

## Role of F-Box Protein $\beta$ Trcp1 in Mammary Gland Development and Tumorigenesis

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**The F-box protein  $\beta$ Trcp1 controls the stability of several crucial regulators of proliferation and apoptosis, including certain inhibitors of the NF- $\kappa$ B family of transcription factors. Here we show that mammary glands of  $\beta$ Trcp1<sup>-/-</sup> female mice display a hypoplastic phenotype, whereas no effects on cell proliferation are observed in other somatic cells. To investigate further the role of  $\beta$ Trcp1 in mammary gland development, we generated transgenic mice expressing human  $\beta$ Trcp1 targeted to epithelial cells under the control of the mouse mammary tumor virus (MMTV) long terminal repeat promoter. Compared to controls, MMTV  $\beta$ Trcp1 mammary glands display an increase in lateral ductal branching and extensive arrays of alveolus-like protuberances. The mammary epithelia of MMTV  $\beta$ Trcp1 mice proliferate more and show increased NF- $\kappa$ B DNA binding activity and higher levels of nuclear NF- $\kappa$ B p65/RelA. In addition, 38% of transgenic mice develop tumors, including mammary, ovarian, and uterine carcinomas. The targeting of  $\beta$ Trcp1 to lymphoid organs produces no effects on these tissues. In summary, our results support the notion that  $\beta$ Trcp1 positively controls the proliferation of breast epithelium and indicate that alteration of  $\beta$ Trcp1 function and expression may contribute to malignant behavior of breast tumors, at least in part through NF- $\kappa$ B transactivation.**

F-box proteins (FBPs) are defined by the presence of an approximately 40-amino-acid domain named the F box after the protein, cyclin F, in which it was originally identified (2). Studies in different species have shown that FBPs play a crucial role in the ubiquitin-mediated degradation of cellular regulatory proteins (e.g., cyclins, cyclin-dependent kinase inhibitors,  $\beta$ -catenin, I $\kappa$ B, etc.) (reviewed in references 11, 29, and 31). Indeed, FBPs are subunits of ubiquitin ligases named SCFs because they comprise Skp1, Cul1, and one of many FBPs.

$\beta$ Trcp1 ( $\beta$ -transducin repeat-containing protein), the mammalian ortholog of *Xenopus*  $\beta$ TrCP (53), was identified by using either Skp1 or the pseudosubstrate Vpu as bait in two-hybrid screens (13, 41). Mammalian  $\beta$ Trcp1 and the paralogous protein  $\beta$ Trcp2 have been reported to be involved in the degradation of I $\kappa$ B (inhibitor of NF- $\kappa$ B [nuclear factor  $\kappa$ B]) family members in response to NF- $\kappa$ B-activating stimuli (17, 21, 24, 25, 32, 45, 49, 52, 59–61). Several studies have reported that  $\beta$ Trcp1 and  $\beta$ Trcp2 also control  $\beta$ -catenin stability in mammalian cultured cells (23, 24, 30, 37, 43, 59). Furthermore, additional  $\beta$ Trcp substrates have been proposed: Atf4/Creb2 (35), Smad3 (18), Smad4 (57), the alpha interferon receptor (33), the prolactin receptor (38), and the

disks large tumor suppressor (40). More recently,  $\beta$ Trcp1 and  $\beta$ Trcp2 have been implicated in cell cycle control. During the S and G<sub>2</sub> phases, these two FBPs keep Cdk1 inactive by inducing the degradation of its activating phosphatase Cdc25a (9, 28). At the G<sub>2</sub>/M transition,  $\beta$ Trcp1 and  $\beta$ Trcp2 change their specificity by targeting the Cdk1-inactivating kinase Wee1 for degradation (58), resulting in Cdc25a accumulation and Cdk1 activation. Finally, in mitosis  $\beta$ Trcp1 and  $\beta$ Trcp2 promote the degradation of Emi1 (22, 42, 47), an inhibitor of the anaphase-promoting complex/cyclosome, thereby attenuating Cdk1 activity via the anaphase-promoting complex/cyclosome-mediated degradation of two activating cyclin subunits (cyclin A and cyclin B). Thus,  $\beta$ Trcp1 and  $\beta$ Trcp2 contribute to “turning off” Cdk1 in the S and G<sub>2</sub> phases, turning it on at the G<sub>2</sub>/M transition, and turning it off again in late mitosis.

Previously, we have shown that  $\beta$ Trcp1 loss of function in mice does not affect viability but induces an impairment of spermatogenesis and reduced male fertility (22). In the present study, we show that mammary glands of  $\beta$ Trcp1<sup>-/-</sup> mice display a hypoplastic phenotype. To investigate further the role of  $\beta$ Trcp1 in mammary gland development, we generated and characterized a transgenic mouse model in which we targeted  $\beta$ Trcp1 expression to epithelial cells using the mouse mammary tumor virus (MMTV) promoter. In addition, the epithelial-tissue-specific phenotype observed in both the loss-of-function and gain-of-function mouse models was confirmed by generating a transgenic mouse mutant in which  $\beta$ Trcp1 expression was targeted to the lymphoid organs using the CD4 promoter and in which no phenotype was observed. The results of these studies are herein presented.

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## MATERIALS AND METHODS

**Generation of MMTV  $\beta$ Trcp1 and CD4  $\beta$ Trcp1 transgenic mice.** The MMTV  $\beta$ Trcp1 transgene construct was generated by cloning the human  $\beta$ Trcp1 cDNA into the pMSG expression vector that contains an MMTV long terminal repeat promoter and a simian virus 40 polyadenylation signal (15, 56). The CD4  $\beta$ Trcp1 transgene construct was generated by cloning the human  $\beta$ Trcp1 cDNA in a plasmid containing the minimal CD4 enhancer, the minimal murine CD4 promoter, the transcriptional initiation site, and 70 bp of the untranslated first exon and part of the first intron of the murine CD4 gene (36). Constructs were injected into the pronuclei of fertilized eggs from B6D2F1 donors, which were subsequently transferred into pseudopregnant mice. The strain was maintained on a BALB/c genetic background. Screening of founder animals and their offspring was performed by genomic PCR and confirmed by Southern hybridization on genomic DNA from tail biopsy samples. The following primers were used for genomic PCR: 5'-CTGGCTATCATCACAAGAGCGGAACGGA-3' and 5'-CCAAGCTTCTCCACAGCATGCCGTC-3'.

