Perturbation of transforming growth factor (TGF)-ß1 association with latent TGF-β binding protein yields inflammation and tumors

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Transforming growth factor-β (TGF-β) activity is controlled at many levels including the conversion of the latent secreted form to its active state. TGF-β is often released as part of an inactive tripartite complex consisting of TGF-β, the TGF-β propeptide, and a molecule of latent TGF-β binding protein (LTBP). The interaction of TGF-β and its cleaved propeptide renders the growth factor latent, and the liberation of TGF-β from this state is crucial for signaling. To examine the contribution of LTBP to TGF-β function, we generated mice in which the cysteines that link the propeptide to LTBP were mutated to serines, thereby blocking covalent association. *Tgfb1*$^{C33S/C33S}$ mice had multiorgan inflammation, lack of skin Langerhans cells (LC), and a shortened lifespan, consistent with decreased TGF-β1 levels. However, the inflammatory response and decreased lifespan were not as severe as observed with *Tgfb1*$^{-/-}$ animals. *Tgfb1*$^{C33S/C33S}$ mice exhibited decreased levels of active TGF-β1, decreased TGF-β signaling, and tumors of the stomach, rectum, and anus. These data suggest that the association of LTBP with the latent TGF-β complex is important for proper TGF-β1 function and that *Tgfb1*$^{C33S/C33S}$ mice are hypomorphs for active TGF-β1. Moreover, although mechanisms exist to activate latent TGF-β1 in the absence of LTBP, these mechanisms are not as efficient as those that use the latent complex containing LTBP.

TGF-β activation

Transforming growth factor (TGF)-β1 is secreted as part of an inactive tripartite complex consisting of the 25-kDa TGF-β1 homodimer, the TGF-β1 dimeric propeptide, and a molecule of the latent TGF-β binding protein (LTBP) (Fig. 1*A*) (1, 2). TGF-β is cleaved from its initial translation product by intracellular proteolytic processing, but the liberated N-terminal propeptide remains associated with TGF-β by noncovalent bonds even after secretion. The union of TGF-β and its propeptide renders the growth factor latent. Thus, the TGF-β propeptide is called the latency-associated protein (LAP). LAP and LTBP are linked by disulfide bonds that form intracellularly between the cysteine 33 residues in each of the two LAP chains and a pair of cysteine residues in the LTBP. Four LTBPs, 1, 2, 3, and 4, are known, but only LTBP-1, -3, and -4 bind LAP (3). The complex of TGF-β1, LAP, and LTBP is called the large latent complex (LLC), whereas the complex of TGF-β1 and LAP is referred to as the small latent complex (SLC).

Within the LLC, the functions of TGF-β and LAP are clear: TGF-β is the potential signaling molecule and LAP confers latency, thereby regulating the time and place of TGF-β action. The release of TGF-β from inhibition by LAP is referred to as latent TGF-β activation. Latent TGF-β can be activated by several different molecules, including proteases, thrombospondin-1, reactive oxygen species, and the integrins αvß6 and αvß8 (1).

The functions of LTBP, however, are not well understood (4, 5). LTBPs may act as intracellular chaperones for the SLC. In the absence of LTBP, SLC is secreted slowly and the liberated complex contains incorrectly paired disulfide bonds, whereas in the presence of LTBP, the secretion of TGF-β is enhanced and disulfide bond formation is correct (6). LTBPs may regulate latent TGF-β sequestration in the extracellular matrix (ECM) (4). Both immunochemical and biochemical approaches have revealed the presence of LLC in the ECM and association of the LTBP with fibronectin and fibrillin (4, 7, 8). Failure of LLC to associate properly with the matrix results in increased active TGF-β and abnormalities in skin and lung (9, 10). LTBP-1 participates in latent TGF-β activation by the integrin αvß6. αvß6 binds to Arg–Gly–Asp (RGD) sequences present in TGF-β1 and TGF-β3 LAPs and activates latent TGF-β via this interaction (11). However, a mutant of TGF-β1 in which cysteine 33, which normally forms the disulfide bonds with LTBP, is mutated to serine, thereby precluding LLC formation but permitting SLC formation, was not activated by αvβ6 (12). This result indicates a requirement for LTBP-1 in αvβ6-mediated latent TGF-β activation. LTBP-1 binds to the ECM protein fibronectin (13), enabling the cells to apply force to the latent complex via integrin binding to LAP, thereby releasing the TGF-β1 (14, 15).

Null or hypomorphic mutations in the *Ltbp-1L*, *Ltbp-3*, and *Ltbp-4* genes have yielded mice with abnormalities in the development of the heart, bones, and lungs, respectively (16–19). The observed phenotypes all have been related to decreased TGF-β activity as described in other mouse models (20–23), further supporting the proposed importance of the LLC in TGF-β function.

