Disruption of the gene encoding the latent transforming growth factor-β binding protein 4 (LTBP-4) causes abnormal lung development, cardiomyopathy, and colorectal cancer

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Transforming growth factor-βs (TGF-βs) are multifunctional growth factors that are secreted as inactive (latent) precursors in large protein complexes. These complexes include the latency-associated propeptide (LAP) and a latent transforming growth factor-β binding protein (LTBP). Four isoforms of LTBPs (LTBP-1–LTBP-4) have been cloned and are believed to be structural components of connective tissue microfibrils and local regulators of TGF-β tissue deposition and signaling. By using a gene trap strategy that selects for integrations into genes induced transiently during early mouse development, we have disrupted the mouse homolog of the human _LTBP-4_ gene. Mice homozygous for the disrupted allele develop severe pulmonary emphysema, cardiomyopathy, and colorectal cancer. These highly tissue-specific abnormalities are associated with profound defects in the elastic fiber structure and with a reduced deposition of TGF-β in the extracellular space. As a consequence, epithelial cells have reduced levels of phosphorylated Smad2 proteins, overexpress c-myc, and undergo uncontrolled proliferation. This phenotype supports the predicted dual role of LTBP-4 as a structural component of the extracellular matrix and as a local regulator of TGF-β tissue deposition and signaling.

[_Key Words_: LTBP; TGF-β; extracellular matrix; colorectal cancer; pulmonary emphysema; cardiomyopathy]

Transforming growth factor-βs (TGF-βs) belong to a protein superfamily whose members are necessary for normal development. They also control cell growth and differentiation in a variety of adult tissues and are involved in a wide range of immune responses (Massague and Wotton 2000; Massague et al. 2000). Most cells secrete TGF-β as a functionally inactive, latent cytokine complexed to its latency-associated propeptide (LAP), which interacts with its receptors only after activation. There are two known, structurally different latent complexes of TGF-β. The small latent complex consists of a mature TGF-β dimer bound to the N-terminal portion of the TGF-β–LAP propeptide. The large latent complex contains, in addition to TGF-β–LAP, one of the latent TGF-β-binding proteins (LTBPs). Unless displaced from the LTBPs after secretion, TGF-β is usually deposited in the extracellular matrix (ECM) as a high-molecular-weight complex with LTBP and LAP (Taipale et al. 1998; Koli et al. 2001).

LTBPs belong to the family of fibrillin/LTBP glycoproteins. More than 60% of the protein structure of the LTBP family is composed of repetitive EGF-like domains and 8-cystein repeats, both of which are believed to mediate protein–protein interactions. Like the fibrillins, LTBPs are structural components of the extracellular matrix (ECM) microfibrils, which associate with elastic fibers. Although LTBPs are not involved directly in TGF-β latency, most cells secrete TGF-β in complex with LTBP and, by targeting TGF-β to the ECM, form extracellular deposits of TGF-β. This is believed to pro-

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vide tissues with a rapidly inducible and highly localized TGF-β signal (Taipale et al. 1998; Koli et al. 2001).

Four isoforms of LTBPs (LTBP-1–LTBP-4) have been cloned thus far (Tsuji et al. 1990; Moren et al. 1994; Yin et al. 1995; Giltay et al. 1997; Saharinen et al. 1998). Each has several splice variants, and at least one (LTBP-1) has two alternative promoters (Koski et al. 1999). The expression of both, isoforms and splice variants, appears tightly controlled in a tissue-specific manner.

Although multiple in vitro studies predicted a dual role for LTBPs as structural components of the extracellular matrix (ECM) and as local regulators of TGF-β signaling (Taipale et al. 1998), their functions at the organismal level are largely unknown. Here we show that severely reduced expression of LTBP-4 (Saharinen et al. 1998), caused by a gene trap integration into the mouse *LTBP-4* gene, causes a severe pulmonary emphysema-like condition associated with cardiomyopathy and colorectal cancer in transgenic mice. These abnormalities are correlated with a major fragmentation of elastin fibers and a defective TGF-β signaling.

Results

A stem cell clone recovered from a gene trap screen generates a complex disease phenotype in transgenic mice

From a larger screen for gene trap integrations into genes induced during embryonic stem (ES) cell differentiation (Thorey et al. 1998), we obtained one integration (3C7) that generated a complex disease phenotype when bred to homozygosity in transgenic mice (Table 1).

By the age of 4 wk, 3C7 mice developed a rectal prolapse induced by an extensive edema in the mucosal layer of the colorectal region (Fig. 1A). Microscopically, the region showed solitary ulcers associated with epithelial hyperplasia and occasional crypts infiltrating the submucosal layer of the colonic wall (Fig. 1C,D). These early lesions are typical for a mucosa prolapse syndrome and most likely reflect some structural abnormalities of the colonic wall (du Boulay et al. 1983). At older ages
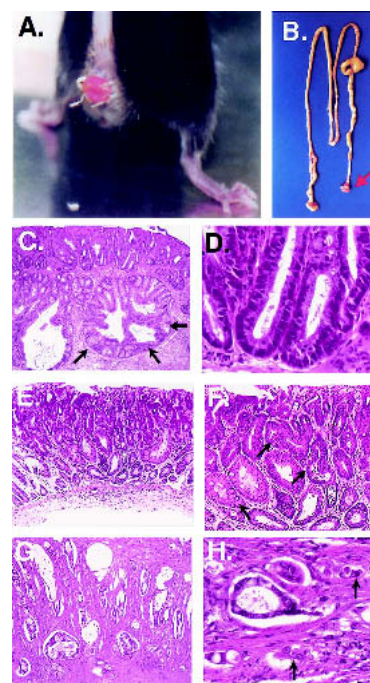
Figure 1. Colorectal cancer in 3C7 mice (–/– = hypomorphic *LTBP-4* alleles). (*A*) Rectal prolapse and (*B*) digestive tract preparation with colorectal tumor (arrow) of a 4-month-old mouse. (*C,D*) Mucosal prolapse syndrome of the rectum. Superficial rectal ulceration and well circumscribed pseudoinvasion of crypts into the submucosa (arrows; *C*, original magnification 100×). Nonneoplastic rectal crypts with regenerative epithelial cells (*D*, H/E stain, original magnification 600×). (*E,F*) Colorectal adenoma showing aberrant crypt foci (*E*, H/E stain, original magnification 100×) with dysplastic crypts (arrows) and increased number of goblet cells (arrow; *F*, detail of *E*). (*G,H*) Invasive rectal adenocarcinoma. Neoplastic tubules invade the muscularis propria (*G*, H/E stain, original magnification 100×), and show dissociation with small tumor cell clusters (arrows) at the invasive front (*H*, H/E stain, original magnification 600×).

