Excessive transforming growth factor-β signaling is a common mechanism in osteogenesis imperfecta

Ingo Grafe¹, Tao Yang¹, Stefanie Alexander¹, Erica P Homan¹, Caressa Lietman¹, Ming Ming Jiang¹,², Terry Bertin¹, Elda Munivez¹, Yuqing Chen¹, Brian Dawson¹,², Yoshihiro Ishikawa³,⁴, Mary Ann Weis⁵, T Kuber Sampath⁶, Catherine Ambrose⁷, David Eyre⁵, Hans Peter Bächinger³,⁴ & Brendan Lee¹,²

Osteogenesis imperfecta (OI) is a heritable disorder, in both a dominant and recessive manner, of connective tissue characterized by brittle bones, fractures and extraskeletal manifestations. How structural mutations of type I collagen (dominant OI) or of its post-translational modification machinery (recessive OI) can cause abnormal quality and quantity of bone is poorly understood. Notably, the clinical overlap between dominant and recessive forms of OI suggests common molecular pathomechanisms. Here, we show that excessive transforming growth factor-β (TGF-β) signaling is a mechanism of OI in both recessive (Crtap−/−) and dominant (Col1a2tm1.1Mcbr) OI mouse models. In the skeleton, we find higher expression of TGF-β target genes, higher ratio of phosphorylated Smad2 to total Smad2 protein and higher in vivo Smad2 reporter activity. Moreover, the type I collagen of Crtap−/− mice shows reduced binding to the small leucine-rich proteoglycan decorin, a known regulator of TGF-β activity. Anti–TGF-β treatment using the neutralizing antibody 1D11 corrects the bone phenotype in both forms of OI and improves the lung abnormalities in Crtap−/− mice. Hence, altered TGF-β matrix-cell signaling is a primary mechanism in the pathogenesis of OI and could be a promising target for the treatment of OI.

Most cases of OI are caused by autosomal dominant mutations in the genes encoding type I collagen (COL1A1 and COL1A2). In recent years, mutations in additional genes encoding the proteins involved in post-translational modifications of collagen have been identified as causing recessive forms of OI. The first described was in cartilage-associated protein (Crtap), a member of the prolyl-3-hydroxylase complex that is responsible for 3-hydroxylation of proline residue 986 of the α1 chain of type I collagen. Hypomorphic Crtap mutations lead to partial loss of 3-hydroxyproline (3Hyp) in fibrillar collagen and overmodification of other residues and result in recessive OI type VII, which clinically overlaps with the dominant forms of OI. The physiological function of 3Hyp is incompletely understood, but biochemical and genetic studies suggest that it is involved in collagen-protein interactions and required for normal bone mineralization. The extracellular matrix is an important reservoir for signaling molecules and their regulators. In bone, TGF-β acts as a central coordinator of bone remodeling by coupling the activity of bone-resorbing osteoclasts and bone-forming osteoblasts. TGF-β is produced by osteoblasts, secreted predominantly in inactive, latent forms and deposited into the bone matrix, where it can be released and activated during bone resorption by osteoclasts. As an additional level of regulation, active TGF-β can be bound by proteoglycans, which modulate its bioactivity in association with collagen fibrils. Because type I collagen is the most abundant component of the extracellular matrix in bone, we hypothesized that alterations of collagen observed in OI can affect the signaling-modulating function of the bone matrix. Consistent with this, Crtap−/− mice show phenotypic overlap with animal models of upregulated TGF-β signaling. For example, TGF-β overexpression results in low bone mass. In addition, Crtap−/− mice exhibit an enlargement in alveolar airspace in lungs, similar to that observed in a model of Marfan’s syndrome, where dysregulated TGF-β signaling contributes to lung pathology. Therefore, we first studied the status of TGF-β signaling in the Crtap−/− mouse model of recessive OI.

Compared with wild-type (WT) samples, calvarial bone of Crtap−/− mice showed a higher expression of the TGF-β targets Cdkn1a (cyclin-dependent kinase inhibitor 1a, P21) and Serpine1 (plasminogen activator inhibitor-1), consistent with elevated TGF-β activity. To confirm activation of the intracellular TGF-β signaling pathway, we evaluated the status of Smad2, a second messenger protein, which becomes phosphorylated after activation of TGF-β receptors. Consistent with higher TGF-β signaling, immunoblot analyses demonstrated a greater ratio of phosphorylated Smad2 (pSmad2) to total Smad2 to total Smad2 in calvarial bone samples of Crtap−/− compared with WT mice. To confirm higher TGF-β activity in vivo, we crossed Crtap−/− mice with reporter mice expressing luciferase in response

¹Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, USA. ²Howard Hughes Medical Institute, Houston, Texas, USA. ³Research Department, Shriners Hospital for Children, Portland, Oregon, USA. ⁴Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon, USA. ⁵Department of Orthopaedics and Sports Medicine, University of Washington, Seattle, Washington, USA. ⁶Genzyme Research Center, Framingham, Massachusetts, USA. ⁷Department of Orthopaedic Surgery, University of Texas Health Science Center at Houston, Houston, Texas, USA. Correspondence should be addressed to B.L. (blee@bcm.edu).