These data indicate that LTBPs may assume multiple roles with regard to TGF-β action—as enhancers of SLC secretion, as SLC matrix localizers, and as participants in the activation of latent TGF-β. However, the interpretation of certain results can

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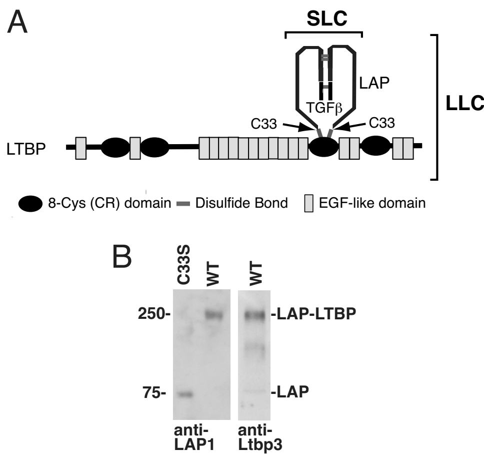

Fig. 1. TGF-β1 latent complexes from *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ mice. (*A*) Structure of latent TGF-β LLC. The LLC of TGF-β1 consists of the mature cytokine dimer, the propeptide dimer (LAP), and LTBP. The LAP residues that bind to LTBP are cysteines 33. Changing this residue to serine prevents formation of the TGF-β1 LLC. (*B*) LAP associations in *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ lung cell culture media as revealed after SDS/PAGE and immunoblotting. Immunoreactive material from Tgfb1$^{C33S/C33S}$ cells is detected only at the position of the LAP dimer (75 kDa). (Left) There is no band at the position of LAP-LTBP. LAP immunoreactive material is present at the position corresponding to 250 kDa, the position of LAP plus LTBP. (Center) Western blot of *Tgfb1*$^{C33S/C33S}$ cell conditioned medium with an antibody to LTBP-3. (Right) The positive reaction indicates that the LLC is composed of LAP and LTBP-3. Blotting with antibodies to LTBP-1 revealed no LLC indicating the absence or undetectable levels of this species in either sample. Antibody to LTBP-4 was not tested.

be criticized for several reasons including problems with overexpression, loss of TGF-β-independent functions, compensation by other LTBPs, and the difficulty of characterizing the effects of LTBP on latent TGF-β activation, if the absence of LTBP impedes SLC secretion.

To address the role of LTBP in TGF-β action and to avoid the caveats described above, we generated a mutant mouse in which the cysteine residue (Cys 33) that binds TGF-β1 LAP to LTBP was mutated to serine. In these *Tgfb1*$^{C33S/C33S}$ mice, TGF-β1 LAP cannot covalently complex with any LTBP, and all TGF-β1 is secreted as SLC. TGF-β1^{C33S} generates a form of SLC that is effectively secreted (12). This ensures that the phenotypes of *Tgfb1*$^{C33S/C33S}$ mice represent a lack of LLC formation and not the loss of either SLC or an LTBP. We reasoned that if LLC formation is essential for TGF-β function, *Tgfb1*$^{C33S/C33S}$ mice should resemble *Tgfb1*$^{-/-}$ mice, which have multiorgan inflammation, die within 3 weeks of birth, and lack epidermal Langerhans cells (LC). Alternatively, if LLC formation is not critical for TGF-β function, *Tgfb1*$^{C33S/C33S}$ mice should resemble wild-type mice.

Results

Generation of *Tgfb1*$^{C33S/C33S}$ Mice. Two lines of *Tgfb1*$^{C33S/C33S}$ mice were generated as described in *Methods* and supporting information (*SI*) Fig. S1. The *Tgfb1*^{C33S} genotype is distinguished experimentally from the *Tgfb1* wild-type allele by the loss of the BfuAI restriction site and/or by the presence of the lox sequence in the first intron of the *Tgfb1* gene.