(12–14 wk) most animals developed adenomas consisting of enlarged and abnormally branched cystic glands with

Table 1. *Summary of abnormalities developing in 3C7 mice*

Age (weeks)	Lung	Heart	Colon	Number of processed mice
4–6	Mild emphysema	Normal	Mucosal prolapse syndrome of the rectum	18
12–14	Moderate emphysema, rupture of septal walls	Moderate cardiomyopathy with right ventricular dilatation (cardiac body weight index increased >10%)	Colorectal adenoma	22
24–32	Severe emphysema with extensive atelectatic areas	Cardiomyopathy with biventricular dilatation (cardiac body weight index increased >30%)	Invasive colorectal carcinoma	14

All the other inspected organs and tissues were microscopically normal. Analysis included aorta, liver, kidney, intestine, lymph nodes, brain, bone marrow, thymus, adrenals, pituitary, thyroid, pancreas, skin, uterus, and testicles.

increased numbers of goblet cells (Fig. 1E,F). By the age of 6–8 mo, all mice developed visible tumors in the distal portion of the colorectum with diameters of 0.7–1.5 cm (Fig. 1B). Histologically, these tumors had all features of colorectal adenocarcinomas and were formed by enlarged and dysplastic crypts lined by neoplastic epithelial cells. Crypts showed various degrees of epithelial cell detachment and infiltrated all layers of the colorectal wall (Fig. 1G,H). Interestingly, these lesions are similar if not identical to the colorectal cancers developing in Smad3 knockout mice (Zhu et al. 1998). Because Smad3 is a downstream target of TGF-β, this similarity suggested defective TGF-β signaling in 3C7 mice.

In addition to colorectal cancer, 3C7 mice developed a pulmonary emphysema-like condition, which, unlike the colonic tumors, was already present at birth. The emphysema worsened with time so that by the age of 6–8 mo the lungs of 3C7 mice exceeded normal size by three-fold (Fig. 2A,B). Histologically, alveolar spaces were enlarged, inflated, and significantly reduced in number. They were surrounded by thin, dysplastic, and frequently incomplete septal walls (Fig. 2B). The lobular connective tissue was diminished and replaced by multifocal atelectatic areas. This resulted in an almost complete loss of pulmonary elasticity. The emphysema was associated with a cardiomyopathy that initially involved only the right heart. However, disease progression eventually led to biventricular dilatation and ubiquitous myofibrillar hypertrophy (Table 1; Fig. 2C). By the age of 6–8 mo the majority of the mutant mice had a cardiac/body weight index exceeding normal values by 30%.

Clinically, the animals were healthy up to 6 mo of age, but thereafter their general condition deteriorated. Most mice had to be killed between 6 and 8 mo of age because of severe dyspnea and rectal prolapse infections that frequently progressed toward ulcerative proctitis. Autopsy revealed massive inflammatory infiltrations of the colonic wall associated with a reactive splenomegaly.

As heterozygous mice were indistinguishable from wild-type mice, and transgene inheritance was consistent with a Mendelian pattern (data not shown), we concluded that the gene trap had disrupted the function of an autosomal recessive gene. Although the described phenotype was stable over 6 generations of back-crossings to C57BL6 mice, abnormalities were more severe in 129/SvJ mice. Accordingly, most homozygous 129/SvJ F_1 mice died during the first 10 d of life.

Figure 2. Pulmonary emphysema-like condition and cardiomyopathy in 3C7 mice. (A) Heart/lung preparations from wild-type (+/+) and homozygous 3C7 (–/–, hypomorphic *LTBP-4* alleles) mice. (B) Lungs, H/E stain at 200× magnification. (C) Hearts, H/E stain at 200× magnification.