Received 13 October 2013; accepted 24 March 2014; published online 4 May 2014; doi:10.1038/nm.3544
Figure 1  Excessive TGF-β signaling in Crtap−/− mice. (a) Quantitative RT-PCR of TGF-β target genes Cdkn1a and Serpinel1 in calvarial bone of WT and Crtap−/− mice at postnatal day 3 (P3). Results are shown as fold change of the mean of WT group ± s.d.; n = 5 per group. (b) Western blot analysis of P3 calvarial protein extracts showing amounts of activated Smad2 (pSmad2) relative to total Smad2 protein in Crtap−/− versus WT mice; n = 3 per group. (c) Quantification of the western blot shown in b. Results are shown as fold change of the mean of WT group ± s.d. (d) Bioluminescence in regions that overlap with skeletal structures in Crtap−/− compared with WT mice that were intercrossed to TGF-β reporter mice (SBE-luc mice). Representative image of 3 litters at P10 is shown (scale bar, 1 cm). (e) TGF-β activity in conditioned medium from WT and Crtap−/− bone marrow stromal cells cultured under osteogenic conditions. Results are shown as fold change of the mean of WT group ± s.d.; n = 6 per group. (f) Immunostaining of lungs (P10) for pSmad2 (red) in WT and Crtap−/− mice, with DAPI (blue) staining of nuclei (40x magnification). Representative images of n = 3 mice per group are shown (scale bars, 20 μm). *P < 0.05 WT versus Crtap−/−, determined by Student’s t-test.

to TGF-β (SBE-luc mice). Compared with WT SBE-luc littermates, Crtap−/− SBE-luc mice showed more intense bioluminescence of areas over skeletal structures, indicating higher TGF-β activity in vivo (Fig. 1d; in three litters, Crtap−/− mice show a mean 2.86-fold higher (±0.34 s.d.) bioluminescence signal at the skull compared with WT mice).

To test whether higher TGF-β signaling is intrinsic to bone, i.e., tissue autonomous, we cultured bone marrow stromal cells (BMSCs) under osteogenic conditions in vitro. By using a TGF-β reporter cell line, we found that conditioned medium from Crtap−/− BMSCs exhibited greater TGF-β activity compared with medium from WT BMSCs (Fig. 1e). Together, these findings indicate that loss of Crtap enhances TGF-β signaling in bone in a tissue-autonomous fashion.

Patients with severe OI can exhibit lung abnormalities, and respiratory failure is one of the leading causes of death in these individuals. Crtap−/− mice show an enlarged alveolar airway space compared with WT mice, a feature associated with higher TGF-β signaling in other models. Accordingly, lungs of Crtap−/− mice showed more intense staining for pSmad2 in alveolar cells compared with lungs of WT mice, indicating that higher TGF-β activity is also present in extraskelatal tissues (Fig. 1f).

To understand whether upregulated TGF-β signaling represents a causal mechanism contributing to the bone and lung phenotypes in Crtap−/− mice, we performed a rescue experiment with a pan-TGF-β neutralizing antibody (1D11). We treated 8-week-old Crtap−/− mice with 1D11 for 8 weeks; all control Crtap−/− and WT mice received a nonspecific control antibody (13C4). Treatment with 1D11 did not change the body weight of Crtap−/− mice, indicating that TGF-β inhibition did not affect their general nutritional status (Supplementary Fig. 1).

Mass spectrometric and collagen cross-links analyses showed that 1D11 did not considerably change the status of P986 3-hydroxylation or collagen cross-links in Crtap−/− mice, suggesting that dysregulated TGF-β signaling is a consequence of the altered collagen and not directly involved in intracellular collagen processing or extracellular fibril assembly (Supplementary Fig. 2).

