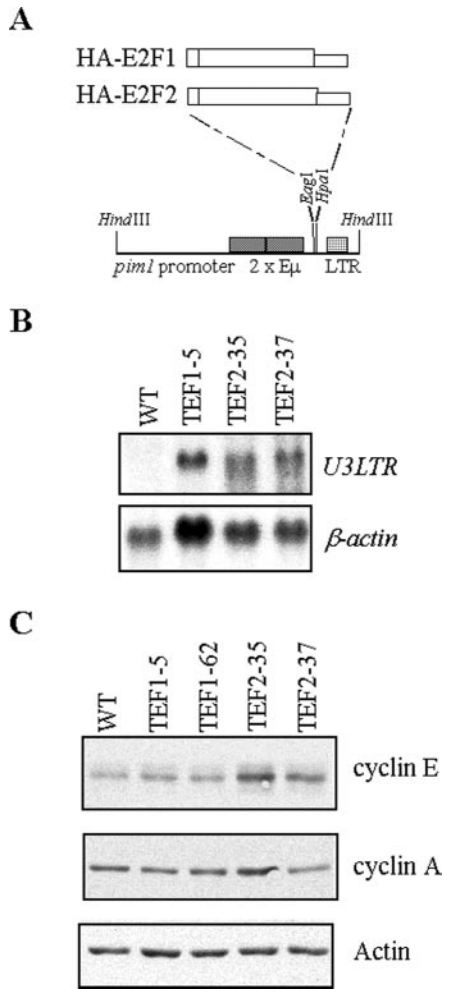


FIG. 1. **Eμ-pp-E2F1 and Eμ-pp-E2F2 transgene construction and expression.** *A*, schematic representation of the Eμ-pp-E2F transgenes, where HA-tagged human E2F1 or E2F2 cDNAs, in conjunction with small t antigen splice donor and splice acceptor sequences, were cloned into the EagI-HpaI enzyme restriction sites of the Eμ-*pim1* promoter transgenic vector. The Moloney murine leukemia virus LTR provides a polyadenylation signal. *B*, Northern blot analysis on total RNA isolated from thymi of 5–8-week-old wild type (*WT*), Eμ-pp-E2F1 (TEF1-5), and Eμ-pp-E2F2 (TEF2-35 and TEF2-37) transgenic mice. Blots were hybridized with *U3LTR* probe to detect transgene expression and β-actin as loading control. *C*, immunoblotting demonstrates cyclin E and cyclin A protein levels in total cell extracts of wild-type, E2F1 (TEF1-5 and TEF1-62), and E2F2 (TEF2-35 and TEF2-37) transgenic thymi, with actin as loading control.

appropriate T cell repertoire on the cell surface of thymocytes (40, 41). Total mRNA was extracted from freshly isolated thymocytes or TECs that were cultured short term *in vitro* to obtain pure epithelial cells in sufficient quantity. Surprisingly, Northern blot analysis indicated that Eμ-pp-E2F2 was highly expressed in purified TECs and very low or nondetectable in primary thymocytes (Fig. 2*A*). Similarly, transgenic E2F2 expression was absent in mature peripheral T cells (data not shown). TECs isolated from Eμ-pp-E2F1 mice displayed reduced viability and were not able to grow *in vitro*, suggesting that E2F1 overexpression promotes apoptosis in these cells. Immunohistochemical analysis performed on thymic sections of Eμ-E2F2 mice confirmed HA-E2F2 nuclear staining in TECs from E2F2 transgenic thymi but not wild-type controls (Fig. 2, *B* and *C*), and high E2F2 protein levels coincided with the network of cytokeratin-positive TECs interspersed between islets of thymocytes (Fig. 2, *D* and *E*). Thus, *pim1* promoter in conjunction with Eμ sequences direct high transgene expression of E2F2 in TECs.

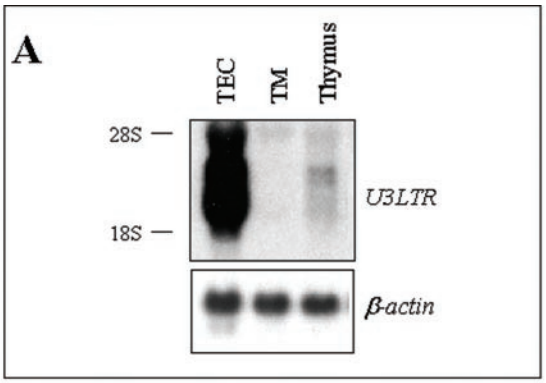
High Incidence of Spontaneous Thymic Tumors in Eμ-pp-E2F2 Transgenic Mice—The predominant phenotype observed in aging Eμ-pp-E2F2 animals of each transgenic line, which became apparent between the age of 5 and 11 months, was respiratory distress and dyspnea. Shortly thereafter, these E2F2 transgenic mice became cachectic and had to be sacrificed (Fig. 3). At autopsy, diseased Eμ-pp-E2F2 animals displayed severe hyperplastic thymi, often occupying the entire thoracic cavity (Fig. 4*A*). There was no evidence of enlarged secondary lymphoid tissues (spleen and lymph nodes) or macroscopic invasion of other organs. In most cases, the normal thymic bilobar structure was preserved in the solid hyperplastic thymi.