The gene trap integration in 3C7 mice is in the fifth intron of the LTBP-4 gene

To identify the gene responsible for the 3C7 phenotype, we first used 5′ RACE to isolate cellular sequence co-transcribed with the provirus (gene trap sequence tag, GTST; Wiles et al. 2000). Normally, the type of retroviral gene trap used in these experiments selects for integrations into exons (exon trap), and in most cases sequences upstream of the integration site are transcribed as cell–provirus fusion transcripts that can be easily identified by RT-PCR (Wempe et al. 2001). However, 5′ RACE was unsuccessful, as was the detection of cell–provirus fusion transcripts by Northern blotting (Fig. 3B). This suggested that in this case the gene trap, which is normally transcribed from an upstream cellular promoter, was activated instead by a nearby cellular enhancer. Although quite infrequent, we have observed enhancer activations with this type of gene trap, as it still contains a minimal promoter in the 5′-LTR (Fig. 3B; Thorey et al. 1998). Taken together, these results indicated that the gene trap had integrated into an intron and was therefore inaccessible to cloning by RT-PCR. To overcome this problem, we used inverse PCR and genome walking to isolate the genomic sequence spanning the provirus integration site. A 781-nt contig was cloned and sequenced and used to find matches in the public databases. As no matches were found after extensive searching, we assumed that the trapped gene was still unknown. To identify the gene, cDNA and genomic libraries were screened with the genomic contig, thus resulting in the isolation of a 12-kb genomic fragment with two short stretches of homology to the human cDNA encoding the latent transforming growth factor-β binding protein 4 (LTBP-4; GenBank accession nos. AF051344, NM_003573.1, AF051345). The matching segments corresponded to exons 6 and 7 encoding the 5′-end of the first EGF repeat, which
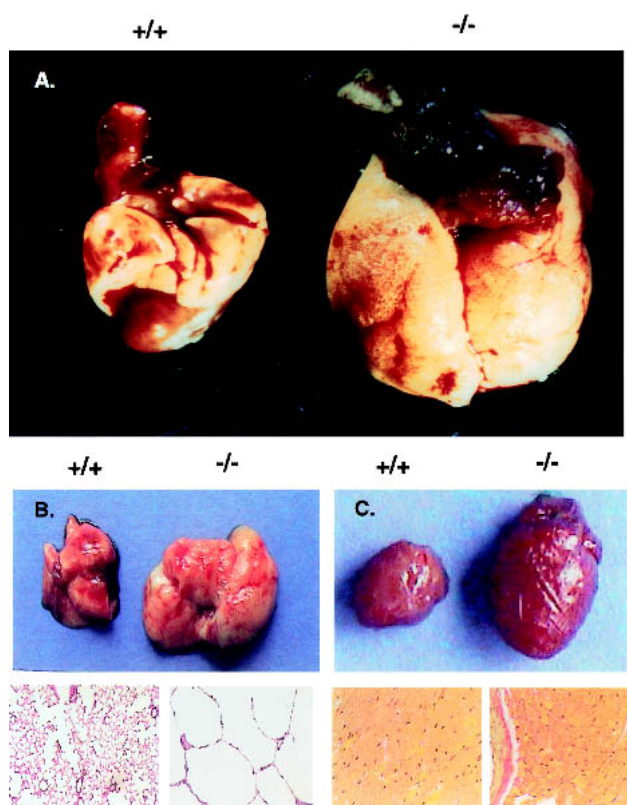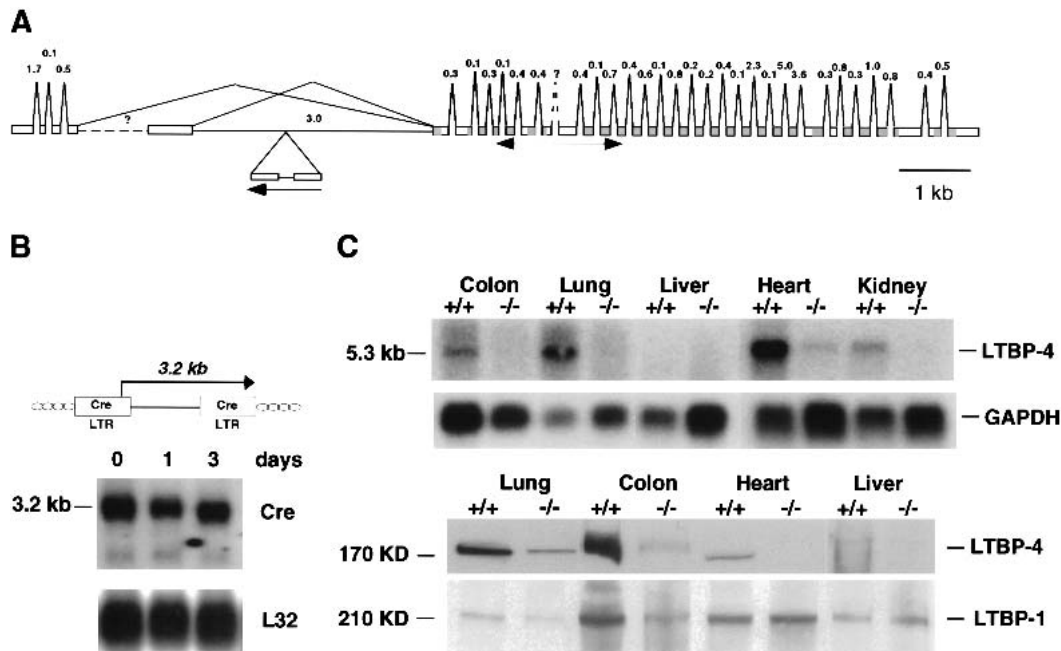
Figure 3. Gene trap disruption of the *LTBP-4* gene locus. (*A*) Predicted intron/exon structure of the mouse *LTBP-4* gene with the gene trap integration site. The exon/intron structure was compiled by using the mouse cDNA sequences for the short and long 5' splice variants (GenBank accession nos. AF410798 and AF410799), the human cDNA sequences for the various 5' splice variants (GenBank accession nos. NM_003573, AF054502, AF054501, AF051345, AF051344, and Y13622), and the available genomic sequence of mouse chromosome 7 (GenBank accession no. AC073713). EGF domains and 8Cy repeats are shaded in dark and light gray, respectively. (*B*) Structure of the U3Cre gene trap provirus (*top*) and Northern blot analysis of 3C7 ES cells (*bottom*). Polyadenylated RNAs from ES cells that were grown for 3 d in the absence of LIF and feeder layers were blotted onto nylon filters and hybridized to ³²P-labeled Cre- or ribosomal protein L32-specific probes. (*C*) LTBP-4 expression in wild-type (+/+) and homozygous 3C7 (–/–, hypomorphic *LTBP-4* alleles) mice. (*Top*) Northern blot analysis. Polyadenylated RNAs from selected mouse tissues were blotted onto nylon filters and hybridized to a ³²P-labeled LTBP-4 probe. The probe was a 505-nt fragment extending between nucleotides 882 and 1367 of the *LTBP-4S* cDNA (GenBank accession no. AF410798; for location, see *A*). This sequence stretch is shared by all known splice variants. (*Bottom*) Immunoblotting with human anti-LTBP-4 and anti-LTBP-1 antibodies. Proteins from selected tissue digests (see Materials and Methods) were separated on denaturing polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes, and reacted with polyclonal anti-LTBP-1 or anti-LTBP-4 antibodies raised against the third and fourth 8-Cystein repeats that are shared between known splice variants (Saharinen et al. 1998).

is shared among known splice variants (Fig. 3A; Saharinen et al. 1998).