As previously reported, Crtap−/− mice exhibited reduced bone mass and abnormal trabecular bone parameters (Fig. 2a,b). Microcomputed tomography (microCT) imaging analysis of vertebrae and femurs demonstrated that compared with control Crtap−/− mice, Crtap−/− mice treated with 1D11 had significantly improved trabecular bone parameters, to near WT levels (Fig. 2a,b and Supplementary Tables 1 and 2). The effects of TGF-β inhibition with 1D11 on the skeleton have been reported previously in WT and E-selectin ligand-1 knockout (Esl1−/−) mice, a model with higher TGF-β activity due to impaired inhibition of TGF-β processing during its production. Whereas WT mice had moderately elevated (33% improvement) bone volume/total volume (BV/TV) after treatment with 1D11, Esl1−/− mice exhibited a 106% improvement after 1D11 treatment. This suggests that targeting TGF-β in a pathophysiological situation where it is upregulated could lead to a relatively more pronounced positive effect. In the present study, 1D11 improved the BV/TV at the spine by 235% in Crtap−/− mice, supporting the hypothesis that dysregulated TGF-β signaling is a major contributor to low bone mass.

At the femur midshaft, the measures of cortical architecture (cortical thickness, diameter, cross-sectional area and cross-sectional moments of inertia) in Crtap−/− mice were lower compared to the same parameters in WT mice. Following 1D11 treatment, these parameters were no longer significantly different from those in WT mice (Supplementary Table 3). To test whether 1D11 treatment resulted in improved bone strength, we performed biomechanical testing of the femurs. TGF-β inhibition resulted in higher maximum load and ultimate strength in WT and Esl1−/− mice, indicating that TGF-β inhibition at the cellular level, as previously reported, leads to a relatively more pronounced positive effect. In the present study, 1D11 improved the fracture strength, we performed biomechanical testing of the femurs. TGF-β inhibition resulted in higher maximum load and ultimate strength in WT and Esl1−/− mice, indicating that TGF-β inhibition at the cellular level, as previously reported, leads to a relatively more pronounced positive effect. In the present study, 1D11 improved the fracture strength.
were elevated in 8-week-old (osteocalcin and CTX) and 16-week-old (CTX only) control Crtap−/− mice compared with levels in WT mice (Supplementary Fig. 3). Similar changes have been described in patients with OI, who show higher osteoclast and osteoblast numbers consistent with elevated bone turnover19,20. Notably, mouse models of upregulated TGF-β signaling also show higher bone resorption and abnormal bone remodeling compared with WT mice8,14. Most reports of the effects of TGF-β on bone cells are consistent with a model where TGF-β stimulates the recruitment and initial differentiation of osteoclast and osteoblast precursors at the site of bone repair8,21,22, followed by insulin-like growth factor–mediated osteoblast differentiation23. However, at persistently high doses, TGF-β can inhibit osteoblast differentiation by repressing Runx2 (ref. 24), the key transcription factor that induces osteoblastic differentiation. Therefore, fine tuning of TGF-β availability is crucial for the local coupling of bone resorption with formation during bone remodeling, and its imbalance can lead to bone pathologies8,21.

In contrast to those from control Crtap−/− mice, bone sections of 1D11-treated Crtap−/− mice showed reduced osteoclast and osteoblast numbers; these numbers were even lower than those observed in bone sections from WT mice, indicating a supraphysiologic suppression of bone remodeling resulting from TGF-β inhibition at the dose of 1D11 used (Fig. 2c). Our findings are different from those of previous studies in WT mice, where 1D11 treatment resulted in lower osteoclast but higher osteoblast numbers25. This may reflect distinct effects of TGF-β inhibition in a pathological situation, with higher TGF-β signaling and bone remodeling compared with normal bone. TGF-β inhibits later differentiation of osteoblast precursors24, and upregulated TGF-β signaling could thereby lead to a higher proportion of immature osteoblastic cells. In contrast, a higher number or proportion of immature osteoblasts could result in higher TGF-β secretion. The finding that TGF-β inhibition reduces the elevated osteoblast numbers in Crtap−/− mice suggests that the upregulated TGF-β signaling causally contributes to the higher number of osteoblasts.

We also observed higher osteocyte numbers per bone area in control Crtap−/− compared with WT mice; these numbers were reduced to WT levels in 1D11-treated Crtap−/− mice (Fig. 2c and Supplementary Table 5). In patients with OI, a higher osteocyte density has been observed in individuals with more severe forms of the disease, probably reflecting the presence of immature primary bone owing to a defect in physiological bone maturation26. Similarly, overexpression of TGF-β in WT mice results in higher osteocyte density14. As a possible explanation, TGF-β has been shown to inhibit osteoblast apoptosis during the transition of osteoblasts to osteocytes27 and could thereby lead to a higher osteocyte density. Collectively, these findings indicate that excessive TGF-β signaling contributes to a high bone turnover status and impaired bone maturation in Crtap−/− mice and that inhibition of TGF-β signaling reverses these alterations.