Hematoxylin and eosin-stained sections indicated that the functional organization of cortex and medulla was still present in all E2F2 thymic tumors examined, but darker stained lymphocyte-rich cortical areas were largely increased in size compared with their normal counterparts (Fig. 4, *B* and *C*). Pale stained medulla-like areas with Hassall corpuscle-like structures were present as isolated islets in enlarged thymi instead of one continuous area within a normal thymic lobe. These macroscopic and histological features recapitulated the characteristics of epithelial thymomas. A few Eμ-pp-E2F2 tumors showed small necrotic areas, but overall there was no evidence for high grade malignancy. None of the 18 Eμ-pp-E2F1 mice that survived into late adulthood developed thymomas or showed an enlarged thymus upon necroscopy. These findings indicate that the induction of thymomas in Eμ-pp-E2F transgenic animals results as a consequence of deregulated E2F2 expression, but not E2F1. This different ability to induce tumors in thymic epithelium may well be related to the differential ability of E2F1 and E2F2 to induce apoptosis.

Phenotypic Analyses of Thymocytes in Eμ-pp-E2F2 Thymomas—To investigate whether thymocytes present in hyperplastic thymi of Eμ-pp-E2F2 transgenic mice showed evidence of transformation, T cell receptor rearrangements were assessed by Southern blotting. T cell lymphomas are normally mono- or oligoclonal in origin, which can be demonstrated by a specific and unique rearrangement pattern at the joining regions of the T cell receptor β locus (Fig. 4*D*, *lane 2*). However, Eμ-pp-E2F2 tumors contained either a regular germ line configuration, as seen in wild-type thymus (Fig. 4*D*, *lanes 1*, *3*, *5*, *6*, and *8*), or polyclonal rearrangements (Fig. 4*D*, *lanes 4* and *7*), arguing against the presence of T lymphoid tumor cells.

These findings were corroborated by flow cytometric analyses, which also showed no indications for clonal outgrowth of specific thymocyte subsets in Eμ-pp-E2F2 thymic tumors as defined by CD4 and CD8 coreceptor expression (Fig. 4*E*). Instead, we found altered ratios of thymocyte subsets in Eμ-pp-E2F2 thymomas, with relatively more immature $CD4^-CD8^-$ double negative pre-T cells as well as a higher proportion of postselected $CD4^+$ or $CD8^+$ single-positive thymocytes (Fig. 4*E* and Table I). Young Eμ-pp-E2F2 mice without thymomas showed normal CD4/CD8 thymocyte distributions (data not shown). These analyses indicate that enforced expression of E2F2 in TECs results in cortical thymomas with concomitant perturbation of T cell development.

Thymomas Express Increased Levels of Transgenic E2F2—We wanted to assess whether the induction of thymic tumors in E2F2 transgenic mice was associated with altered E2F2 expression levels. Northern blot analysis demonstrated that the occurrence of thymomas correlated with more abundant Eμ-pp-E2F2 transgene expression. All tumor samples showed enhanced levels of transgenic *E2F2-LTR* transcripts compared with nontumorigenic counterparts (Fig. 5*A*). Similar results were observed when E2F2 protein levels were analyzed by immunoblotting. Human HA-E2F2, which migrates slower