**RNA extraction and reverse transcription-PCR (RT-PCR) analysis.** Total RNA was extracted using RNeasy (QIAGEN) according to the manufacturer's instructions. RNA was quantified, and its purity was determined by standard spectrophotometric methods. cDNA was synthesized from 1  $\mu$ g of the total RNA by using the Omniscript RT kit (QIAGEN). The following primers specific to human  $\beta$ Trcp1 were used: F, 5'-AATTCCTCAGAGAGAGAAGACT-3', and R, 5'-TCTGGCAAAACACTAAATATAT-3'. To amplify mouse GAPDH, the following primers were used: F, 5'-GGGTGGAGCCAAACGGGTC-3', and R, 5'-GGAGTTGCTGTTGAAGTCGCA-3'. cDNA was amplified using 1 U of *Taq* DNA polymerase (Fermentas Inc., Hanover, Md.), and amplification was performed in a PTC-100 thermal cycler (MJ Research Inc.) for 35 cycles (denaturation at 94°C for 1 min, annealing for 1 min, and extension at 72°C for 2 min). The annealing temperatures were as follows: for human  $\beta$ Trcp1, 55°C; for mouse GAPDH, 58°C. The amplification reaction products were resolved on 2.0% agarose-Tris-acetate-EDTA gels, electrophoresed at 100 V, and visualized by ethidium bromide staining.

**Histology and whole-mount preparation.** The animals were monitored daily. Necropsies were performed on all animals that died spontaneously during the observation period. For histological analysis, mammary glands and other tissues were fixed overnight or longer in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned. They were stained with hematoxylin and eosin for histological analysis. For whole-mount tissue preparations, mammary glands were processed as described previously (27).

**Immunohistochemistry and bromodeoxyuridine (BrdU) incorporation.** For immunohistochemical staining, deparaffinized sections were immersed in methanol containing 0.03% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The slides were subjected to microwaving in 10 mM citrate buffer three times for 5 min and then incubated with Protein Block Serum-Free (Dako, Tucson, Ariz.) for 30 min to block the nonspecific antibody binding sites. The slides were treated with an anti-p65/RelA polyclonal antibody (1:100; Santa Cruz) at 4°C overnight and then with EnVision+ (Dako) for 30 min. Peroxidase activity was developed using 3,3'-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl (pH 7.5) containing 0.001% hydrogen peroxidase. The sections were slightly counterstained with Mayer's hematoxylin. For the negative control, the primary antibodies were replaced with normal mouse serum.

For BrdU incorporation, a sterile solution containing 10 mg of BrdU (Sigma)/ml and 0.6 mg of fluorodeoxyuridine (Sigma)/ml in phosphate-buffered saline was injected intraperitoneally (100  $\mu$ l/10 g of body weight). After 2 h, the mice were sacrificed, mammary glands were collected and fixed, and 5- $\mu$ m sections were prepared. BrdU incorporation was visualized by immunohistochemistry using a BrdU detection kit (Zymed Laboratories), and the nuclei were counterstained with hematoxylin. For quantification, 10 random fields per section at a magnification of 40 $\times$  were documented by photomicroscopy, and the percentage of BrdU-positive epithelial-cell nuclei relative to the total number of epithelial-cell nuclei was calculated.

**Electrophoretic mobility shift assay.** The electrophoretic mobility shift assay was performed as described previously (4, 46). Briefly, 3  $\mu$ l (approximately 3  $\mu$ g) of cell extract was incubated for 20 min at room temperature in 20  $\mu$ l of buffer (20 mM Tris [pH 7.4], 5% glycerol, 0.1% Tween 20, 0.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM EDTA, 50 mM KCl) containing 2  $\mu$ g of poly(dI-dC) and a  $\kappa$ B probe (100,000 cpm) labeled using Klenow fill-in. The probe was the palindromic  $\kappa$ B probe previously described (6). The mixture was then separated on a native polyacrylamide gel that was dried and exposed for autoradiography.

**Immunoblotting and Northern blotting.** Immunoblotting and Northern blotting were performed as previously described (12, 22). Antibodies to cyclin A (3), Emi1,  $\beta$ -catenin, and I $\kappa$ B $\alpha$  (22) were previously described. Antibodies to I $\kappa$ B $\beta$ ,

NF- $\kappa$ B1/p105, and keratin 18 were from Santa Cruz Biotechnology, antibody to c-Myc was from Sigma, and antibody to cyclin D1 was from Zymed.

## RESULTS

**Hypoplastic phenotype of  $\beta$ Trcp1<sup>-/-</sup> mammary gland.** To study the function of  $\beta$ Trcp1 in the development of the mammary gland, we analyzed whole-mount preparations of mammary glands from wild-type and  $\beta$ Trcp1-deficient female virgin mice. In comparison with those of wild-type mice, mammary glands from  $\beta$ Trcp1<sup>-/-</sup> mice showed a hypobranching phenotype (Fig. 1a, b, d, and e). Histological examination confirmed the hypoplasia of  $\beta$ Trcp1<sup>-/-</sup> mammary glands (Fig. 1c and f). In virgin adults (approximately 6 months of age), this phenotype was highly penetrant (found in 9 of 10 mice). In contrast, no significant differences between the two phenotypes were observed during pubertal ductal elongation, pregnancy, and lactation, the times of maximal hormonal stimulation.

To determine whether the observed defects in mammary gland development were the result of reduced cell proliferation, DNA synthesis was measured in an in vivo BrdU incorporation assay. Six-month-old virgin mice were administered BrdU, whose incorporation into DNA was then detected by immunohistochemistry (Fig. 1g). In  $\beta$ Trcp1<sup>-/-</sup> mammary glands, the proliferation index, calculated as the percentage of BrdU-positive cells out of the total epithelial cells, was approximately 50% of that measured in estrus-matched wild-type mice (5.6%  $\pm$  1.9% versus 9.9%  $\pm$  2.2%;  $P = 0.001$ ). In contrast, no differences in BrdU incorporation were detected in other organs, such as intestine and skin (Fig. 1g). Apoptosis, measured as the percentage of cells positive by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL), at different ages was not different between the two genotypes (data not shown). The presence of a hyperplastic phenotype in the mammary glands of  $\beta$ Trcp1<sup>-/-</sup> mice was not the result of a lack of  $\beta$ Trcp2 expression in mammary epithelial cells, as shown by Northern blot analysis (Fig. 1h).

Thus, we conclude that  $\beta$ Trcp1 is necessary for the proper development of the mouse mammary gland under conditions of low hormonal stimuli.

**Targeted expression of  $\beta$ Trcp1 induces hyperplasia and transformation in epithelial organs.** To further analyze the role of  $\beta$ Trcp1 in the development of the mammary gland epithelium, we generated  $\beta$ Trcp1 transgenic mice by placing the human  $\beta$ Trcp1 cDNA under the control of the MMTV long terminal repeat promoter (Fig. 2A to C). Using this construct, three independent  $\beta$ Trcp1 transgenic lines of mice were obtained, but all the experiments described herein were performed with two MMTV  $\beta$ Trcp1 transgenic lines (lines 2 and 19) that expressed similar  $\beta$ Trcp1 levels in the breast epithelium (Fig. 2A).