We next investigated whether TGF-β inhibition affected the lung abnormalities in Crtap−/− mice and found that 1D11 treatment resulted in amelioration of this phenotype. Whereas lungs of control antibody-treated Crtap−/− mice showed a 6.9-µm increase of the mean distance between alveolar structures compared to WT mice, lungs of Crtap−/− mice treated with 1D11 demonstrated only a 2.7-µm increase...
compared to WT mice (Fig. 2d,e). This finding indicates that excessive TGF-β signaling is also a contributor to the lung abnormalities in Crtap−/− mice. Upregulated TGF-β signaling has been linked to developmental pulmonary abnormalities and disease in mature lungs. For example, TGF-β overexpression in lungs results in impaired lung development and enlarged airway space28, and upregulated TGF-β signaling is a pathomechanism of emphysema and bronchial asthma29,30.

Given the partial rescue of the lung phenotype with 1D11 in Crtap−/− mice, it is possible that dysregulated TGF-β signaling affects lung development when anatomic structures are established, in addition to maintaining pulmonary tissue at later stages.

We next asked how collagen alterations due to loss of Crtap result in dysregulated TGF-β signaling. Biochemical analyses have indicated that collagen prolyl-3-hydroxylation does not fundamentally affect the stability of collagen molecules and instead may affect collagen–protein interactions6. An attractive hypothesis is that loss of 3Hyp and/or collagen overmodification could affect collagen interaction with small leucine-rich proteoglycans (SLRPs). SLRPs bind to both type I collagen and type II collagen and thereby modulate TGF-β activity3,4. For example, the SLRP decorin inhibits distinct effects of TGF-β in osteosarcoma cells, such as the upregulation of biglycan synthesis31, whereas it enhances TGF-β activity in preosteoblastic cells4. The decorin–binding region on type I collagen has been suggested to center at residues 961 and 962 of the triple helical domain32, which is located in close proximity to the sequestration of TGF-β signaling, even if no major changes in absolute TGF-β levels are present (Supplementary Figs. 5 and 6). This notion is supported by the finding that COL1A1 and COL1A2 mutations in severe forms of dominant OI cluster in regions that are known to bind proteoglycans33, further supporting the relevance of proteoglycan-collagen interactions for normal bone homeostasis. This implies that other proteoglycans that are competing with decorin for the binding site on collagen35 may also contribute to

Hence, we hypothesize that decorin binding to collagen is critical for TGF-β regulation and that this binding is disrupted with altered collagen structure in OI. We found that although loss of Crtap did not alter the expression of decorin and other SLRPs in calvarial bone (Fig. 3a) or the qualitative abundance of decorin in trabecular bone (Supplementary Fig. 4), it did reduce binding of decorin to type I collagen (Fig. 3b; mean reductions of decorin binding to Crtap−/− versus WT type I collagen at 3, 5 and 12 μM of decorin were 28.5%, 33.5% and 38.1%, respectively). On the basis of the requirement of collagen binding for decorin to effectively reduce TGF-β bioactivity3, it is possible that this altered binding in OI affects decorin’s ability to sequester mature TGF-β in the matrix and modulate TGF-β function. This could contribute to dysregulated TGF-β signaling, even if no major changes in absolute TGF-β levels are present (Supplementary Figs. 5 and 6).

Figure 3 Reduced decorin binding to type I collagen of Crtap−/− mice. (a) Quantitative RT-PCR of calvarial bone of P3 mice for the small leucine-rich proteoglycans decorin (Dcn), biglycan (Bgn) and asporin (Aspn) in WT and Crtap−/− mice. Results given as fold change of the mean of WT group ± s.d.; n = 5 per group. NS, not significant, determined by Student’s t-test. (b) Surface plasmon resonance analysis measuring the binding of recombinant decorin core protein to type I collagen of WT and Crtap−/− mice. Three technical replicates at each of the indicated concentrations of decorin were performed in two independent biological replicates (for each biological replicate, collagen was pooled from three mice per genotype). Results are shown as the percentage of the mean of WT (bars indicate mean per group). RU, response units.