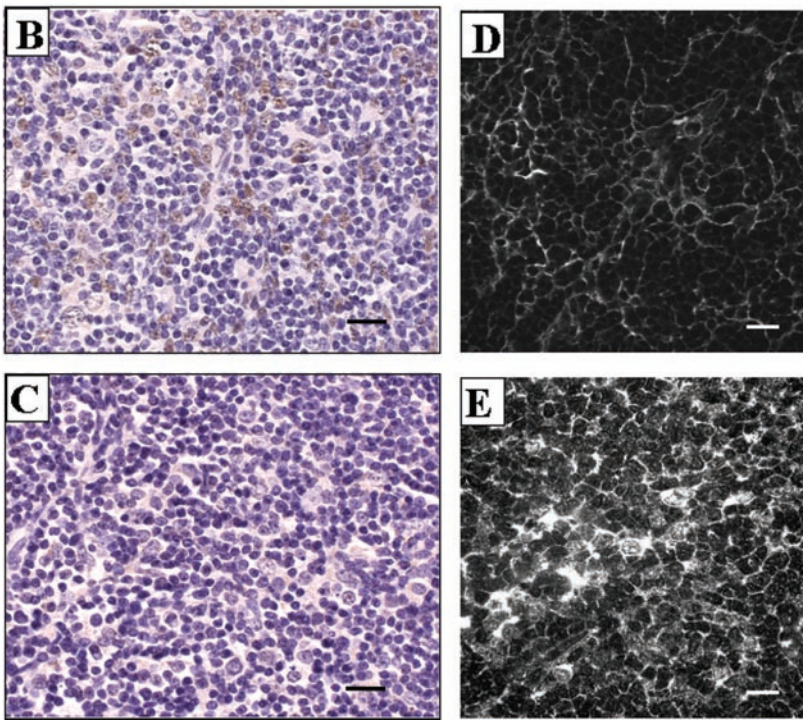


FIG. 2. **Expression of Eμ-pp-E2F2 transgene in TECs.** *A*, Northern blot shows RNA expression levels of Eμ-pp-E2F2 transgene as detected with *U3LTR* probe in total thymus, TECs cultured for short term *in vitro*, and freshly isolated thymocytes (*TM*). Northern blot was subsequently hybridized with β-actin probe for loading control. The positions of 28 S and 18 S ribosomal bands are indicated. *B* and *C*, HA-E2F2 protein is expressed in TECs of Eμ-pp-E2F2 transgenic animals. Immunohistochemistry was performed on paraffin-embedded thymic tissue sections of Eμ-pp-E2F2 (*B*) or wild-type (*C*) mice using HA-antibody and conventional DAB staining. *D* and *E*, E2F2 overexpression is localized in characteristic network of cytokeratine-positive TECs. Immunohistochemistry on cryosections of Eμ-pp-E2F2 thymus stained with pan-cytokeratin antibody (*D*) or E2F2 antibody (*E*) is shown. Primary antibodies are visualized by fluorescein-isothiocyanate-conjugated secondary antibodies. *Scale bars* represent 20 μm.

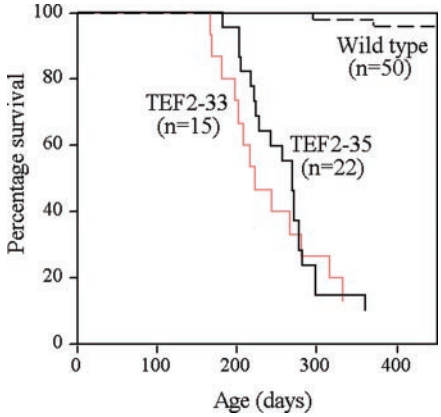


FIG. 3. **Kaplan-Meier survival graph of Eμ-pp-E2F2 transgenic mice.** Survival rates of mice from two Eμ-pp-E2F2 transgenic lines TEF2-33 ($n = 15$) and TEF2-35 ($n = 22$) and wild-type (*WT*) FVB/N mice ($n = 50$) are depicted. The age at which ill animals were sacrificed is indicated.

than murine E2F2 during SDS-PAGE, was strongly up-regulated in total cell extracts of Eμ-pp-E2F2 thymomas compared with nontumorigenic thymi of young E2F2 transgenic animals (Fig. 5*B*). In contrast, endogenous levels of mouse E2F2 remained similar in normal and hyperplastic thymi from Eμ-pp-E2F2 mice of each transgenic line tested (Fig. 5*B*). Thus, thymomas that arise in Eμ-pp-E2F2 mice show an increase in transgenic E2F2 expression levels, which most likely results from the expansion of non-T lymphoid E2F2-expressing TECs.

E2F2-induced Thymomas Arise from ER-TR4$^+$ Thymic Cortical Epithelial Cells—To confirm that E2F2-induced thymic tumors indeed originated from cytokeratin-positive thymus epithelium, immunohistochemistry was performed on normal and Eμ-pp-E2F2 hyperplastic thymi. In wild-type thymus, cortical and medullary areas could easily be distinguished morphologically and by pan-cytokeratin staining, with thymic cortical epithelial cells oriented perpendicularly toward the capsule that envelops the thymus (Fig. 6*A*). The cortical areas in E2F2 thymomas, however, displayed a more disoriented architecture, notably at subcapsular areas and cortical-medullary junctions (Fig. 6*B*). Overall, epithelial cells were increased in numbers and formed small clusters, giving rise to more intense cytokeratin staining.