Wild-type (*Tgfb1*$^{+/+}$), heterozygous (*Tgfb1*$^{+/C33S}$), and *Tgfb1*$^{C33S/C33S}$ mice are born at essentially normal Mendelian ratios (Table S1). The two lines appeared equivalent in all of the analyses described below. *Tgfb1*$^{C33S/C33S}$ mice can survive for 5 months, although 50% of the animals die within 30 days (Fig. S2*A*). The fact that all *Tgfb1*$^{C33S/C33S}$ mice are born and many survive for several months is in contrast to *Tgfb1*$^{-/-}$ mice that either die *in utero* or within 3 weeks of birth (24). The cause of death in *Tgfb1*$^{C33S/C33S}$ mice is unknown, but at necropsy some of the *Tgfb1*$^{C33S/C33S}$ animals had thrombi in their right atria. Interestingly, a mutant form of LAP-β1 with Cys33Ser substitution overexpressed in mouse heart yields atrial fibrosis and delayed myocardial healing (25). We did not observe this in our mice, perhaps because we did not use overexpression of *Tgfb1*$^{C33S/C33S}$. *Tgfb1*$^{C33S/C33S}$ animals appeared physically normal at birth, but by 4 weeks, differences in weight were apparent between homozygous mutant vs. *Tgfb1*$^{+/C33S}$ and *Tgfb1*$^{+/+}$ mice (Fig. S2*B*). These differences persisted for the remainder of the life of the mice (Fig. S2*B*).

The prolonged survival of *Tgfb1*$^{C33S/C33S}$ mice compared to *Tgfb1*$^{-/-}$ mice suggested that association of LTBP with SLC was dispensable for TGF-β1 function. To ensure that *Tgfb1*$^{C33S/C33S}$ mice produced only SLC, we characterized TGF-β1 from cultures of immortalized cells derived from *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ lungs (26). We verified the lack of TGF-β1 LAP association with LTBP by Western blot analysis of conditioned media from *Tgfb1*$^{+/+}$ and mutant cell cultures after nonreducing SDS/PAGE (Fig. 1*B*). In the *Tgfb1*$^{+/+}$ sample we detected a single 250-kDa band by both anti-LAP and anti-LTBP antibodies. This band corresponds to the covalent complex of LAP and LTBP, as SDS dissociates TGF-β1 from the LLC; therefore, no LLC or SLC is visible after SDS/PAGE. Immunoreactive protein from *Tgfb1*$^{C33S/C33S}$ cells was observed at the position corresponding to 75 kDa, the molecular mass of the TGF-β1 LAP dimer, but there was no immunoreactive material at the 250-kDa position. Thus, covalent complexes did not form between the mutant LAP and LTBP in *Tgfb1*$^{C33S/C33S}$ mice.

Langerhans Cells in *Tgfb1*$^{C33S/C33S}$ Mice. The early death of *Tgfb1*$^{C33S/C33S}$ mice compared to *Tgfb1*$^{+/+}$ or *Tgfb1*$^{+/C33S}$ animals was reminiscent of the early mortality of *Tgfb1*$^{-/-}$ mice. Therefore, we examined the *Tgfb1*$^{C33S/C33S}$ mice for additional phenotypes associated with TGF-β1 deficiency. A striking phenotype of *Tgfb1*$^{-/-}$ mice is the absence of LC in the epidermis (27). To test whether *Tgfb1*$^{C33S/C33S}$ mice contain LC in their epidermis, we stained sheets of back skin epidermis from 21-week-old *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ mice for LC (28). No LC were detected in the epidermis of the mutant mice (Fig. S3), consistent with decreased active TGF-β1 generation in *Tgfb1*$^{C33S/C33S}$ mice.

Inflammation in *Tgfb1*^{C33S} Mice. Another early phenotype in *Tgfb1* null mice is multiorgan inflammation (29, 30). Therefore, we analyzed multiple tissues from *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ mice of different ages for inflammation. Histological analysis of tissues of *Tgfb1*$^{C33S/C33S}$ mice revealed inflammation of the heart, lungs, and rectum (Fig. 2) and the stomach, colon, cecum, and anus (data not shown) by 10 weeks of age. There was no observable inflammation of the skin, liver, prostate, or pancreas (data not shown). In the GI tract, inflammation was observed as early as 5 weeks after birth. There was some variability in the degree of inflammation, and animals that survived for long periods (3–4 months) exhibited little to no inflammation. Within the lungs, inflammation was seen around large blood vessels and large airways (data not shown). In the rectum, inflammatory cells were observed in the lamina propria and surrounding the crypts. As TGF-β1 is a potent suppressor of inflammation (31), the inflammation in the *Tgfb1*$^{C33S/C33S}$ mice probably reflects decreased active TGF-β1 produced in the affected organs. However, the inflammation in *Tgfb1*$^{C33S/C33S}$ mice was not as severe as that seen in *Tgfb1*$^{-/-}$ mice (29, 30) suggesting that the *Tgfb1*$^{C33S/C33S}$ mice are hypomorphs, rather than nulls, for TGF-β1 formation.