Figure 4 Inhibition of upregulated TGF-β signaling improves the bone phenotype in a mouse model of dominant OI (Col1a2tm1.1Mcbr). (a) Quantitative RT-PCR of TGF-β target genes Cdkn1a and Serpine1 in calvarial bone of P3 WT and Col1a2tm1.1Mcbr mice. Results are shown as fold change of the mean of WT group ± s.d.; n = 3 per group. (b) Western blot analysis showing activated Smad2 (pSmad2) relative to total Smad2 protein in P3 calvaria of WT and Col1a2tm1.1Mcbr mice; n = 3 per group. (c) Quantification of the western blot shown in B. Results are shown as fold change of the mean of WT group ± s.d. (d) MicroCT images of L4 vertebral bodies of 16-week-old WT, control antibody-treated Col1a2tm1.1Mcbr and 1D11-treated Col1a2tm1.1Mcbr mice after treatment for 8 weeks (scale bars, 500 μm). (e) MicroCT analysis results of L4 vertebral bodies for bone volume/total volume (BV/TV), trabecular number (Tb.N) and thickness (Tb.Th) in WT, control Col1a2tm1.1Mcbr and 1D11-treated Col1a2tm1.1Mcbr mice. Results are shown as means ± s.d.; n = 6 per group. *P < 0.05. NS, not significant, determined by Student’s t-test (a and c) and one-way ANOVA (e).
dysregulated TGF-β activity and that additional signaling pathways could be altered35. Because of the clinical overlap of some recessive and dominant forms of OI, it is possible that dysregulation of TGF-β signaling is a common disease mechanism. To address this hypothesis, we investigated the status of TGF-β signaling in a mouse model of dominant OI. Knock-in mice carrying a G610C mutation in the Col1a2 gene (Col1a2tm1.1Mcbr) phenocopy a dominantly inherited moderate form of OI that was identified in an Amish population. Compared with bone samples of WT mice, bone samples from Col1a2tm1.1Mcbr mice showed higher expression of the TGF-β target genes Cdkn1a and Serpine1, indicating upregulation of TGF-β signaling (Fig. 4a). Consistent with these results, immunoblot analyses showed a greater ratio of pSmad2 to total Smad2 in bone of Col1a2tm1.1Mcbr compared with WT mice, similar to our observation in Crtap−/− mice (Fig. 4b,c).

To test whether excessive TGF-β signaling also represents a causal mechanism in dominant OI, we treated 8-week-old Col1a2tm1.1Mcbr mice with the TGF-β neutralizing antibody 1D11 and control Col1a2tm1.1Mcbr and WT mice with the control antibody 13C4 for 8 weeks. Similar to 1D11 treatment in Crtap−/− mice, 1D11 treatment in Col1a2tm1.1Mcbr mice restored the trabecular bone parameters at the spine compared to control-treated Col1a2tm1.1Mcbr mice to WT levels, including bone volume, trabecular number and trabecular thickness (Fig. 4d,e and Supplementary Table 6). Together, these findings indicate that dysregulated TGF-β signaling is also a key contributor to the pathogenesis of dominant OI and that anti-TGF-β therapy corrects the bone phenotype in dominant OI.

From a clinical-translational perspective, potential negative effects of systemic TGF-β inhibition in patients with OI have to be considered. Whereas Tgfb1−/− mice develop a severe dysregulation of the immune system with inflammatory disease within the first weeks of life36, we did not observe obvious negative effects on general health, behavior or growth in either Crtap−/− or Col1a2tm1.1Mcbr mice treated with 1D11, suggesting that the effects of partial pharmacological inhibition of TGF-β in adult mice are different from those of a complete loss of TGF-β1 during development. In humans, fresolimumab, which is similar to 1D11 in its affinity and specificity to the three isoforms of TGF-β, has been tested in phase 1 clinical studies in patients with primary focal segmental glomerulosclerosis37, idiopathic pulmonary fibrosis38 and cancers39. In these studies, fresolimumab was, in general, well tolerated, with possible dose-related adverse events including skin rashes or lesions, epistaxis, gingival bleeding and fatigue.