Three classes of epithelium can be phenotypically distinguished in thymus: subcapsule (ER-TR5$^+$), cortex (ER-TR4$^+$), and medulla (ER-TR5$^+$), even though many different subtypes of epithelial cells exist in each of these areas (41, 42). Immunohistochemical analysis indicated that E2F2-induced thymomas showed more intensive staining and larger areas of ER-TR4$^+$ epithelial cells compared with normal thymus (Fig. 6, *C*

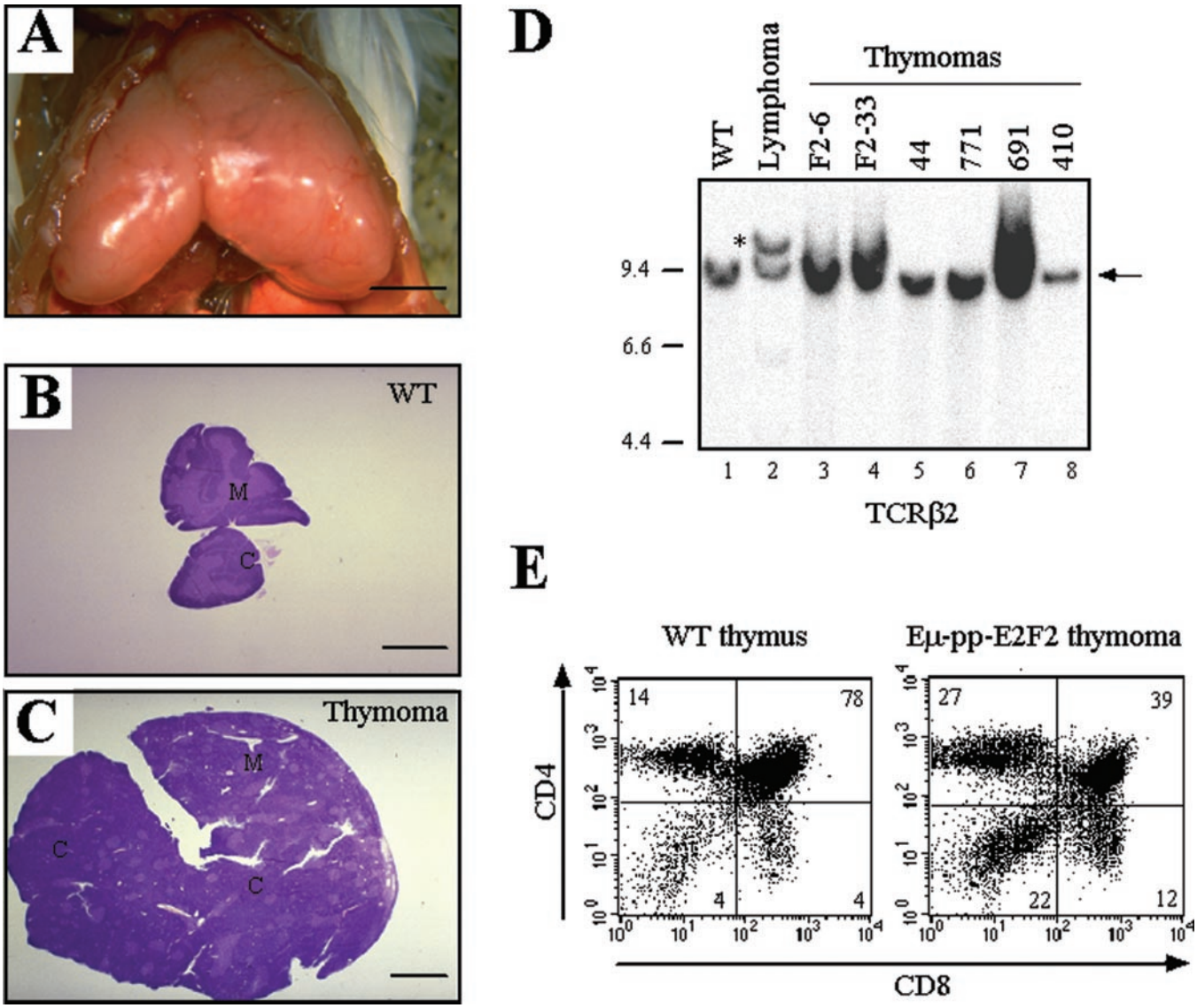


FIG. 4. **Characterization of thymomas in Eμ-pp-E2F2 transgenic animals.** *A*, macroscopic representation of severe hyperplastic thymus filling thoracic cavity of TEF2-35 animal at age of 8 months. The lungs and heart are almost completely obscured by the thymic tumor. The *scale bar* represents 8 mm. *B* and *C*, hematoxylin and eosin-stained paraffin sections of wild-type (*WT*) thymus at age of 4 weeks and TEF2-33 thymoma at age of 7 months. Medulla (*M*) and cortical (*C*) areas are indicated. *Scale bars* represent 5 mm. *D*, Southern blot analysis on genomic DNA isolated from thymi of wild-type (*WT*), Moloney murine leukemia virus-induced T cell lymphoma, Eμ-pp-E2F2 thymomas of founder TEF2-6 (*F2-6*), and TEF2-33 (*F2-33*), and E2F2 transgenic animals *44*, *771* (both TEF2-33), *691*, and *410* (both TEF2-35). Each genomic DNA sample is digested with PvuII, blotted, and hybridized with a T cell receptor β2 probe to detect T cell receptor rearrangements. The *arrow* indicates the position of the germ line band. The *asterisk* indicates the monoclonal rearranged T cell receptor β allele. *E*, flow cytometry was performed on a single cell suspension of thymocytes isolated from wild-type thymus and Eμ-pp-E2F2 thymoma of founder TEF2-6, stained with anti-CD4 and anti-CD8 antibodies. The percentage of cells in each quadrant is indicated.

TABLE I

Latency and distribution thymocyte subsets of Eμ-pp-E2F2 thymomas