MMTV  $\beta$ Trcp1 mice appeared normal at birth, and their growth was indistinguishable from that of their wild-type littermates. Compared to those of wild-type virgin female mice, the mammary glands of MMTV  $\beta$ Trcp1 mice (six mice, approximately 10 weeks of age, for each genotype) were found by whole-mount analysis to display an increase in lateral ductal branching and extensive arrays of alveolus-like hyperplasia (Fig. 3A, panels a and d). At a higher magnification, the ducts were shorter, wider, and covered with small alveolus-like pro-

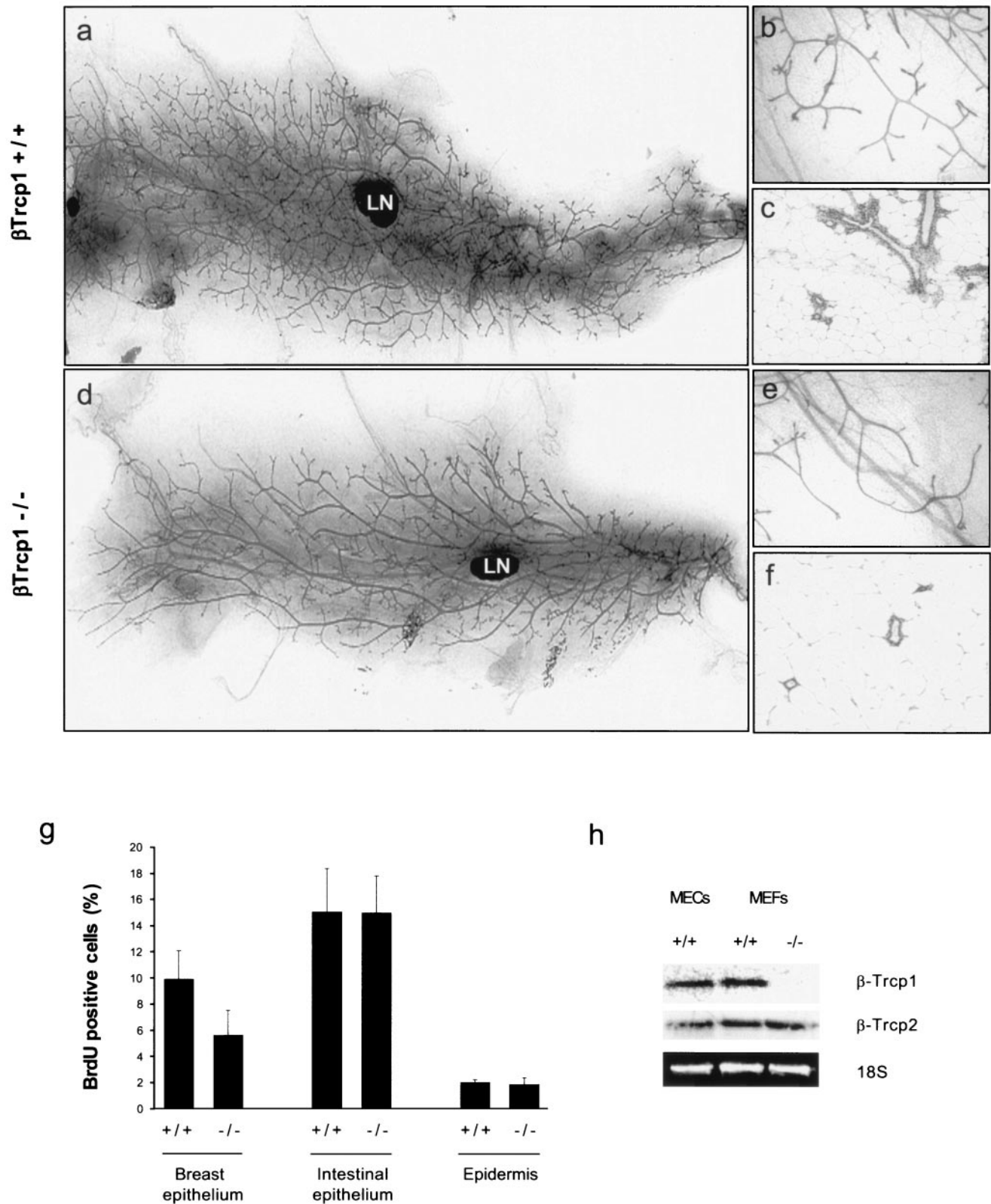


FIG. 1. Mammary gland hypoplasia in  $\beta$ Trcp1<sup>-/-</sup> mice. (a to f) Whole-mount preparations (a, b, d, and e) and hematoxylin and eosin-stained sections (c and f) of inguinal mammary glands from 6-month-old virgin  $\beta$ Trcp1<sup>-/-</sup> mice (d to f) and wild-type littermates (a to c). LN, lymph node. (g) BrdU incorporation in the indicated organs of virgin  $\beta$ Trcp1<sup>-/-</sup> mice and wild-type littermates. The percentages of BrdU-positive nuclei were calculated. Error bars represent standard errors of the means. (h) Levels of  $\beta$ Trcp1 and  $\beta$ Trcp2 mRNAs in mammary epithelial cells (MECs) and mouse embryonic fibroblasts (MEFs).