The molecular mechanisms of OI are incompletely understood. As a result, current treatment options for patients with OI are mainly limited to antosteoporosis therapies with antiresorptive drugs. Of note, a recent randomized controlled trial of the anabolic agent teriparatide showed that adult patients with severe OI responded differently than those with mild OI40. This suggests that there are genotypic differences in response to therapies targeted at modifying cell signaling and that TGF-β inhibition may be a promising target in severe OI owing to mutations in the genes encoding collagen and proteins involved in its post-translational modification. Overall, our data support the concept of dysregulated matrix-cell signaling as a mechanism in the pathogenesis of different forms of brittle bone disease and point to a disease-specific mechanism–based strategy for the treatment of OI by neutralizing overactive TGF-β activity.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank D. Spencer and his lab (Baylor College of Medicine) for providing the IVIS camera system and training, M. Starbeck and F. Gannon for consultation and advice in bone histomorphometry, M. Warman and C. Jacobsen (Boston Children’s Hospital) for providing the Col1a2tm1.1Mcbr mice and helpful information. We also thank L. Fisher (US National Institute of Dental and Craniofacial Research) for providing the decorin antibody LF-113. We thank M. Bagos for help with microCT analyses, A. Abraham for help with the biomechanical testing, W. Song and S. Liu for their help with serum bone turnover analyses, the US National Institutes of Health (NIH) for providing the Image software and A. Choi and H. Liang (Harvard Medical School) for providing the Image modification for lung morphometry, which was generated by P. Thompson. Also, we thank D. Rikfin (New York University Medical Center) for providing PAI-luciferase reporter mink lung epithelial cells. In addition, we thank R. Mertello, G. Sule, D. Baldridge and G. Ghosal for their helpful discussions and P. Fonseca for editorial assistance. This work was supported by a research fellowship from the German Research Foundation/Deutsche Forschungsgemeinschaft (I.G.), a Michael Geisman Fellowship from the Osteogenesis Imperfecta Foundation (I.G.), grant support from Shriners Hospitals for Children (H.P.B.), NIH grants S51DE020954 (E.P.H.), R37AR072518 and R01 AR036794 (D.E.) and P01 HD70394 (B.L. and D.E.) and the Howard Hughes Medical Institute Foundation (B.L.). This work was also supported by the Baylor College of Medicine Intellectual and Developmental Disabilities Research Center (HD024064), the Eunice Kennedy Shriver US National Institute of Child Health & Human Development and the Rolante and Berdon Lawrence Bone Disease Program of Texas.

AUTHOR CONTRIBUTIONS

I.G. and B.L. conceptualized the study, T.Y., T.K.S., C.A., D.E. and H.P.B. contributed to the design of the study and experiments. I.G., T.Y., S.A., E.P.H., C.L., M.M.J., T.B., E.M., Y.C., B.D., Y.I., M.A.W. and C.A. performed and analyzed the experiments. I.G., T.Y. and B.L. wrote the manuscript with contributions from all authors. B.L. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

© 2014 Nature America, Inc. All rights reserved.

1. Rauch, F. & Glorieux, F.H. Osteogenesis imperfecta. Lancet 363, 1377–1385 (2004).

2. Baldridge, D.L. et al. CRTAP and LEPRE1 mutations in recessive osteogenesis imperfecta. Hum. Mutat. 29, 1345–1347 (2008).

3. Markmann, A., Hauser, M., Schonherr, E. & Kresse, H. Influence of decorin expression on transforming growth factor-β-mediated collagen gel retraction and biglycan induction. Matrix Biol. 19, 631–636 (2000).

4. Takeuchi, Y., Kodama, Y. & Matsumoto, T. Bone matrix decorin binds transforming growth factor-β and enhances its bioactivity. J. Biol. Chem. 269, 32634–32638 (1994).

5. Morell, R. et al. CRTAP is required for prolyl 3-hydroxylation and mutations cause recessive osteogenesis imperfecta. Cell 127, 291–304 (2006).

6. Mizuno, K., Peyton, D.H., Hayashi, T., Engel, J. & Bächinger, H.P. Effect of the -Gly-3(3-hydroxyprolyl-4(R)-hydroxyprolyl): tripeptide unit on the stability of collagen model peptides. FEMS J. 275, 5830–5840 (2008).

7. Homan, E.P. et al. Differential effects of collagen prolyl 3-hydroxylation on skeletal tissues. PLoS Genet. 10, e1004121 (2014).

8. Tang, Y. et al. TGF-β1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. Nat. Med. 15, 757–765 (2009).

9. Yang, T. et al. E-selectin ligand 1 regulates bone remodeling by limiting bioactive TGF-β1 in the bone microenvironment. Proc. Natl. Acad. Sci. USA 110, 736–741 (2013).

10. Dallas, S.L. et al. Characterization and autoregulation of latent transforming growth factor β (TGF-β) complexes in osteoblast-like cell lines. Production of a latent complex lacking the latent TGF-β binding protein. J. Biol. Chem. 269, 6815–6821 (1994).

11. Hering, S. et al. TGFβ1 and TGFβ2 mRNA and protein expression in human bone samples. Exp. Clin. Endocrin. Diabetes 109, 217–226 (2001).

12. Bacro, R.D., Mundy, G.R., Seyedin, S.M. & Boneuad, L.F. Activation of the bone-derived latent TGF β complex by isolated osteoclasts. Biochem. Biophys. Res. Commun. 158, 817–823 (1989).