Figure 3A shows that the gene trap integration had occurred into the fifth intron of the mouse *LTBP-4* gene in a reverse transcriptional orientation relative to the gene. This inverted orientation explains the absence of a cell–provirus fusion transcript and supports a gene trap activation by a cellular enhancer.

*The proviral integration in* LTBP-4 *severely interrupts gene expression*

To investigate whether the proviral intron disruption interfered with gene expression, mRNA from a variety of mouse tissues was hybridized on Northern blots to an *LTBP-4*-specific probe. As shown in Figure 3C (top), mice homozygous for the gene trap integration expressed <2% of normal *LTBP-4* mRNA. Transcript levels in wild-type mice were significantly higher in the lung, heart, and colon than in other tissues, suggesting that tissues that normally express high levels of LTBP-4 are also the most affected by the mutation (Fig. 3C).

To test whether the protein levels were similarly decreased, the same range of tissues was analyzed by immunoblotting using an anti-LTBP-4-specific antibody (Saharinen et al. 1998). As shown in Figure 3C (bottom), the protein was hardly detectable in 3C7 mice, but the levels in various tissues of wild-type mice closely followed the pattern of mRNA expression. Taken together, the results indicate that the gene trap integration into the fifth intron of the *LTBP-4* gene resulted in a nearly null allele.

Elastic fibers are fragmented in the lung and colon of 3C7 mice

Together with fibrillins, LTBPs are believed to be components of the sheath of microfibrils that surrounds the elastic fiber's elastin core (Saharinen et al. 1999). Using immuno-electron microscopy (IEM) and immunogold labeling, LTBP-1 and LTBP-2 were shown to associate with the extracellular matrix microfibrils (Taipale et al. 1996; Shipley et al. 2000). Although IEM studies have not yet been performed with the other LTBPs, mainly because of

the lack of high-avidity antibodies, in vitro immunofluorescence studies indicate that LTBP-4 has an ECM distribution similar to LTBP-1 and LTBP-3 (K. Koli and J. Keski-Oja, unpubl.). Because some evidence suggests that microfibrils regulate elastic fiber formation by guiding tropoelastin deposition during embryogenesis and early postnatal life (Pereira et al. 1997), we thought that lack of LTBP-4 might alter the ECM's microfibril structure and thus the integrity of elastic fibers, particularly in the lung and colon, which both lost elasticity in 3C7 mice. To test this, we visualized elastic fibers using an elastin-specific histological stain. Figure 4 shows that the lung and colon of 3C7 mice lacked normal elastic fibers and, instead, showed multiple patches of fragmented and condensed elastin. Unexpectedly, elastic fibers in the heart appeared unaltered by the mutation, suggesting that the cardiomyopathy is mostly secondary to the emphysema (Fig. 4). However, because LTBP-4 expression is comparatively high in the heart (Fig. 3C), we cannot exclude a contribution of the mutation to the cardiac phenotype. Taken together, the results suggest that in selected tissues, LTBP-4 interferes with the nor-
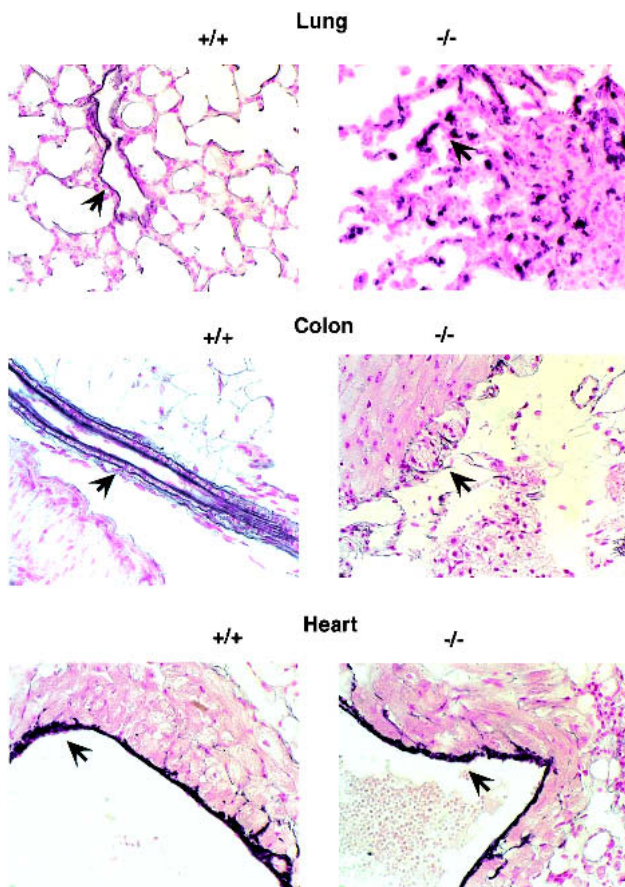
Figure 4. Fragmentation of elastic fibers in 3C7 mice. To visualize elastic fibers (arrows), lung sections of 12-week-old mice were stained with Weigert's resorcin-fuchsin and photographed at 400× magnification (+/+, wild-type mice; −/−, 3C7 mice with hypomorphic *LTBP-4* alleles).

mal development of elastic fibers in a tissue-specific manner.

Deposition of TGF-β1 to the ECM requires LTBP-4

We found recently that of all three TGF-β isoforms, only TGF-β1 covalently associates with LTBP-4 in vitro (Saharinen and Keski-Oja 2000). Because LTBPs are responsible for the export and deposition of TGF-β in the ECM, this isoform-specific binding suggested that mice lacking LTBP-4 would also lack extracellular TGF-β1. To test this, we used anti-TGF-β1 antibodies that recognize TGF-β1 epitopes selectively displayed inside (LC) or outside (CC) the cell (Flanders et al. 1989; Thompson et al. 1989). Immunohistochemical analysis showed that in 3C7 mice, the lung, colon, and heart contained no detectable extracellular TGF-β1 (Fig. 5). As intracellular levels were essentially normal, the result suggests a defect in export and/or deposition of small latent TGF-β1 (TGF-β1–LAP) in the ECM. This defect appears to be tissue-specific because the kidneys of 3C7 mice had normal levels of extracellular TGF-β1 (Fig. 5).