The age at which moribund Eμ-pp-E2F2 animals were sacrificed is indicated together with individual mouse identification numbers and transgenic founder lines of origin. Fraction of $CD4^-CD8^-$ double negative, $CD4^+CD8^+$ double positive, $CD4^+CD8^-$ single positive, and $CD4^-CD8^+$ single positive thymocyte subsets are shown as determined by flow cytometric analysis on freshly isolated thymic tumor single cell suspensions. For reference, the normal distribution in wild-type (WT) FVB mice is indicated. For some thymomas, the distribution of thymocyte subsets was not determined (ND).

Animal	Eμ-pp-E2F2 line	Age	$CD4^-CD8^-$ (WT, 4%)	$CD4^+CD8^+$ (WT, 78%)	$CD4^+CD8^-$ (WT, 14%)	$CD4^-CD8^+$ (WT, 4%)
		weeks				
35	TEF2–6 founder	36	22%	39%	27%	12%
44	TEF2–33 founder	48	14%	48%	27%	11%
771	TEF2–33 F_1	24	6%	71%	19%	6%
773	TEF2–33 F_1	30	ND	ND	ND	ND
409	TEF2–35 F_1	32	9%	67%	19%	5%
691	TEF2–35 F_1	31	6%	55%	30%	9%
354	TEF2–37 F_1	36	ND	ND	ND	ND
496	TEF2–37 F_1	30	5%	64%	24%	7%

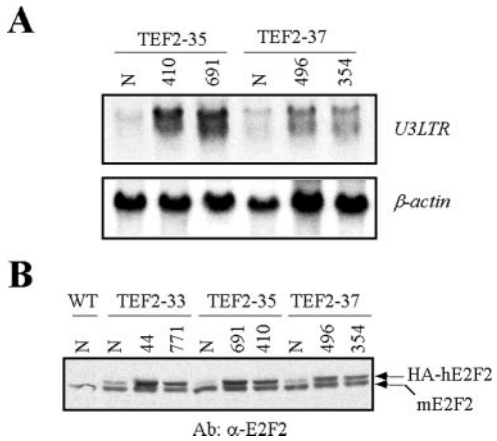


FIG. 5. **Transgenic E2F2 expression levels in thymomas.** *A*, Eμ-pp-E2F2 RNA levels in nontumorigenic thymus (*N*) and thymomas of TEF2-35 and TEF2-37 animals as detected with *U3LTR* probe. Northern blot was subsequently hybridized with a β-actin probe as a loading control. *B*, immunoblotting with E2F2 antibody detects endogenous mouse E2F2 and transgene-derived human HA-E2F2 protein levels in normal (*N*) wild-type thymus (*WT*) and nontumorigenic thymi (*N*) or thymomas of TEF2-33, TEF2-35, and TEF2-37 animals.

and *D*). In contrast, ER-TR5$^+$ medullary and subcapsule epithelial cells in thymomas exhibited an architecture similar to and expression level equal to those in wild-type thymus (Fig. 6, *E* and *D*). Similarly, there was no enrichment of ER-TR7$^+$ mesenchymal fibroblasts in thymomas (Fig. 6, *G* and *H*), implying that Eμ-pp-E2F2 thymomas are primarily related to ER-TR4$^+$ cortical epithelial cells.