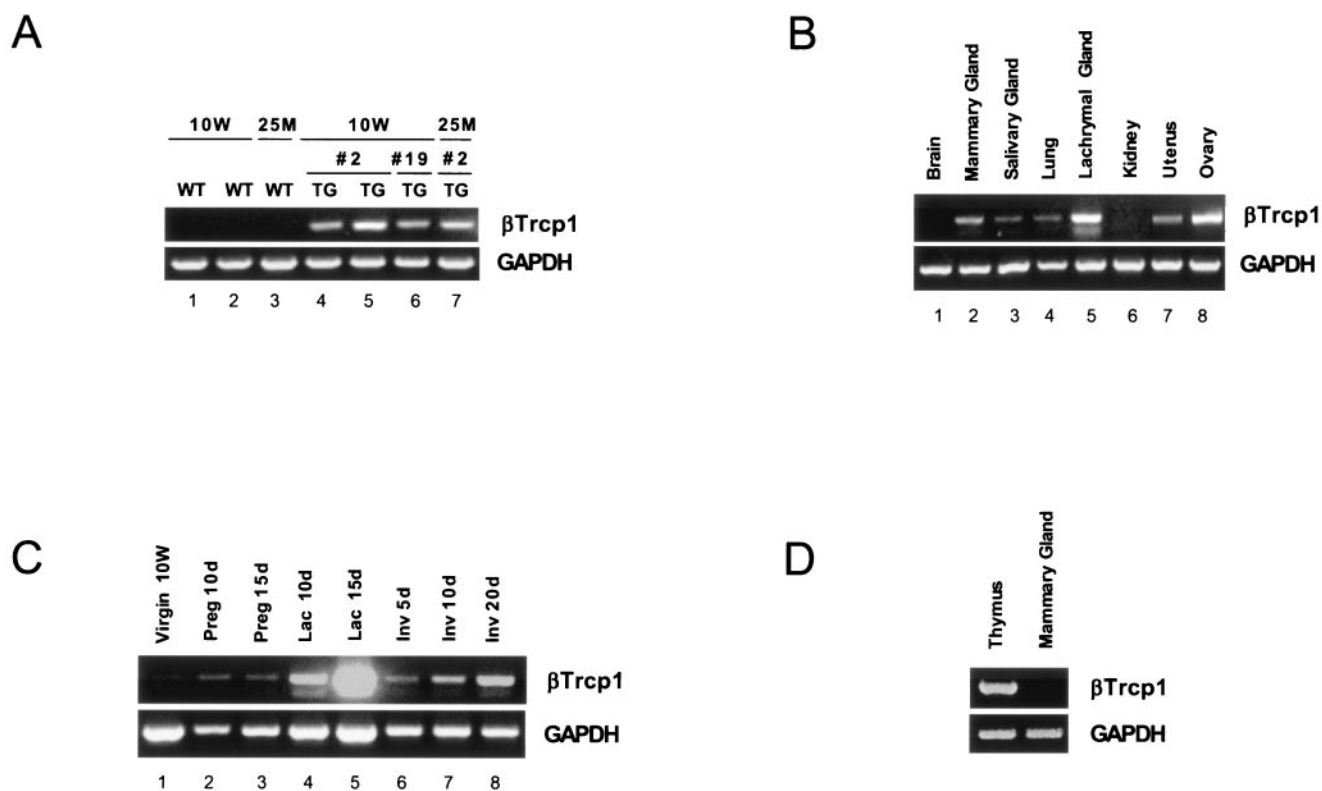


FIG. 2. Characterization of MMTV  $\beta$ Trcp1 and CD4  $\beta$ Trcp1 transgene expressions. (A) RT-PCR analysis of the  $\beta$ Trcp1 transgene expression in mammary glands. RT-PCR was performed with primers specific to human  $\beta$ Trcp1 or to mouse GAPDH; 1  $\mu$ g of total RNA prepared from mammary glands of the indicated lines of 10-week-old (10W) and 25-month-old (25M) transgenic (TG) and wild-type (WT) virgin mice. (B) RT-PCR analysis of MMTV  $\beta$ Trcp1 transgene expression in the indicated organs. (C) RT-PCR analysis of transgene expression in mammary glands of 10W virgin mice or during pregnancy (Preg), lactation (Lac), or involution (Inv) at different days (d). (D) RT-PCR analysis of the CD4- $\beta$ Trcp1 transgene expression in thymus and mammary gland.

tuberances (Fig. 3A, panels b and e). In histological sections the number of ducts was increased in comparison to wild-type glands. In addition, the ductal epithelial-cell layers of MMTV  $\beta$ Trcp1 glands were thicker than those from wild-type mice (Fig. 3A, panels c and f), confirming the pervasive ductal hyperplasia. The observed hyperplasia of the mammary gland correlated with a twofold increase in cell proliferation, as measured by an in vivo BrdU incorporation assay ( $28.1\% \pm 5.2\%$  versus  $14.4\% \pm 2.9\%$ ;  $P = 0.016$ ) (Fig. 3A, panel g). In contrast, apoptosis, measured as the percentage of TUNEL-positive cells, was not significantly different in either virgin (data not shown) or breeder transgenic mice during mammary involution (Fig. 3A, panel h), although it appeared somehow delayed (values at day 10 for transgenic mice were similar to values at day 5 for wild-type animals, and vice versa).

The aberrant hyperplasia of mammary glands of  $\beta$ Trcp1 transgenic mice was maintained throughout life. In older animals (14 to 22 months of age), the difference with the controls was dramatic (Fig. 3B, panels a to f). This mammary-gland hyperplasia was observed in 8 out of 10 virgin aged  $\beta$ Trcp1 transgenic mice examined, but not in wild-type virgin aged mice (0 out of 8). In addition, malignant transformation of the mammary gland was observed in two  $\beta$ Trcp1 transgenic animals ( $n = 47$ ) but was never observed in the control mice ( $n = 43$ ). Histopathological analysis indicated that these two mam-

mary tumors were adenocarcinomas, one showing both a tubular pattern (well-formed duct-like structures) and a papillary pattern and the other displaying a solid tubular pattern (Fig. 4A). Overall, 38% of MMTV  $\beta$ Trcp1 females developed epithelial tumors, including mammary, ovarian, and uterine tumors, at an incidence significantly higher than that in wild-type mice (Fig. 4B and D). The presence of tumors in other epithelial tissues is not surprising since the MMTV promoter targets  $\beta$ Trcp1 expression not only to the mammary gland but also to uterine, ovarian, and other epithelial cells, such as those of the salivary glands, lachrymal glands, and lung (Fig. 2B).

These results suggest that targeted expression of  $\beta$ Trcp1 induces hyperplasia and transformation in epithelial organs, particularly those devoted to reproduction and lactation. To confirm this specificity, we generated transgenic mice expressing  $\beta$ Trcp1 targeted to the T-lymphoid lineage by using the murine CD4 promoter to drive the expression in thymic T cells (Fig. 2D). Despite a  $\beta$ Trcp1 expression similar to that achieved in breast cells, no difference in cell proliferation (data not shown) and tumor incidence (Fig. 4C) was observed between CD4 transgenic mice and controls.

**$\beta$ Trcp1 activates NF- $\kappa$ B in breast epithelium.**  $\beta$ Trcp1 mediates the ubiquitination of several cellular substrates including I $\kappa$ B family members (i.e., I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ ) resulting in their proteasome-dependent degradation and consequent ac-