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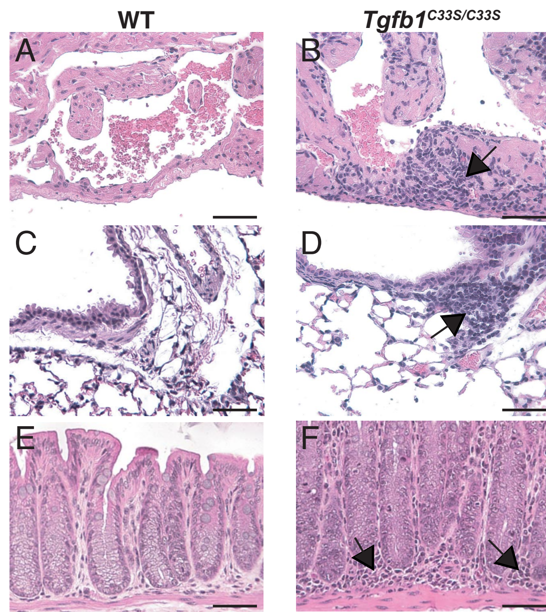

Fig. 2. Inflammation in $Tgfb1^{+/+}$ and $Tgfb1^{C33S/C33S}$ tissues. Samples were taken from WT and $Tgfb1^{C33S/C33S}$ fixed, sectioned, and stained. (*A*, *C*, and *E*) $Tgfb1^{+/+}$ tissues. (*B*, *D*, and *E*) $Tgfb1^{C33S/C33S}$ tissues. (A and *B*) Heart. There is no inflammation in the $Tgfb1^{+/+}$ heart, but inflammatory cells (arrows) are present in the mutant heart. (*C* and *D*) Lung. Although the numbers of inflammatory cells in the $Tgfb1^{+/+}$ lung are low, in the mutant tissue significant numbers of inflammatory cells surround certain blood vessels (arrows). (*E* and *F*) Terminal colon/rectum. The tissue from the $Tgfb1^{+/+}$ mouse displays few inflammatory cells. However, the tissue from the $Tgfb1^{C33S/C33S}$ animal had large numbers of inflammatory cells in the lamina propria. The heart and lung tissues were from 4-week-old animals and the terminal colon/rectal tissue from 12-week-old mice. (Scale bar: 50 μM.)

Tumors in $Tgfb1^{C33S/C33S}$ Mice. Histological examination of tissues from $Tgfb1^{C33S/C33S}$ mice revealed tumors in multiple organs (Table 1). Neoplasms were observed in ≈40% of the mutant animals 8 weeks of age (Table 1). We observed papillomas in the forestomach (data not shown) and adenocarcinomas in the glandular stomach (Fig. 3*A*), adenosquamous carcinomas in the terminal colon/rectum (Fig. 3*B*), and squamous cell carcinomas in the anus (data not shown). A few animals had tumors at multiple sites (Table 1). We found no tumors in the more proximal colon, skin, or lungs. The absence of tumors at more proximal sites in the colon of $Tgfb1^{C33S/C33S}$ mice may reflect a longer latency period for tumor development, as colon tumors in $Tgfb1^{-/-}$ mice develop significantly later than the tumors we observed. Tumor development correlated with the intensity of inflammation, as animals with mild inflammation rarely had tumors, whereas mice with strong inflammation almost always had tumors. The appearance of tumors in $Tgfb1^{C33S/C33S}$ mice is consistent with decreased levels of active TGF-β1 and a requirement for LLC formation for proper active TGF-β formation.

Table 1. Incidence of neoplasms in $Tgfb1^{C33S/C33S}$ mice

	Genotype		
Tumors	$Tgfb1^{+/+}$	$Tgfb1^{+/C33S}$	$Tgfb1^{C33S/C33S}$
+	0	0	21
−	29	24	30

Tumor frequency in $Tgfb1^{+/+}$, $Tgfb1^{+/C33S}$, and $Tgfb1^{C33S/C33S}$ mice. All mice were >8 weeks old when killed. There were 12 gastric papillomas, 4 gastric adenocarcinomas, 5 anal squamous cell carcinomas, 4 rectal adenocarcinomas, and 1 cecal adenocarcinoma. Four mice had both a squamous cell carcinoma and a rectal adenocarcinoma, two mice had a gastric adenocarcinoma and a rectal carcinoma, one mouse had a gastric papilloma and a cecal adenocarcinoma.