Secondary Mutations Are Required for Tumor Phenotype in E2F2 Mice—Overexpression of cyclin D1 and D2 in mouse thymic epithelium induces severe hyperplasia, which is already evident at the age of 8 weeks (43, 44). To investigate the extent of thymic hyperplasia in young Eμ-pp-E2F2 mice and study the development of E2F2-induced thymomas in time, thymi from transgenic animals and wild-type siblings from line TEF2-35 were harvested at ages of 8, 12, 16, and 20 weeks, and thymus wet weight was determined. In normal FVB mice, involution of the thymus became apparent between 8 and 12 weeks, and average thymi weight declined from 0.058 g at 12 weeks, to 0.044 g and 0.035 g by 16 and 20 weeks, respectively (Fig. 7). In contrast, Eμ-pp-E2F2 mice showed no significant thymic involution, with an average thymus weight of 0.08 g between 8 and 16 weeks of age. Although it was evident that thymus size displayed more variability among E2F2 transgenic mice, up to the age of 3 months most E2F2 transgenic thymi were on average 2-fold larger in size than wild-type thymi (Fig. 7). This was also accompanied by increased thymocyte counts in Eμ-pp-E2F2 mice of each founder line (data not shown).

After the initial stage of mild thymic epithelial hyperplasia in Eμ-pp-E2F2 mice, the first significant hyperplastic thymi started to appear at the age of 20 weeks, displaying a 20-fold increase in thymus weight and histological evidence of malignant transformation. Thus, there was a clear lag phase before gross hyperplastic thymi could be observed. Furthermore, cortical thymomas developed stochastically in E2F2 transgenic animals, between the age of 5 and 11 months. These data imply that cortical thymomas in Eμ-pp-E2F2 mice most likely arise after the accumulation of additional (epi-) genetic alterations.

DISCUSSION

E2F transcription factors have important functions in controlling cell cycle progression, apoptosis, differentiation, and replicative senescence (45, 46). Ectopic expression of E2F2, like E2F1, induces S phase entry in immortalized quiescent rodent fibroblasts (47, 48), primary adult rat sensory neurons (49),

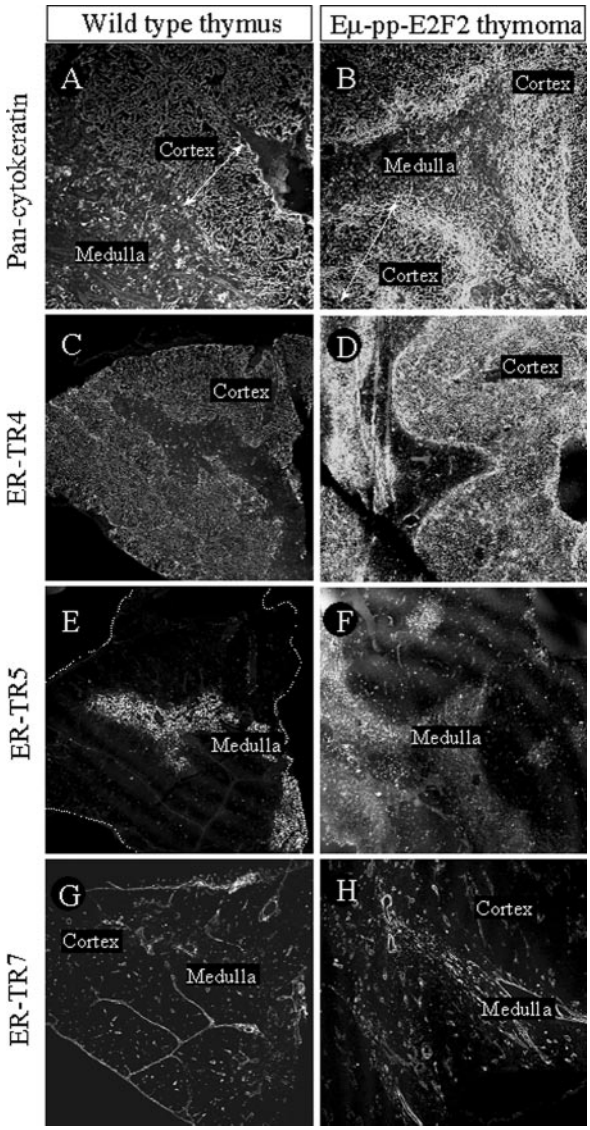


FIG. 6. **Immunohistochemical analyses on E2F2-induced thymomas.** Cryosections of wild-type thymus (*A*, *C*, *E*, and *G*) and Eμ-pp-E2F2 thymoma (*B*, *D*, *F*, and *H*) were stained with antibodies against cytokeratin (pan-cytokeratin) (*A* and *B*), cortical epithelium (ER-TR4) (*C* and *D*), medullary epithelium (ER-TR5) (*E* and *F*), and reticular fibroblasts (ER-TR7) (*G* and *H*). Primary antibodies were visualized with fluorescein-isothiocyanate-conjugated secondary antibodies.