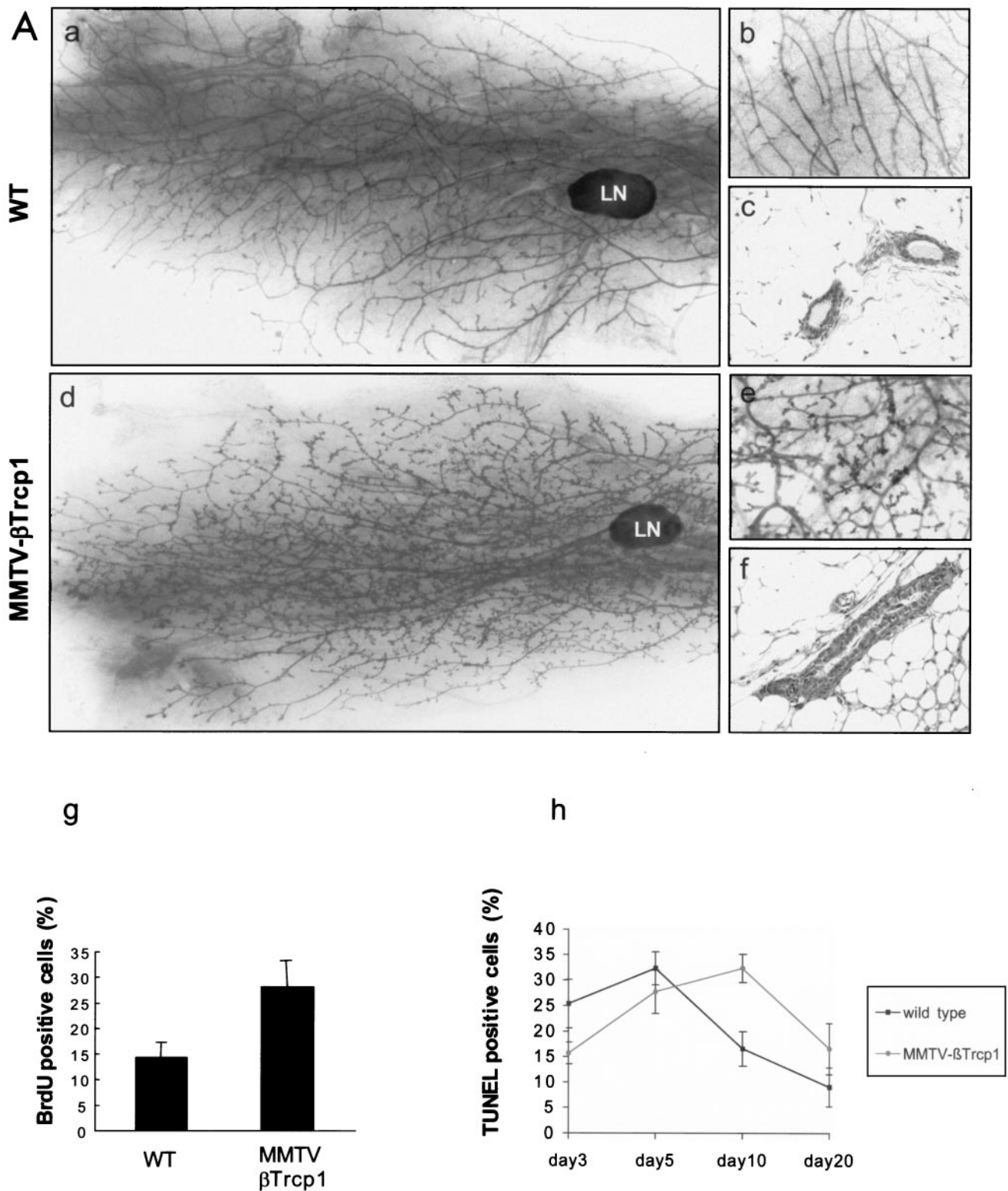


FIG. 3. Mammary gland hyperplasia in MMTV  $\beta$ Trcp1 transgenic mice. (A) Whole mounts (a, b, d, and e) and hematoxylin and eosin-stained sections (c and f) of inguinal mammary glands from 10-week-old virgin MMTV  $\beta$ Trcp1 transgenic mice (d to f) and wild-type (WT) littermates (a to c). LN, lymph node. (g) BrdU incorporation in breast epithelium of 10-week-old virgin WT and estrus-matched MMTV  $\beta$ Trcp1 transgenic littermates. The percentages of BrdU-positive nuclei were calculated. (h) Percentages of TUNEL-positive nuclei at different days after removal of pups. Error bars represent standard errors of the means. (B) Whole mounts (a, b, d, and e) and hematoxylin and eosin-stained (c and f) sections of inguinal mammary glands from 22-month-old WT mice (a to c) and MMTV  $\beta$ Trcp1 transgenic littermates (d to f).

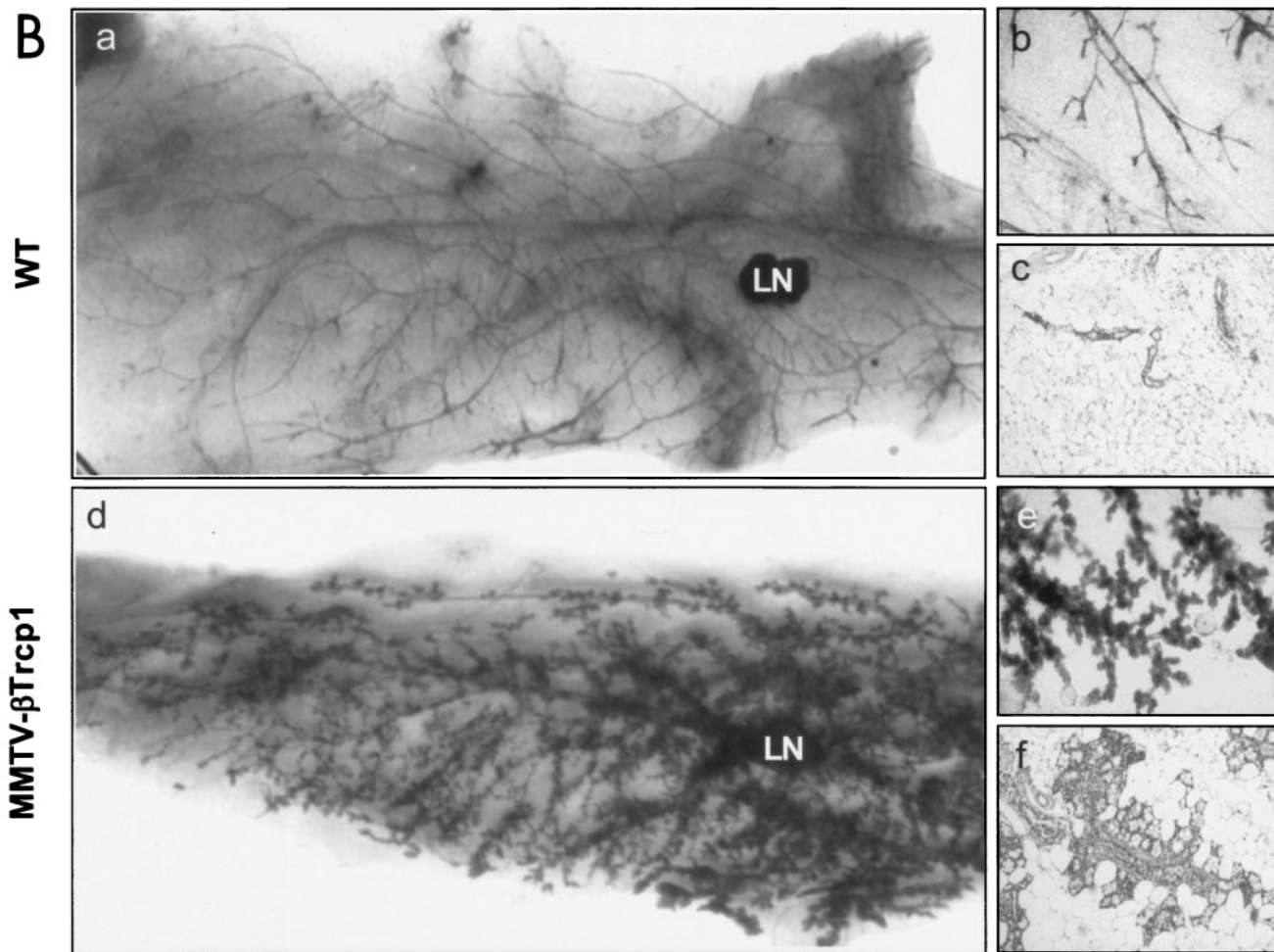


FIG. 3—Continued.

tivation of the NF- $\kappa$ B transcription activity. To determine whether  $\beta$ Trcp1 expression in mammary glands was associated with an increased activation of NF- $\kappa$ B, we examined the DNA binding activity of NF- $\kappa$ B in the mammary, lachrymal, and salivary glands, in the uterus, and in the ovary. Mammary gland, ovary, and uterus of 10-week-old virgin transgenic females showed higher NF- $\kappa$ B activity in comparison with that of wild-type females (Fig. 5A and data not shown). This increase was also evident in mammary glands of older mice (6 and 22 months old) and in breast tumors in MMTV  $\beta$ Trcp1 transgenic mice (Fig. 5A, panel b).