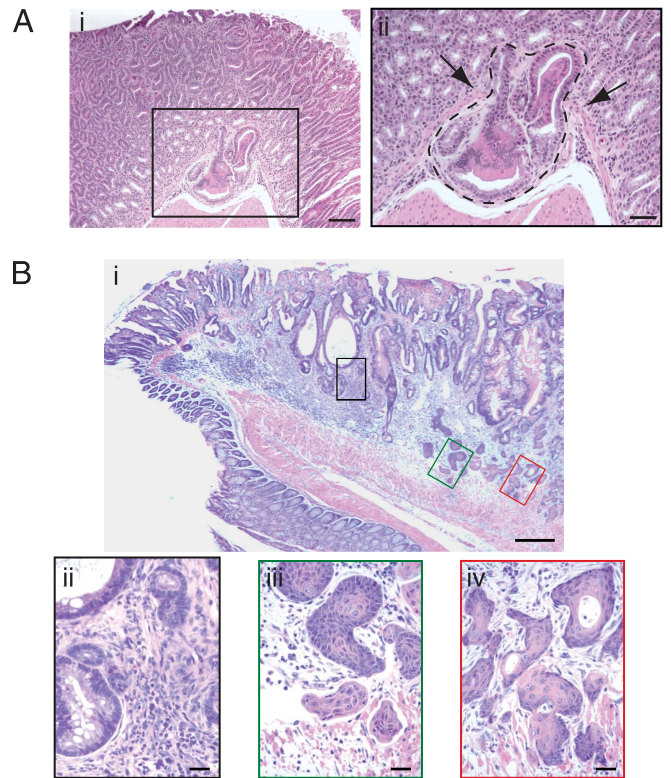

Fig. 3. Tumors in $Tgfb1^{C33S/C33S}$ mice. (*Ai*) Section from the glandular stomach mucosa illustrating an early gastric adenocarcinoma. (*ii*) Higher magnification of the enclosed box in *Ai*. Arrows point to invasive dysplastic epithelial cells that have breached the basement membrane and muscularis mucosa. (*B*) (*i*) Section near the rectal–anal junction illustrating an adenosquamous carcinoma. (*ii–iv*) Higher magnification of regions comprising the boxed areas shown in *B*. (*Biii*) Tumor with adenomatous histology. (*iii–iv*) Tumor with squamous cell carcinoma histology. [Scale bars: 100 μM (*Ai*); 50 μM (*Aii*); 200 μM (*Bi*); 50 μM (*Bii*); and 20 μM (*B iii* and *iv*).]

TGF-β Signaling. The phenotypes of $Tgfb1^{C33S/C33S}$ mice indicate decreased levels of TGF-β signaling. Therefore, we next examined the tissues of $Tgfb1^{C33S/C33S}$ mice for impaired TGF-β signaling activity. TGF-β1 signaling is propagated intracellularly by Smad2 and -3, which upon TGF-β receptor activation are phosphorylated, complex with Smad4, relocate in the nucleus, and form transcription complexes at TGF-β-responsive genes (2). Nuclear localization of P-Smad2 and -3, therefore, reflects TGF-β signaling within tissues. We examined the distribution of P-Smad2 in the stomachs of $Tgfb1^{+/+}$ and $Tgfb1^{C33S/C33S}$ mice at 11 weeks in animals with little inflammation and found that the nuclear staining of the mutant cells was less abundant and less intense than that of $Tgfb1^{+/+}$ cells, indicating decreased TGF-β signaling (Fig. 4*A*). We observed similar differences in the lungs at 8 weeks (data not shown). TGF-β acts as a suppressor of cell division in many epithelia. Therefore, we examined stomach and rectal epithelia of $Tgfb1^{C33S/C33S}$ mice for dividing cells with an antibody to Ki67 (32). We observed increased numbers of positively stained cells in the mutant compared to $Tgfb1^{+/+}$ in both the stomach (Fig. 4*B*) and the rectal (data not shown) epithelium consistent with decreased levels of TGF-β in the mutant tissue. We measured Myc expression in mutant rectal tissue using Q-RT-PCR (Fig. 4*C*), as C-Myc expression is often suppressed by TGF-β (33). C-Myc levels were enhanced in the