and postmitotic epithelial lens fiber cells (35). However, it has been suggested that in primary diploid fibroblasts, entry into S phase by E2F1 or E2F2 overexpression requires the additional inactivation of pRb- or p53-mediated G_1 checkpoint control (50). Interestingly, E2F2 seems to have opposing roles in regulating cell division of hematopoietic cells because antigen-stimulated *E2f2*$^{-/-}$ splenic T cells show accelerated G_1-S phase progression (15, 16), whereas S phase is stalled and prolonged in *E2f2*$^{-/-}$ hematopoietic progenitor cells (17).

In this study, we demonstrate that enforced expression of E2F2 results in mild hyperproliferation of the thymus in young Eμ-pp-E2F2 transgenic mice and the subsequent development of thymomas. The Eμ-pp-E2F2 thymic tumors all show characteristics reminiscent of human type B1 thymoma ("organoid" thymoma). The tumor mimics normal thymus with areas of cortical and medullary differentiation, however with architectural disorganization between the distinct compartments and within the cortical compartment. This is reflected by concentrated patches of ER-TR4$^+$ cortical TECs, without an in-

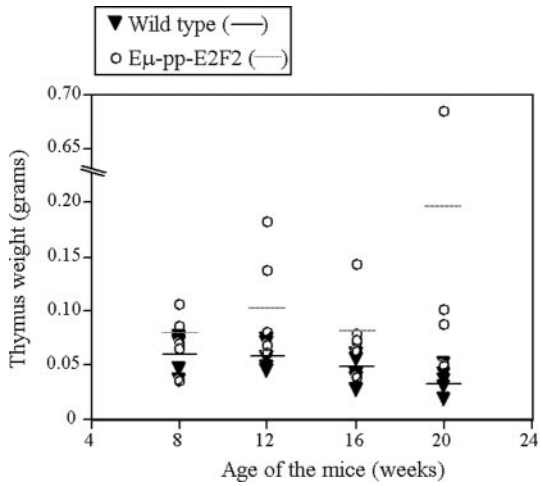


FIG. 7. **Evolvement of thymic hyperplasia in Eμ-pp-E2F2 mice.** Thymi from E2F2 transgenic mice of line TEF2-35 and wild-type littermates were collected and weighed. The *scatterplot* indicates individual wet weight as well as average for wild-type (*solid line*) and E2F2 thymi (*dotted line*) at 8, 12, 16, and 20 weeks of age.

crease of ER-TR5$^+$ medullary TECs and ER-TR7$^+$ reticular fibroblasts.

Transgenic E2F2 levels appear to be quite high in epithelial cells of the thymus, as shown by RNA expression analysis on isolated TECs cultured *in vitro* as well as immunohistochemical staining on sections of E2F2 transgenic thymi. In contrast, Eμ-pp-derived *E2F2* mRNA is almost nondetectable in primary thymocytes and peripheral T lymphocytes. Accordingly, there is no evidence for the presence of malignant lymphocytes within E2F2-induced thymic tumors, supporting the notion that the enlargement of E2F2 transgenic thymi primarily results from the expansion of cytokeratin-positive epithelial cells. At present it is not clear why the Eμ-pp transgene does not drive detectable *E2F2* mRNA expression in the T cell lineage, which is normally the case. Similarly, we have not been able to detect Eμ-pp-E2F1 mRNA expression in isolated thymocytes (data not shown). Therefore, we speculate that the introduction of artificial splice donor and acceptor sequences, consisting of SV40 small t antigen intervening sequences at the 3′-end of the transgenic cDNA inserts, may have modified the expression pattern of Eμ and *pim1* promoter. Alternatively, thymocytes expressing high levels of human HA-E2F1/2 could have been negatively selected.

Some epithelial cell lineages, like skin epidermis or gut epithelium, have a rapid cell turnover and are characterized by the presence of stem cells that give rise to progenitor and terminally differentiated cells (51). It has been demonstrated that E2F expression and activity are tightly regulated during development and differentiation of these epithelial tissues (52, 53), suggesting a critical role of E2F transcription factors in controlling epithelial cell cycle kinetics. Thymic epithelium originates from the third pharyngeal pouch endoderm, and recently a common epithelial precursor cell has been identified in embryonic thymus (54, 55). However, it has not yet been established whether this embryonic thymic precursor cell harbors the capacity for self-renewal and whether it represents an adult thymic epithelial stem cell. A unique feature of the thymus is its involution quite early in life, which correlates with deterioration of thymic stromal structures, including epithelium. This observation may argue against the existence of true epithelial stem cells within the adult thymus and suggests the presence of progenitors with more restricted self-renewal capacity and high differentiation probability (transit-amplifying cells).