To determine whether the tissue-specific reduction in TGF-β1 matrix deposition would correlate with a reduced LTBP-4 transcription, we performed in situ hybridization experiments using oligonucleotide probes. As expected, the 3C7 bronchioalveolar and colonic crypt epithelial cells expressed significantly less LTBP-4 than the corresponding wild-type cells, suggesting a link between TGF-β1 matrix deposition and LTBP-4 expression (data not shown).

To investigate this further, we analyzed ECMs isolated from the colon and the kidneys of 3C7 mice by immunoblotting using an anti-TGF-β1–LAP antibody. As shown in Figure 6, TGF-β1–LAP/LTBP complexes were significantly below normal levels in the colonic ECM but not in the renal ECM, suggesting that TGF-β1 matrix deposition requires LTBP-4 only in the colon. Interestingly, although the anti-β1–LAP antibody recognizes any LTBP/LAPβ1 complex, it produces hardly any signal in the colonic ECM of 3C7 mice. In contrast, it readily visualizes LTBP/LAPβ1 complexes in the kidney ECM, which are similar to, or only slightly below wild-type levels (Fig. 6, bottom). Because only minimal LTBP-4 is transcribed in the 3C7 kidney (Fig. 3), it is very likely LAPβ1 is complexed to another LTBP in this organ. LTBP/LAPβ1 complex intensities are similarly weak in the mutant colonic ECM, regardless of whether they are visualized by anti-LTBP-4 (Fig. 6, upper left panel) or anti-LAPβ1 (Fig. 6, upper right panel) antibodies. Thus, it is quite plausible that in the colon, LTBP-4 is the only isoform responsible for TGF-β1 matrix deposition. Taken together, the results confirm the immunohistochemical analysis and underscore the tissue specificity of LTBP-4.

Lack of extracellular TGF-β is associated with reduced TGF-β signaling

As TGF-β is a strong inhibitor of epithelial cell proliferation and defective TGF-β signaling has been shown to
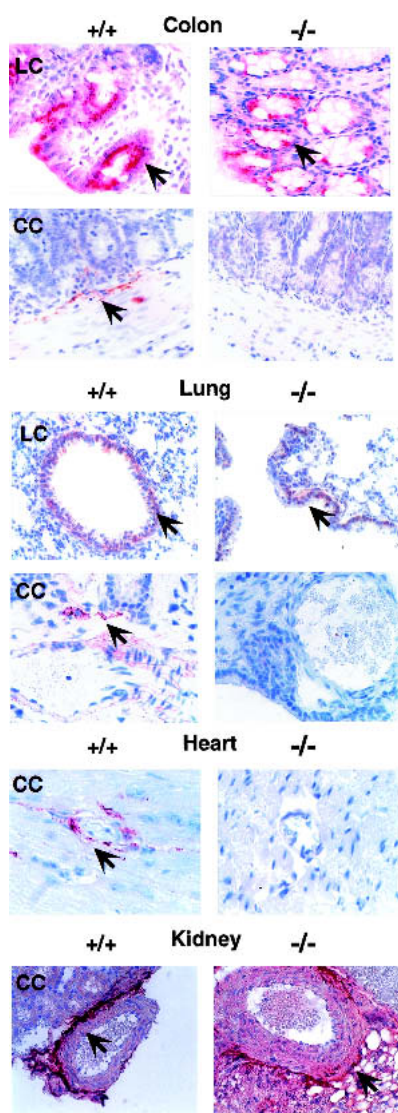
Figure 5. Lack of extracellular TGF-β1 in 3C7 mice. To visualize the TGF-β1 deposition in the ECM, tissue sections from 8-week-old mice were stained with polyclonal TGF-β antibodies specific for the intracellular (LC) or extracellular (CC) form of TGF-β1 (see Materials and Methods) and photographed at 200× magnification (+/+, wild-type mice; –/–, 3C7 mice with hypomorphic *LTBP-4* alleles). Positive cells and matrix components are reddish-brown (arrows).

play a role in the pathogenesis of cancer (Derynck et al. 2001), we hypothesized that the absence of TGF-β1 from the extracellular space might negatively interfere with TGF-β signaling.

To test this, we analyzed the proliferative status of colorectal tumor cells by immunohistochemistry using antibodies against the cell proliferation markers Ki-67 and c-myc. As shown in Figure 7, both markers were highly expressed in the colorectal region of mutant mice, which is consistent with increased proliferation. More importantly, c-myc was already elevated in the normal mucosa adjacent to the prolapse at 4 wk of age (Fig. 7),

suggesting a more specific alteration of c-myc expression. As TGF-β is a potent inhibitor of c-myc expression (Alexandrow and Moses 1995; Massague and Wotton 2000), we presumed that its elevation is the result of a defective TGF-β signaling in the mutant mice. To investigate this further, we directly assessed the activation level of the TGF-β signal transduction pathway by estimating the levels of phosphorylated Smad2 proteins (Heldin et al. 1997) in the epithelial cells of various tissues. Immunohistochemical analysis with specific anti-Smad2 antibodies showed that P-Smad levels were decreased significantly in the lung and colon but not in the kidney of mutant mice (Fig. 8A). Because the tissue-specific pattern of TGF-β activation correlated with its deposition in the ECM, we conclude that LTBP-4 is critical for TGF-β signaling in the affected tissues.