13. Hildebrand, A. et al. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor β. Biochem. J. 302, 527–534 (1994).
14. Erlebacher, A. & Derynck, R. Increased expression of TGF-β 2 in osteoblasts results in an osteoporosis-like phenotype. J. Cell Biol. 132, 195–210 (1996).
15. Neptune, E.R. et al. Dysregulation of TGF-β1 activation contributes to pathogenesis in Marfan syndrome. Nat. Genet. 33, 407–411 (2003).
16. Baldridge, D. et al. Generalized connective tissue disease in Crtap−/− mouse. PLoS ONE 5, e10560 (2010).
17. Thiele, F. et al. Cardiopulmonary dysfunction in the osteogenesis imperfecta mouse model Aga2 and human patients are caused by bone-independent mechanisms. Hum. Mol. Genet. 21, 3535–3545 (2012).
18. McAllion, S.J. & Paterson, C.R. Causes of death in osteogenesis imperfecta. J. Clin. Pathol. 49, 627–630 (1996).
19. Rauch, F., Travers, R., Parfitt, A.M. & Glorieux, F.H. Static and dynamic bone histomorphometry in children with osteogenesis imperfecta. Bone 26, 581–589 (2000).
20. Ward, L.M. et al. Osteogenesis imperfecta type VII: an autosomal recessive form of brittle bone disease. Bone 31, 12–18 (2002).
21. Janssens, K., ten Dijke, P., Janssens, S. & Van Hul, W. Transforming growth factor-β1 to the bone. Endocr. Rev. 26, 743–774 (2005).
22. Fuller, K., Leun, J.M., Bayley, K.E., Wani, M.R. & Chambers, T.J. A role for TGFβ1 in osteoclast differentiation and survival. J. Cell Sci. 113, 2445–2453 (2000).
23. Xian, L. et al. Matrix IGF-1 maintains bone mass by activation of mTOR in mesenchymal stem cells. Nat. Med. 18, 1095–1101 (2012).
24. Alliston, T., Choy, L., Ducy, P., Karsenty, G. & Derynck, R. TGF-β1–induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. EMBO J. 20, 2254–2272 (2001).
25. Edwards, J.R. et al. Inhibition of TGF-β signaling by TDFI antibody treatment increases bone mass and quality in vivo. J. Bone Miner. Res. 25, 2419–2426 (2010).
26. Sarathchandra, P., Pope, F.M., Kayser, M.V. & Ali, S.Y. A light and electron microscopic study of osteogenesis imperfecta bone samples, with reference to collagen chemistry and clinical phenotype. J. Pathol. 192, 385–395 (2000).
27. Karsdal, M.A. et al. Matrix metalloproteinase–dependent activation of latent transforming growth factor-β controls the conversion of osteoblasts into osteocytes by blocking osteoblast apoptosis. J. Biol. Chem. 277, 44061–44067 (2002).
28. Gauldie, J. et al. Transfer of the active form of transforming growth factor-β 1 gene to newborn rat lung induces changes consistent with bronchopulmonary dysplasia. Am. J. Pathol. 163, 2575–2584 (2003).
29. Morty, R.E., Konigshoff, M. & Eickelberg, O. Transforming growth factor-β signaling across ages: from distorted lung development to chronic obstructive pulmonary disease. Proc. Am. Thorac. Soc. 6, 607–613 (2009).
30. Marwick, J.A. et al. Cigarette smoke-induced oxidative stress and TGF-β1 increase p21waf1/cip1 expression in alveolar epithelial cells. Ann. NY Acad. Sci. 973, 278–283 (2002).
31. Haussler, H., Groning, A., Hasilik, A., Schonherr, E. & Kresse, H. Selective inactivity of TGF-β1/decorin complexes. FEBS Lett. 353, 243–245 (1994).
32. Keene, D.R. et al. Decorin binds near the C terminus of type I collagen. J. Biol. Chem. 275, 21801–21804 (2000).
33. Marini, J.C. et al. Consortium for osteogenesis imperfecta mutations in the helical domain of type I collagen: regions rich in lethal mutations align with collagen binding sites for integrins and proteoglycans. Hum. Mutat. 28, 209–221 (2007).
34. Schönherr, E. et al. Interaction of biglycan with type I collagen. J. Biol. Chem. 270, 2776–2783 (1995).
35. Nikitovic, D. et al. The biology of small leucine-rich proteoglycans in bone pathophysiology. J. Biol. Chem. 287, 33926–33933 (2012).
36. Christ, M. et al. Immune dysregulation in TGF-β 1–deficient mice. J. Immunol. 153, 1936–1946 (1994).
37. Trachtman, H. et al. A phase 1, single-dose study of fresolimumab, an anti-TGF-β antibody, in treatment-resistant primary focal segmental glomerulosclerosis. Kidney Int. 79, 1236–1243 (2011).
38. Lonning, S., Mannick, J. & McPherson, J.M. Antibody targeting of TGF-β in cancer patients. Curr. Pharm. Biotechnol. 12, 2176–2189 (2011).
39. Morris, J.C. et al. Phase I study of GC1008 (fresolimumab): a human anti-transforming growth factor-β (TGFβ) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma. PLoS One 11, e90353 (2014).
40. Orwoll, E.S. et al. Evaluation of teriparatide treatment in adults with osteogenesis imperfecta. J. Clin. Invest. 124, 491–498 (2014).
In vivo bioluminescence imaging. We injected P10 Crtap−/− mice and WT littermates that expressed the TGF-β reporter transgene (SBE-luc mice) with β-luciferin (GoldBio, 150 mg kg−1, i.p.), anesthetized them with isoflurane and performed imaging 10 min after injection using a bioluminescence imaging system (Xenogen).