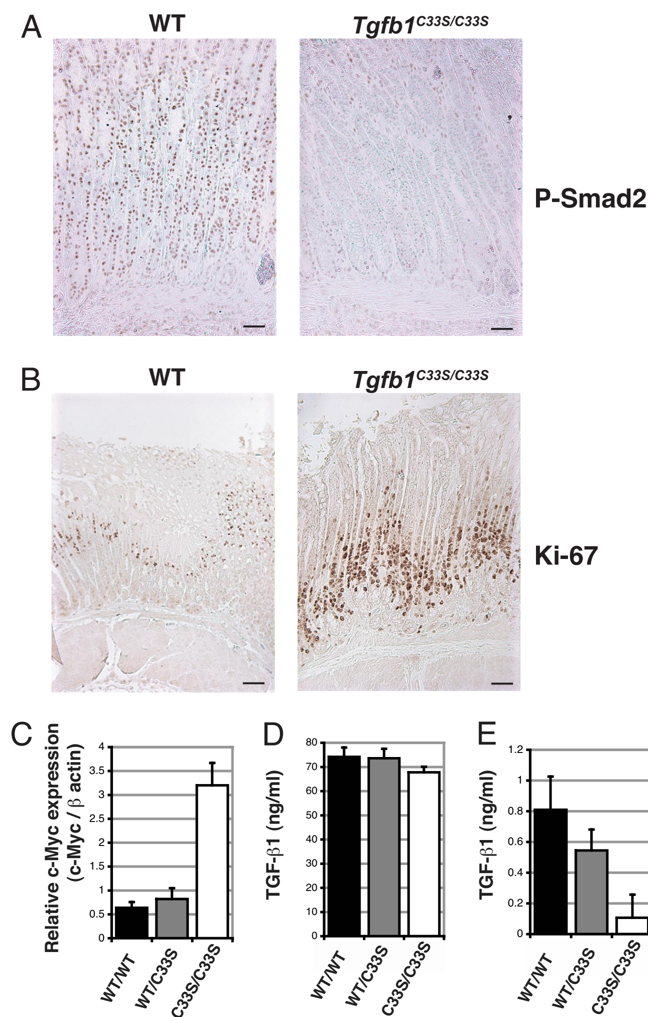

Fig. 4. TGF-β signaling and mitosis in tissues from *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ mice. (*A*) P-Smad2 staining of stomach from *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ of 11-week-old mice. The more intense P-Smad2 nuclear staining in the *Tgfb1*$^{+/+}$ vs. the *Tgfb1*$^{C33S/C33S}$ sample indicates enhanced TGF-β activity. (*B*) Staining of stomach tissue from *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ 21-week-old mice with antibody to Ki67, a marker of mitosis. The number of positive Ki67 cells is higher in the mutant sample compared to the *Tgfb1*$^{+/+}$ sample, consistent with decreased TGF-β1 in the tissue, as TGF-β1 is a suppressor of growth of most epithelial cells. (*C*) Q-RT-PCR of stomach epithelium for Myc from 12-week-old mice. *Tgfb1*$^{C33S/C33S}$ tissue had higher expression of Myc than did control tissue consistent with decreased TGF-β1 in the mutant tissue. (*D* and *E*) TGF-β1 in sera from *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ mice. TGF-β1 in sera from *Tgfb1*$^{+/+}$, *Tgfb1*$^{+/C33S}$, and *Tgfb1*$^{C33S/C33S}$ mice was measured by an ELISA specific for active TGF-β1. (*D*) Total TGF-β1 in sera after acidification. Acidification releases all active TGF-β from its latent complex. The total TGF-β1 from the three genotypes was equivalent. (*E*) Active TGF-β1 in sera. TGF-β1 was measured directly in sera without acidification. The amount of active TGF-β1 in the *Tgfb1*$^{+/+}$ and *Tgfb1*$^{+/C33S}$ samples is significantly higher than that found in samples from *Tgfb1*$^{C33S/C33S}$ mice. Samples from 10–12 animals of each genotype were assayed for TGF-β. Assays on each serum sample were done in triplicate. Values are presented as SEM.

mutant vs. the *Tgfb*$^{+/+}$ tissue. The expression of two other genes *Pai-1* and *Ctgf*, which are regulated by TGF-β, was not altered in mutant compared to normal tissue (data not shown), perhaps because of opposing effects of the inflammatory response. Overall, these results indicate decreased active TGF-β1 generation in *Tgfb1*$^{C33S/C33S}$ mice.

Decreased TGF-β1 signaling in *Tgfb1*$^{C33S/C33S}$ mice might result from decreased synthesis, processing, or secretion of TGF-β1^{C33S}. Therefore, we assayed sera from *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ mice for TGF-β1 by ELISA to assess total and active TGF-β1 levels *in vivo*. When we measured the total amount of TGF-β1 in each sample after acid treatment, a process that releases all latent TGF-β from LLC and SLC, we found only a slight difference between *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ samples, indicating no significant inhibition of synthesis, processing, or release of the mutant protein (Fig. 4*D*). Untreated sera from either *Tgfb1*$^{+/+}$ or *Tgfb1*$^{C33S/C33S}$ mice contained $<1\%$ active TGF-β1 compared to the acid-treated samples. But the *Tgfb1*$^{+/+}$ and *Tgfb1*$^{+/C33S}$ samples contained significantly more active TGF-β1 than did the *Tgfb1*$^{C33S/C33S}$ samples (Fig. 4*E*). Therefore, we conclude that the mutant mice produce approximately normal levels of latent TGF-β1, but the lack of LLC formation impairs latent TGF-β1 activation.