As an independent way to test the status of the NF- $\kappa$ B pathway (7), we examined by immunohistochemistry the nuclear localization of p65/RelA, which increases in response to NF- $\kappa$ B activation. The nuclear localization of p65/RelA in mammary epithelial cells of 10-week-old MMTV  $\beta$ Trcp1 transgenic mice was much higher than that in wild-type animals (Fig. 5B).

Lastly, we analyzed by immunoblotting the expression of cell cycle regulatory proteins and members of the NF- $\kappa$ B and  $\beta$ -catenin pathways in mammary glands from 10-week-old virgin wild-type and  $\beta$ Trcp1 transgenic mice (Fig. 5C). In 75% of

the cases (eight mice for each genotype), we observed a significant increase in the levels of cyclin D1 in mammary glands from  $\beta$ Trcp1 transgenic mice compared with those in wild-type glands. In contrast, the levels of I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , NF- $\kappa$ B1/p105, NF- $\kappa$ B2/p100,  $\beta$ -catenin, cyclin A, Emi1, Wee1, and c-Myc were identical in wild-type and  $\beta$ Trcp1 transgenic mammary glands (Fig. 5C and data not shown).

### DISCUSSION

A  $\beta$ Trcp1-deficient mouse mutant was previously generated, and what at that time was an unsuspected role for  $\beta$ Trcp1 in controlling both meiosis and mitosis was found (22). In the present study, we have found that in the absence of elevated hormonal stimuli, loss of  $\beta$ Trcp1 induces a hypoplastic phenotype in mammary glands (Fig. 1). In addition, cell proliferation is decreased in breast epithelium but not in other organs, such as skin and intestine (Fig. 1). Similarly, we were unable to find alterations in the lymphoid compartments of  $\beta$ Trcp1<sup>-/-</sup> mice. We investigated the development of T and B cells and extensively tested for the presence of alterations in the immune response and defects in the apoptotic response without finding

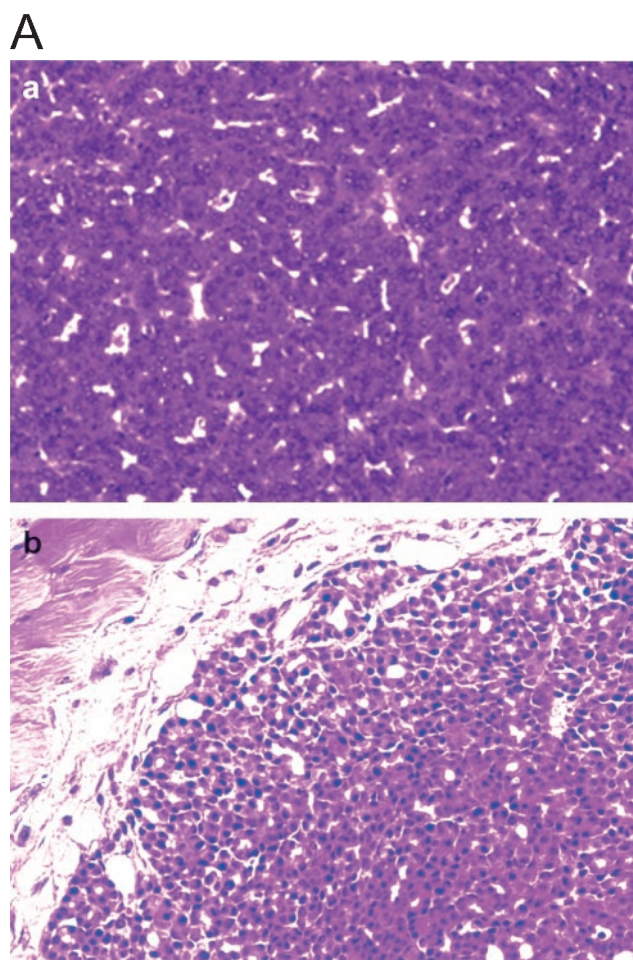
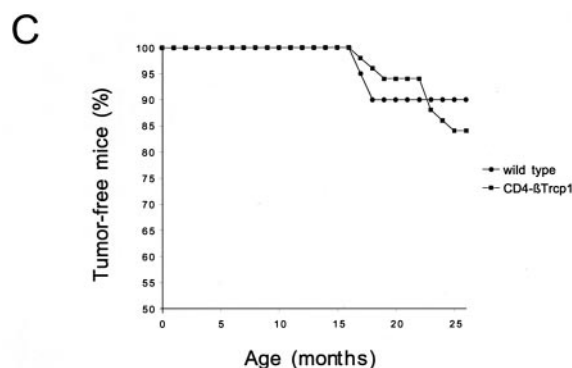
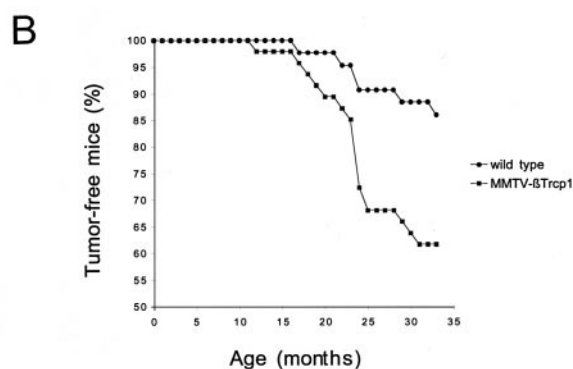


FIG. 4.  $\beta$ Trcp1 expression induces tumorigenesis in epithelial organs. (A) Hematoxylin and eosin-stained section of two mammary adenocarcinomas found in MMTV  $\beta$ Trcp1 mice, one with a papillo-tubular pattern (a) and the other displaying a solid tubular pattern (b). (B) Graph of tumor incidence in MMTV  $\beta$ Trcp1 transgenic mice and control wild-type littermates. The percentage of animals in each cohort remaining free of palpable tumors was plotted as a function of age for  $\beta$ Trcp1 transgenic mice ( $n = 47$ ) and wild-type mice ( $n = 43$ ). (C) Graph of tumor incidence in CD4  $\beta$ Trcp1 transgenic mice and control wild-type littermates. The percentage of animals in each cohort remaining free of palpable tumors was plotted as a function of age for  $\beta$ Trcp1 transgenic mice ( $n = 49$ ) and wild-type mice ( $n = 20$ ). (D) Table showing tumors arising in MMTV  $\beta$ Trcp1 mice (TG) and control littermates (WT). There was no significant difference in the development of tumors between virgin and breeder MMTV  $\beta$ Trcp1 females. The difference between total number of tumors and total number of mice in the TG column (indicated by an asterisk) is due to the presence of two tumors found in the same mouse.

any significant difference between mutant and wild-type mice (data not shown). Accordingly, we did not observe any aberrant response of thymocytes and macrophages of  $\beta$ Trcp1<sup>-/-</sup> mice to a variety of stimuli or stresses (22).