Our data suggest that enforced E2F2 expression prevents the onset of thymic involution because thymus size does not decline in E2F2 transgenic animals beyond the age of 12 weeks as normally occurs in wild-type mice. Between the age of 8 and 12 weeks, Eμ-pp-E2F2 mice display mild thymic hyperplasia with on average a 2-fold increase in thymus weight compared with control littermates. One likely explanation for the observed thymic hyperplasia is an increased proliferative capacity of E2F2 transgenic TECs, which correlates with our finding of up-regulated cyclin E expression in Eμ-pp-E2F2 thymi. Furthermore, E2F2 transgenic TECs demonstrate a higher plating efficiency when cultured *in vitro* compared with wild-type control cells (data not shown). Therefore, it is plausible that enforced E2F2 expression could also extend the replicative potential of these TECs. Additional studies will be performed in the near future to address these issues in more detail. In addition, it remains to be established whether cortical epithelial cells are more susceptible than medullary TECs for E2F2-induced hyperproliferation or if this is just the result of differential transgene expression. Remarkably, E2F1 transgenic TECs display reduced viability and cannot be expanded *in vitro*, showing earlier on hallmarks of senescence. Thus, E2F1 and E2F2 transgenic TECs display opposite phenotypes, arguing that different transcriptional targets are regulated by E2F1 and E2F2 in these TECs.

After the initial stage of mild thymic hyperplasia which is observed in all Eμ-pp-E2F2 mice of three independent founder lines (TEF2-33, TEF2-35, and TEF2-37), there is a significant delay of about 5 months before overt thymomas arise from which the mice eventually succumb. These findings suggest that additional genetic mutations need to accumulate to achieve complete immortalization of Eμ-pp-E2F2 cortical epithelial cells. The course of thymic hyperplasia is in this respect rather different from K5-cyclin D1 and D2 mice, where gross TEC hyperplasia is already evident at a much earlier age of 8–10 weeks (43, 44). Thus, deregulated E2F2 expression alone seems not to be sufficient for epithelial cell transformation. Disruption of both p16^{INK4A}/pRb and p53 pathway is essential to bypass the senescence checkpoint in human epithelial cells (56), and inactivation of both pathways contributes to the onset of thymic epithelial tumors in SV40 large T and human papillomavirus 16 E6/E7 transgenic mice (57, 58). These data suggest that mutation of p53/MDM2/p19ARF pathway could be a rate-limiting step for the induction of cortical thymomas in Eμ-pp-E2F2 mice. Alternatively, activation of proto-oncogenes like c-*myc* (59) or c-*fos* (60) may contribute to transformation of TECs.

Our data show that Eμ-pp-E2F1 and Eμ-pp-E2F2 mice display completely distinct phenotypes. Overexpression of E2F1 inhibits chondrocyte differentiation and delays endochondral ossification, resulting in dwarf mice with severely reduced postnatal viability (61). On the other hand, enforced expression of E2F2 has no appreciable effect on endochondral bone formation but selectively induces TEC tumors. Although we have not been able to perform extensive analysis on Eμ-pp-E2F1 mice because of the small numbers of viable adult mice, it is clear that none of the 18 adult E2F1 transgenic animals, subjected to postmortem examination, displayed evidence of thymic hyperplasia. These findings are corroborated by the lack of thymic epithelial hyperplasia in K5-E2F1 transgenic mice (29, 30). Our results support an emerging theme that E2F1 and E2F2 have several different nonredundant roles *in vivo* (11). E2F1 is required for efficient negative selection of thymocytes (12, 14) and impairs pre-B cell development (17). E2F1 inhibits terminal differentiation of various mesenchymal cell lineages, including chondrocytes (61), and suppresses the formation of

lymphomas, hemangio- and histiocytic sarcomas (21). On the other hand, E2F2 is essential for proper erythropoiesis and S phase progression of hematopoietic progenitor cells (17) and regulates peripheral immune tolerance (15).

In conclusion, the presented data show that E2F2 acts as a positive regulator of cell proliferation in TECs and induces thymic epithelial tumors (cortical thymomas), thereby establishing for the first time oncogenic activity of E2F2 *in vivo*.

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