Discussion

We generated *Tgfb1*$^{C33S/C33S}$ mice to test for a requirement for LLC formation in TGF-β action. The absence of the cysteine 33 in TGF-β1 LAP precluded covalent association of any LTBP with the TGF-β1 SLC yielding exclusively the TGF-β1 SLC. The inability of the mutant SLC to bind to LTBP was demonstrated in conditioned medium from cultured cells derived from the lungs of *Tgfb1*$^{C33S/C33S}$ mice (Fig. 1*B*). Analysis of active TGF-β1 levels in serum from control and *Tgfb1*$^{C33S/C33S}$ animals indicated that mutant sera contained less active TGF-β1 than did *Tgfb1*$^{+/+}$ sera supporting a role for LLC formation in latent TGF-β1 activation. Although noncovalent interactions can occur between LAP and LTBP (34), we think that this type of association is unlikely to result in biologically significant extracellular interaction, as *Tgfb1*$^{C33S/C33S}$ has been shown to yield defective activation in two systems (12, 35). Therefore, we hypothesize that the hypomorphic, rather than null, phenotype of this mutation reflects activation of the SLC by activators such as TSP-1 (36) or αvβ8 (37).

Tgfb1$^{C33S/C33S}$ mice display a phenotype consistent with systemic decreased levels of active TGF-β1 compared to *Tgfb1*$^{+/+}$ animals. Like *Tgfb1*$^{-/-}$ mice, *Tgfb1*$^{C33S/C33S}$ mice have multiorgan inflammation and lack LC in the epidermis. *Tgfb1*$^{C33S/C33S}$ mice also display decreased levels of nuclear P-Smad2 in the stomach and lungs, and increased C-Myc expression and increased numbers of mitotic cells, all properties associated with decreased TGF-β. However, *Tgfb1*$^{C33S/C33S}$ mice have a milder multiorgan inflammation than *Tgfb1*$^{-/-}$ mice and have a longer life span. *Tgfb1*$^{C33S/C33S}$ animals develop neoplasms of the GI tract without suppression of the immune response, a phenotype absent in *Tgfb1*$^{-/-}$ mice (38). Tumor occurrence in *Tgfb1*$^{C33S/C33S}$, but not in *Tgfb1*$^{-/-}$, mice may relate to the longer survival of the *Tgfb1*$^{C33S/C33S}$ animals, as *Tgfb1*$^{-/-}$ mice develop tumors when their life span is extended (38). However, the tumor spectrum in *Tgfb1*$^{C33S/C33S}$ mice is different from that reported in immunosuppressed *Tgfb1*$^{-/-}$ mice, which develop colon carcinomas (38). Therefore, although *Tgfb1*$^{C33S/C33S}$ mice resemble *Tgfb1*$^{-/-}$ mice, they are not phenocopies. Rather *Tgfb1*$^{C33S/C33S}$ mice appear to be hypomorphic, not null, for active TGF-β1.

The phenotype of the *Tgfb1*$^{C33S/C33S}$ mice should be compared to *Tgfb1*$^{RGE/RGE}$ mice, which produce TGF-β1 that has an Arg–Gly–Glu (RGE) rather than RGD sequence in LAP (39). Absence of the RGD sequence precludes binding of either of the two integrins, αvß6 and αvß8, known to activate latent TGF-β1 (11, 37), to the TGF-β1 LAP. *Tgfb1*$^{RGE/RGE}$ mice are phenocopies of *Tgfb1*$^{-/-}$ mice, with strong multiorgan inflammation, lack of epidermal LC, and early death (39). These results indicate that the early *Tgfb1*$^{-/-}$ phenotypes derive from the absence of TGF-β1 generated by the action of αvβ6 and αvβ8 integrins. We reported that activation of latent TGF-β1 by αvβ6 required the participation of LTBP-1, and that TGF-β1$^{C33S/C33S}$ SLC was not activated by this integrin (12). We hypothesize that the lack of

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LC and the multiorgan inflammation observed in *Tgfb1*$^{C33S/C33S}$ mice result from decreased SLC compared to LLC activation. The fact that *Tgfb1*$^{C33S/C33S}$ mice are not identical to *Tgfb1*$^{-/-}$ or *Tgfb1*$^{RGE/RGE}$ mice may relate to the ability of the integrin $\alpha v\beta 8$ to activate SLC (37). It will be interesting to determine the consequence of blocking $\alpha v\beta 8$-mediated activation of latent TGF-β in *Tgfb1*$^{C33S/C33S}$ mice.