To confirm a role for  $\beta$ Trcp1 in the development of the mammary gland, we generated an MMTV  $\beta$ Trcp1 transgenic mouse mutant (Fig. 2A). We found that targeting expression of  $\beta$ Trcp1 to the mammary gland enhances proliferation and induces hyperbranching and hyperplastic nodules (Fig. 3A), a



Tumor type	No. of tumors	
	WT (n=43)	TG (n=47)
Mammary gland adenocarcinoma	0	2
Ovary tumor	0	4
Uterine tumor	2	7
Lung adenocarcinoma	2	4
Lymphoma	2	2
<b>Total tumors</b>	<b>6</b>	<b>19</b>
<b>Total mice with tumors</b>	<b>6 (14%)</b>	<b>18 (38%) *</b>

phenotype exactly opposite to that observed in the loss-of-function model. The phenotype observed in MMTV  $\beta$ Trcp1 mice is most evident in aged animals, in which mammary hyperplasia is observed in 80% of cases (Fig. 3B). Moreover, 38% of MMTV  $\beta$ Trcp1 mice developed tumors (Fig. 4D) localized to the breast, ovary, uterus, and lung, all organs in which the MMTV promoter induces  $\beta$ Trcp1 expression (Fig. 2B). In contrast, targeting  $\beta$ Trcp1 expression to lymphoid organs using the T-cell-specific promoter CD4 did not produce any effect on either cell proliferation (data not shown) or tumor development (Fig. 4C).

NF- $\kappa$ B is the generic term for a family of transcription factors that regulates key genes involved in cell proliferation, apoptosis, immune responses, and inflammation (19). NF- $\kappa$ B is activated by a wide variety of different stimuli, including proinflammatory cytokines such as tumor necrosis factor alpha

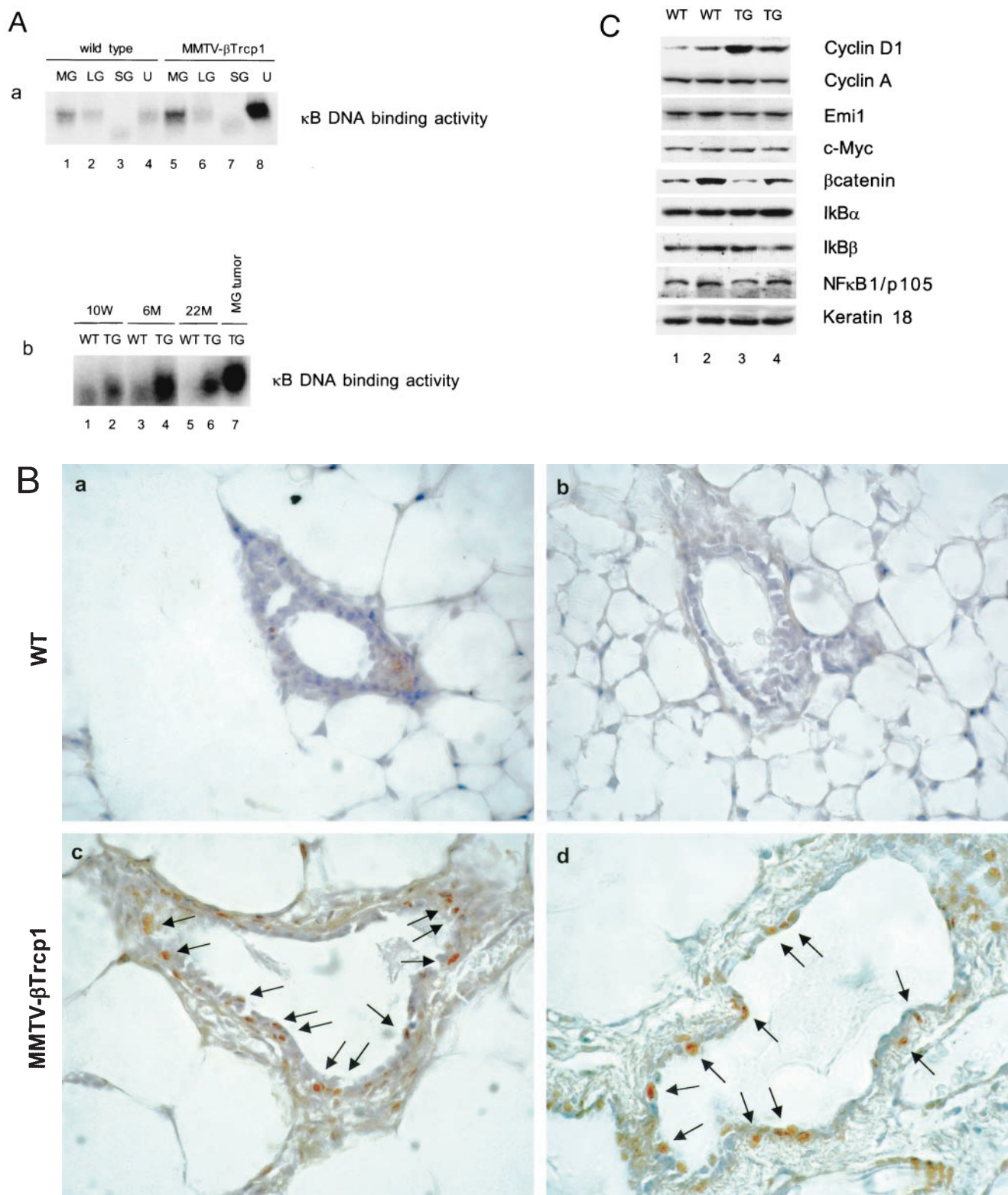


FIG. 5. NF- $\kappa$ B activity is increased in MMTV  $\beta$ Trcp1 transgenic mice. (A) DNA binding activity of NF- $\kappa$ B in mammary gland (MG), lachrymal gland (LG), salivary gland (SG), and uterine horn (U) (a). NF- $\kappa$ B activity during the development of mammary gland (virgin mice at 10 weeks and 6 and 22 months of age) and in a breast tumor (MG tumor) in an MMTV  $\beta$ Trcp1 transgenic mouse (b). (B) Nuclear expression of p65/RelA in mammary glands of 10-week-old wild-type and MMTV  $\beta$ Trcp1 transgenic mice. Arrows show representative immunopositive cells with nuclear staining. (C) Extracts from mammary glands of two representative 10-week-old wild-type (WT) and two  $\beta$ Trcp1 transgenic (TG) mice were subjected to immunoblotting with the indicated antibodies (keratin 18 is shown as a control for the epithelial content of the sample).