Finally, to rule out the possibility that an intrinsic defect of the TGF-β signal transduction pathway could have been responsible for the reduced Smad activation, we tested the ability of TGF-β to induce Smad2 phosphorylation in cultured fibroblasts derived from 3C7 lungs. The immunoblot depicted in Figure 8B shows that the mutant fibroblasts responded normally to exogenous TGF-β, indicating that the TGF-β signaling defect is solely the result of its reduced deposition in the extracellular space.
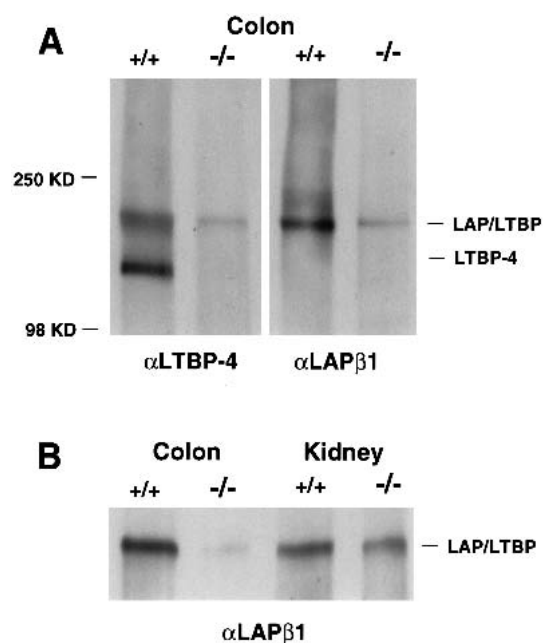
Figure 6. Levels of large latent TGF-β1 complexes in 3C7 mice. Proteins from colon and kidney tissue digests (see Materials and Methods) were separated on denaturing polyacrylamide gels under nonreducing conditions, transferred to nitrocellulose membranes, and reacted with anti-LTBP-4 (αLTBP-4) or anti-LAPβ1 (αLAPβ1) antibodies. (*A*) Free LTBP-4 (*left* panel) and large latent LTBP complexes (*right* panel) in the colonic ECM of wild-type (+/+) and mutant 3C7 (–/–, hypomorphic *LTBP-4* alleles) mice. (*B*) Levels of large latent LTBP complexes in the colonic and renal ECMs of 3C7 mice.
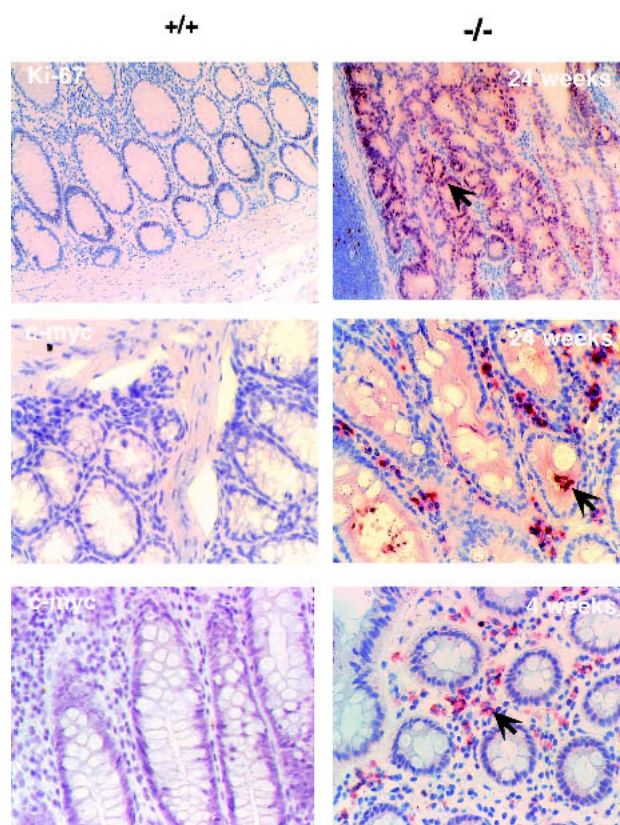
Figure 7. Increased cell proliferation and c-myc induction in the colon of 3C7 mice. Tissue sections were stained with polyclonal rabbit anti-mouse Ki-67 (*top*) or monoclonal anti-mouse c-myc antibodies (*center* and *bottom*) and photographed at 200× magnification (+/+, wild-type mice; –/–, 3C7 mice with hypomorphic *LTBP-4* alleles). Positive cells are reddish-brown (arrows).

Discussion

A strong hypomorphic allele of the mouse *LTBP-4* gene was generated by gene trap mutagenesis in embryonic stem cells. Mice homozygous for the gene trap integration expressed only trace amounts of LTBP-4 and developed severe pulmonary emphysema, cardiomyopathy, and colorectal cancer. The abnormalities were associated with fragmented and disintegrated elastic fibers and with a defective TGF-β deposition in the extracellular space.

Interestingly, the hypomorphic allele was generated by an inverse orientation exon trap insertion into an intron of the *LTBP-4* gene, which theoretically should not interfere with gene expression. However, intron insertions have been shown previously to cause mutations. Examples include the ob2J mutation induced by a retrotransposon insertion into the first intron of the mouse *leptin* gene (Moon and Friedman 1997), the mdr-3 mutation induced by a retroviral LTR insertion into the 22nd intron of the mouse multi-drug resistance gene *mdr-3* (Jun et al. 2000), and the Mbp mutation induced by a retrotransposon insertion into the third intron of the rat myelin basic protein gene *Mbp* (O'Connor et al. 1999). In each case, aberrant splicing precluded the expression of a

fully processed, full-length transcript. We found this to be similar in 3C7 cells, in which RT-PCR detected multiple atypical *LTBP-4* transcripts (data not shown), indicating that the mutation was caused by aberrant splicing.

The disease model described here is consistent with a dual function of the TGF-β-binding proteins (Taipale et al. 1994). Whereas the absence of intact elastic fibers in the lung and colon underscores the structural requirement of LTBP-4, the lack of extracellular TGF-β implicates LTBP-4 in TGF-β signaling. As TGF-β inhibits epithelial cell proliferation, particularly in the colon, we believe that the lack of extracellular TGF-β is the major oncogenic trigger in a colorectal region already predisposed to cancer by a mucosa prolapse syndrome.