Finally, it is noteworthy that *Tgfb1*$^{C33S/C33S}$ mice develop tumors in multiple locations in the GI tract, a particularly sensitive region for tumor induction upon decreased TGF-β signaling. Colon tumors occur in *Tgfb1*$^{-/-}$ mice, if the inflammatory response is eliminated by crossing the null mutation onto a *Rag2*$^{-/-}$ background (38). Colon tumors also occur in other mouse models, such as *Smad3*$^{-/-}$ and *Apc*$^{\Delta 716}$ *Smad4*$^{+/-}$ mice, in which TGF-β signaling is impaired (40, 41). In addition, human colon carcinomas are often associated with mutations in components of the TGF-β signaling pathway (42–44). Previously, it was assumed that TGF-β acted directly on the epithelial cells and the loss of TGF-β signaling permitted initiated cells to proliferate. Recently, mutant mice were described in which TGF-β signaling was suppressed either in stromal cells or in T cells, but the mice develop tumors of the epithelia of the stomach or the intestine (45, 46), i.e., in tissues that have normal TGF-β signaling. Therefore, the effects of TGF-β in the tumor microenvironment may be critical in controlling tumor growth. We do not know whether tumors in the GI tract of *Tgfb1*$^{C33S/C33S}$ mice result from decreased TGF-β1 produced by the epithelial cells, the surrounding cells, or both. This will be an interesting question to answer in future experiments.

Materials and Methods

Plasmids. The pKS10xPNT plasmid was a gift of A. Joyner (Memorial Sloan-Kettering Institute).

Construction of the *Tgfb1* targeting vector. See *SI Methods*.

Generation of *Tgfb1*$^{C33S/C33S}$ mice. See *SI Methods*.

Primary Fibroblasts. Primary fibroblasts were obtained from the minced lungs of 6-day-old genotyped mice. Cells were immortalized by Sv40 large T as described (26).

TGF-β Assays. TGF-β1 concentrations were determined using the TGF-β1 Quantikine kit (R&D Systems). For detect active TGF-β1 in serum, samples were assayed without acid treatment. To detect total TGF-β1, latent TGF-β1 in serum was activated by acid treatment according to the manufacturer's instructions. All samples were quantified using the linear portion of a standard curve generated with the same kit and recombinant TGF-β1 (R&D Systems). Sera were prepared from anesthetized mice by heart puncture with a 27-g needle and a 1-ml syringe. Blood was immediately placed in a glass tube, allowed to clot at 37 °C for 4 h, and the sera obtained after centrifugation at 4 °C for 20 min at 14,000 g.

Immunohistochemistry. Three-micrometer sections of fresh formalin-fixed tissues were used. Unless otherwise noted, all staining was with hematoxylin and eosin. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in 100% methanol for 30 min. Antigen retrieval was performed using a Dako Cytomation Target Retrieval Solution for 20 min at 95 °C or in Na-citrate (0.01 M, pH 6), heated for 10 min in a microwave oven. Primary antibodies used were: phospho-Smad2 (1:300 dilution; Chemicon), Ki67 (1:400; Novocastra), and I-A/I-E (Clone 269; Pharmagen). Secondary staining used biotinylated secondary antibodies (Vector Laboratories) followed by Elite Vectastain ABC kit (Vector Laboratories) and peroxidase substrate DAB kit (Vector Laboratories).

LC Immunostaining. Preparation of dorsal trunk epidermal sheets and staining was done as described (27, 28).

Quantitative Real-Time RT-PCR Analysis. RNA was extracted from 10 pairs of *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ rectums using TRIzol (Invitrogen). Reverse transcription (RT) was performed using 1 μg of RNA and the SuperScript III Reverse Transcriptase (Invitrogen) (50 °C, 60′). The resulting cDNA was used for quantitative real-time RT-PCR (Q-RT-PCR) analysis (47). Q-RT-PCRs were performed using specific primers and QuantiFast SYBR Green PCR Kit (Qiagen) on an iCycler Thermal Cycler (Bio-Rad). Each target transcript expression was quantified by comparing the threshold cycle (TC) with that of hypoxanthine guanine phosphoribosyl transferase by using the comparative TC method (19). Primers used: *Actb* sense AGC CTT CCT TCT TGG GTA TGG, antisense GCC ACC GAT CCA CAC AGA GTA; *Myc* sense GCT GCT GTC CTC CGA GTC CTC, antisense GGG GTT TGC CTC TTC TCC ACA.

Statistical Analyses. Descriptive statistics were performed with StatView J-4.5 program (SAS Institute). The Kaplan–Meier method was used to estimate all survival curves from mouse studies. The log-rank statistic was used to compare the overall survival distributions of *Tgfb1*$^{+/+}$ and *Tgfb1*$^{C33S/C33S}$ mice. Mouse survival curves were compared with the log-rank statistic.

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