and interleukin-1, bacteria, and bacterial lipopolysaccharide, viruses, viral proteins, double-stranded RNA, and physical and chemical stresses. After stimulation, the I $\kappa$ B kinase, IKK, mediates phosphorylation of I $\kappa$ B on two critical serine residues, leading to the immediate recognition by  $\beta$ Trcp and consequent polyubiquitinylation of I $\kappa$ B via the SCF $^{\beta$ Trcp complex (21, 24, 25, 32, 45, 49, 52, 59–61). The consequent rapid degradation of I $\kappa$ B by the 26S proteasome exposes the nuclear localization sequence of NF- $\kappa$ B family members (e.g., p50 and p65/RelA), resulting in their translocation to the nucleus, where they induce the transcription of target genes. NF- $\kappa$ B1/p105 functions both as a precursor of NF- $\kappa$ B/p50 and as a cytoplasmic inhibitor of NF- $\kappa$ B, and its degradation is also mediated by  $\beta$ Trcp (26, 34).

We found that the increase in cell proliferation observed in MMTV  $\beta$ Trcp1 mice associates with an activation of the NF- $\kappa$ B pathway determined by measuring both  $\kappa$ B DNA binding activity and the abundance of nuclear p65/RelA (Fig. 5A and B). The positive regulation of NF- $\kappa$ B activity by  $\beta$ Trcp1 is likely to represent one of the reasons for the changes in cell proliferation observed in the mammary glands of our mouse models. Consistent with this hypothesis, an increase or a decrease in BrdU incorporation was observed in breast epithelium lacking I $\kappa$ B $\alpha$  (7) or expressing an inactive IKK $\alpha$  (10), respectively. These two former studies also agree with ours on another issue: in all three cases, no significant differences in apoptosis rates were observed in mammary glands upon NF- $\kappa$ B activation, despite the well-established role of this transcription factor in inhibiting apoptosis. Since NF- $\kappa$ B is particularly activated during pregnancy, peaking around days 15 to 16 post coitum and during involution, it is possible that a further increase in activity is not achievable because of a plateau. Finally, the work by Cao and colleagues shows that cyclin D1 is a critical effector of the network that controls mammary epithelial proliferation in response to NF- $\kappa$ B activation (10). Similarly, we found elevated levels of cyclin D1 in mammary glands of  $\beta$ Trcp1 transgenic mice (Fig. 5C), which display high NF- $\kappa$ B activity.

NF- $\kappa$ B activation not only plays an important role in the development of the mouse mammary gland (7, 8, 10), but it is also known as a key player in oncogenesis, promoting proliferation and inhibiting apoptosis (reviewed in reference 39). Elevated and/or constitutive NF- $\kappa$ B activation is found in a variety of tumors (20), including breast cancers (14, 44, 50, 51). In addition, transgenic mice overexpressing the NF- $\kappa$ B family member c-Rel in breast epithelium develop late-onset mammary carcinomas (48). Our data confirm and extend this notion by showing a role for  $\beta$ Trcp1, an activator of NF- $\kappa$ B, in controlling proliferation of breast epithelial cells and in contributing to their transformation. Given the well-established role of cyclin D1 in the growth control and transformation of breast epithelium, it is possible that the effect of  $\beta$ Trcp1 on breast tumorigenesis is, at least in part, mediated by the increase in cyclin D1 levels (Fig. 5C). In agreement with our studies of breast tumors,  $\beta$ Trcp1 has been found to be overexpressed in human colorectal carcinomas, and Wnt signaling induces the expression of  $\beta$ Trcp1 in colon cancer cell lines with a consequent upregulation of NF- $\kappa$ B transactivation (54). Furthermore,  $\beta$ Trcp2 is upregulated in tumor cell lines (including breast cancer lines) (55) and in chemically induced mouse

papillomas and squamous-cell carcinomas, and this overexpression is associated with constitutive activation of NF- $\kappa$ B (5).

How does  $\beta$ Trcp1 activate NF- $\kappa$ B in the MMTV  $\beta$ Trcp1 mice? Are other substrates affected by the lack or overexpression of  $\beta$ Trcp1 in breast and other epithelial cells? The SCF $^{\beta$ Trcp complex targets several substrates for degradation, including I $\kappa$ B family members, NF- $\kappa$ B1/p105,  $\beta$ -catenin, Emi1, Cdc25a, Wee1, Atf4/Creb2, Smad3/4, the alpha interferon receptor, the prolactin receptor, and the disks large tumor suppressor. In addition,  $\beta$ Trcp1 activates NF- $\kappa$ B by inducing the processing of NF- $\kappa$ B2/p100 (1, 16). We could not observe in MMTV  $\beta$ Trcp1 or  $\beta$ Trcp1 $^{-/-}$  mice any significant changes in the levels of  $\beta$ Trcp1 substrates by either immunoblotting (for I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , NF- $\kappa$ B1/p105, NF- $\kappa$ B2/p100,  $\beta$ -catenin, Emi1, and Wee1) or immunohistochemistry ( $\beta$ -catenin) (Fig. 5C and data not shown). However, we could observe a clear effect on NF- $\kappa$ B activation. A possible explanation for the lack of changes in  $\beta$ Trcp1-deficient mice is the redundancy with  $\beta$ Trcp2, which is well expressed in breast epithelial cells (Fig. 1h). In transgenic animals the explanation is more difficult; however, it needs to be reiterated that while on one hand  $\beta$ Trcp induces I $\kappa$ B $\alpha$  degradation, on the other, by activating NF- $\kappa$ B, it induces the synthesis of I $\kappa$ B $\alpha$ , obscuring I $\kappa$ B $\alpha$  proteolysis. In addition, it is possible that levels of endogenous  $\beta$ Trcp1 are limiting and that the forced expression of  $\beta$ Trcp1 induces small perturbations in the degradation of I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , and NF- $\kappa$ B1/p105 and in the processing of NF- $\kappa$ B2/p100, the sum of which produces, as a final result, a prolonged and enhanced activation of NF- $\kappa$ B.

So far, of more than 70 human FBPs, only 4 have been well characterized and matched to downstream substrates:  $\beta$ Trcp1,  $\beta$ Trcp2, Skp2, and Fbw7. Of these, Skp2 is the product of a protooncogene, while Fbw7 is a tumor suppressor protein. The results herein demonstrate a role for  $\beta$ Trcp1 in the development of the mammary gland. Furthermore, our results indicate that  $\beta$ Trcp1 might contribute to the malignant behavior of breast and other epithelial cells by providing a gain of function, at least in part via the overactivation of NF- $\kappa$ B.

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