Several studies have associated defects in TGF-β signaling with colorectal cancer. For example, mice with null mutations in the TGF-β signal transducing protein Smad-3, develop tumors that are similar if not identical to the tumors growing in 3C7 mice (Zhu et al. 1998). Although the Smad-3 knockout phenotype could not be replicated by other groups (Datto et al. 1999; Yang et al. 1999), the link between TGF-β signaling defects and colorectal cancer is still compelling because it has been recognized in many other systems, including humans. Accordingly, mutations in the TGF-β signal transducing proteins Smad2 and Smad4 or mutations in the TGF-β II receptors are very common in human colorectal cancers, suggesting that TGF-β and its downstream targets have tumor suppressor functions (Markowitz et al. 1995; Riggins et al. 1997; Zhou et al. 1998, 1999). Whether LTBP-4 itself has such a function remains to be established, but its importance for the integrity of the ECM is likely to prevent cancer cell invasion and metastatic spread.

Whether LTBP-4 has a direct role in elastic fiber formation remains to be established. Because other LTBPs colocalize with fibrillins in the ECM (Taipale et al. 1996; Shipley et al. 2000), it is likely that LTBP-4 is also a component of the microfibril network. In vitro immunofluorescence studies showing similar ECM distribution patterns for all LTBPs, including LTBP-4, support this assumption (K. Koli and J. Keski-Oja, unpubl.). The microfibril network is believed to be crucial for elastic fiber formation because it appears to guide tropoelastin deposition during embryogenesis and early postnatal life (Pereira et al. 1997). Indeed, as has been shown recently, at least two components of the microfibril network, fibrillin 1 and fibrillin 2, are capable of binding tropoelastin, suggesting that the fibrillins (and, by inference, the microfibrils) play an important role in the assembly of elastic fibers (Trask et al. 2000).

One of the most interesting observations made in this study is the tissue selectivity of the *LTBP-4* mutation. Only the lung, the colon, and possibly the heart developed disease directly related to the mutation, indicating that LTBP-4 is a highly tissue-specific protein. Such tissue specificity would predict that despite the extensive structural similarities, latent TGF-β-binding proteins have little or no overlapping functions. Accordingly, we found that mutant mice express almost unaltered levels
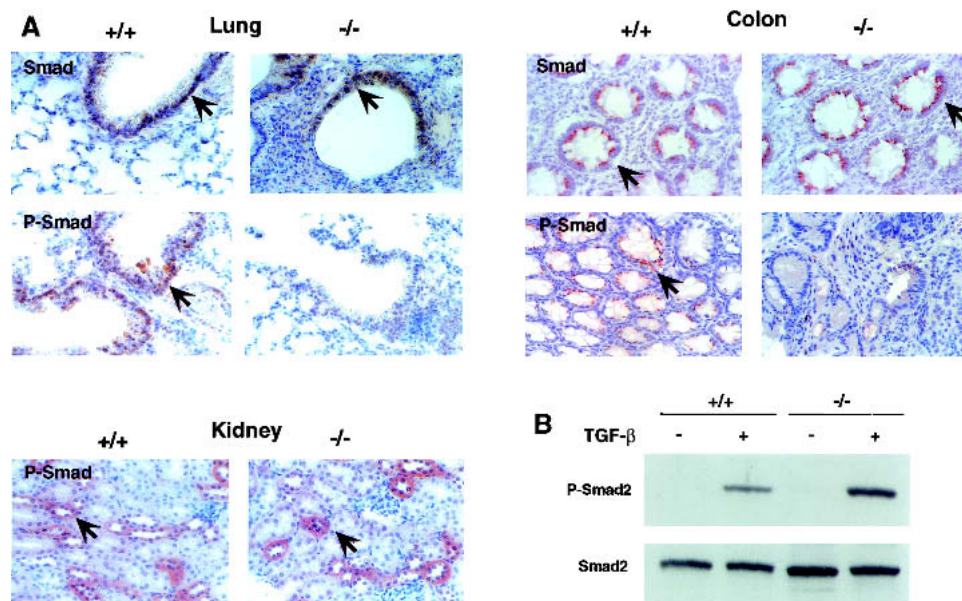
Figure 8. Smad2 phosphorylation in 3C7 mice. (*A*) Decreased Smad2 phosphorylation in the epithelial cells of 3C7 mice. Tissue sections from 8-week-old mice were stained with monoclonal anti-Smad2,3 (Smad) or polyclonal anti-phosphorylated Smad2 (P-Smad) antibodies and photographed at 200× magnification (+/+, wild-type mice; −/−, 3C7 mice with hypomorphic *LTBP-4* alleles). Positive cells are reddish-brown (arrows). (*B*) Induction of Smad2 phosphorylation by TGF-β1 in cultured lung fibroblasts. Fibroblasts were exposed for 1 h to 1 ng/mL TGF-β1. Cell lysates from semiconfluent cultures were separated on denaturing polyacrylamide gels, transferred to nitrocellulose membranes, and reacted with polyclonal anti-P-Smad2 or monoclonal anti-Smad2,3 antibodies.

of LTBP-1 and LTBP-2 proteins, suggesting that these isoforms cannot substitute for LTBP-4 (Fig. 3C; data not shown). Moreover, mice expressing null alleles of *LTBP-2* die very early in development, most likely because of defective implantation (Shipley et al. 2000), and mice expressing null alleles of *LTBP-3* develop skeletal malformations (Dabovic et al. 2002).

In conclusion, we have developed a mouse model of human disease that reveals some crucial functions of LTBP-4. In view of these functions, the *LTBP-4* gene seems a likely candidate gene for human connective tissue disorders and cancer.

Materials and methods

ES cell injections, breeding, and genotyping

The 129/SvJ (D3) ES cell-derived chimeras were generated by injecting C57BL/6 blastocysts. The resulting male chimeras were bred to C57BL/6 females, and agouti offspring were tested for transgene transmission by tail blotting (see below). Animals heterozygous for the gene trap insertion were back-crossed to C57BL/6 mice for at least six generations before analyzing the phenotypes in heterozygous and homozygous offspring.

Cloning of provirus flanking sequences and screening of nucleic acid libraries

Inverse PCR from genomic 3C7 ES cell DNA was performed by using the blunt-end restriction enzyme *Ssp*I and Cre-specific primers as described previously (Wempe et al. 2001). A 210-nt amplification product was cloned into the p-GEMT-vector (Promega) and sequenced using an ABI 310 Genetic Analyzer (Per-

kin-Elmer). The upstream provirus flanking sequence was extended by genome walking using the mouse GenomeWalker kit (Clontech) according to the manufacturer's instructions and the specific flanking sequence forward primers 5′-AAGAGG GAGAAGAAACTGGGCTGG-3′ and 5′-TCCTGTAGTGTTT GCCCCTG-3′. A 781-nt genomic contig spanning the proviral integration site was amplified from the kit's *Eco*RV library and used as a probe to screen the λ-TripleEx 5′-Stretch PLUS mouse kidney cDNA library (Clontech) and the λ EMBL3 mouse liver genomic library (Clontech). Positive plaques were purified by standard methodology, and inserts were sequenced after subcloning into Bluescript vectors.

Nucleic acid hybridization analyses and PCR

Mouse-tail DNA was cleaved with *Bgl*II, which does not cut the provirus. The DNA was fractionated on 1% agarose gels, blotted onto Hybond N nylon filters (Amersham/Pharmacia), and hybridized to a ^{32}P-labeled provirus flanking sequence probe. For Northern blots, 2.5 μg of poly(A)$^+$ mRNAs purified with the FastTrack 2.0 mRNA purification kit (Invitrogen) were fractionated in 1% formaldehyde-agarose gels, transferred onto Hybond N nylon filters, and hybridized to a [^{32}P]dCTP-labeled LTBP-4-specific probe. The probe was a 505-nt fragment extending from nucleotides 862 to 1367 of the mouse LTBP4S cDNA (GenBank accession no. AF410798). All probes were prepared by random priming using the Rediprime kit (Amersham/Pharmacia). Blots were exposed to Kodak-Biomax autoradiography film.

Immunoblotting

Equal amounts of selected tissues were homogenized in ice-cold RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate in 50 mM Tris-HCl at pH 7.2). Following centrifugation,

the deoxycholate insoluble pellet was washed in PBS and digested with plasmin as described previously (Taipale et al. 1994). After adding protease inhibitors (Roche Diagnostics), the supernatants were concentrated in Microcon 30 concentrators (Amicon, Millipore Corp.). Proteins were fractionated by SDS/PAGE (7% acrylamide monomer concentration) and transferred to Protran nitrocellulose membranes (Schleicher & Schuell). Immunodetection after treatment with polyclonal anti-LTBP-1 or anti-LTBP-4 antibodies was performed as described previously (Saharinen et al. 1998).

For Smad2,3 detection, cultured lung fibroblasts were exposed to 1 ng/mL TGF-β1 (Oncogen) for 1 h and then lysed with ice-cold buffer (120 mM NaCl, 0.5% NP-40, 100 mM NaF, 1 mM Na_3Vo_4 in 20 mM Tris-HCl at pH 8.0; protease inhibitors added as Protease Inhibitor Cocktail, Roche Diagnostics). The lysates were cleared by centrifugation, and equal amounts of protein were separated by polyacrylamide gel (7% Tris-HCl) electrophoresis in the presence of SDS. Proteins were then electrophoretically transferred to Protran nitrocellulose membranes (Schleicher & Schuell). Immunodetection was performed as described (Dooley et al. 2001), using a polyclonal rabbit antibody raised against phosphorylated smad2 (kindly provided by Peter ten Dijke, The Netherlands Cancer Institute). The monoclonal mouse anti-Smad2,3 antibody and the LAP-β1 antibody (AF-246-NA) were purchased from Transduction Laboratories and from R&D Systems, respectively.

Database searches

Cellular sequences flanking the proviral integration site (gene trap sequence tag; GTST) were searched in the NCBI/NIH genomic databases (http://www.ncbi.nlm.nih.gov) using the BlastN algorithm.

Histology, histochemistry, and immunohistochemistry

Paraffin sections of mouse tissues were prepared and stained using standard histology procedures. To visualize elastic fibers, microscopic slides were stained with Weigert's resorcin-fuchsin solution (70% [v/v] ethanol, 1% [v/v] HCl, and 0.2% [v/v] resorcin-fuchsin; Chroma, Münster). For immunostainings, deparaffinized and rehydrated tissue slides were first treated for 30 min with 3% H_2O_2 to inactivate the endogenous peroxidase. After rinsing in ddH_2O and soaking in PBS for 5–10 min, slides were treated with 10% (w/v) BSA in PBS to saturate nonspecific protein binding sites. The slides were then exposed at 4°C overnight to the specific monoclonal or polyclonal antibodies (see below). After removing excess antibody, slides were treated with appropriate biotin-labeled secondary antibodies (Dianova) at 37°C for 30 min and finally with horseradish peroxidase (HRP)-labeled streptavidine (Zymed) at 37°C for 20 min. After washing, slides were incubated in diaminobenzidine (Sigma) at room temperature for 10 min. Slides were examined under a conventional microscope after removing the excess substrate in ddH_2O.

For immunohistochemical detection, pretested dilutions of the following antibodies were used: polyclonal mouse anti-TGF-β1-CC (1–30; Flanders et al. 1989), polyclonal mouse anti-TGF-β1-LC (1–30; Flanders et al. 1989), rabbit anti-mouse K-67 (Dianova), monoclonal anti-c-myc (Santa Cruz), monoclonal mouse anti-Smad2,3 (Transduction Laboratories), and rabbit polyclonal anti-P-Smad2 (kindly provided by Peter ten Dijke, The Netherlands Cancer Institute).

Acknowledgments

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