Primary osteoblast culture and TGF-β reporter cells. We isolated bone marrow cells from tibiae and femurs of approximately 2-month-old Crtap−/− and WT mice and cultured them in α-MEM supplied with 10% FBS, 100 U mL−1 penicillin and 100 μg mL−1 streptomycin. We changed the medium every second day and discarded unattached cells. After 7 d, we reseeded the attached cells, defined as bone marrow stromal cells (BMSCs), to 24-well plates at 2.5 × 10⁴ cells per cm² and cultured them in osteogenic medium (α-MEM, 10% FBS, 500 μM ascorbic acid and 10 mM β-glycerophosphate) for 3 d. We collected conditioned medium and incubated this with plasminogen activator inhibitor-1–luciferase reporter mink lung epithelial cells (these cells were obtained from the laboratory of D. Rifkin; they were not recently further profiled or tested for mycoplasma contamination). After 24 h, we collected the cell lysates for luciferase activity assays, which were measured using the Dual-Luciferase Reporter System (Promega). The results were normalized to the total protein amount quantified using the Micro BCA reagent (Pierce).

MicroCT and bone histomorphometry. We scanned lumbar vertebrae and femurs using a Scanco μCT-40 microCT for quantification of trabecular and cortical bone parameters. We analyzed vertebral and femoral trabecular bone parameters using the Scanco analysis software by manually contouring the trabecular bone of vertebral body L4 as well as the distal metaphyseal section of the femur. The cortical bone parameters at the center of the femoral midshaft were quantified using the automated thresholding algorithm included in the software. Scanned undecalcified Crtap−/− mouse spine samples were then embedded in plastic for sectioning. We performed toluidine blue staining and TRAP staining using standard protocols for visualization and quantification of osteoblasts and osteoclasts, respectively, using the Bioquant Osteo Image Analysis System.

Immunostaining and histology. For immunohistochemistry, we collected hind limbs of P5 mice, fixed them overnight in 4% paraformaldehyde and embedded them in paraffin. After deparaffinization and rehydration, we performed heat-induced antigen retrieval (Dako, S1700) followed by treatment with hyaluronidase for 30 min (2 mg mL−1; Sigma). Endogenous peroxidase was blocked using 3% hydrogen peroxide for 10 min. After incubation with blocking solution (3% normal goat serum, 0.1% BSA, 0.1% Triton X-100 in PBS), we incubated sections in antibodies specific for TGF-β1 (G1221, Promega) and decorin (LF-113, provided by L. Fisher) for 60 min (1:25 dilution each in PBS, control samples were incubated in PBS only) at 37 °C in plastic. We subsequently incubated them with secondary antibody (SuperPiciTure Ploymer Detection kit, Invitrogen, 87-9663, undiluted following the manufacturer’s protocol). We added substrate DAB according to the manufacturer’s recommendations and dehydrated and mounted samples using Cytoseal XYL xylene-based mounting medium (Thermo Scientific). We processed sections of WT and mutant littermates at the same time. Images of the trabecular bone were taken with a light microscope (Axioplan 2, Zeiss) using identical exposure times for WT and mutant littermates.

We equally inflated lungs of P10 and 16-week-old Crtap−/− mice during tissue collection, fixed them in 4% paraformaldehyde and embedded paraffin embedding. We used lungs of P10 Crtap−/− and WT mice for immunostaining for pSmad2. Briefly, we treated paraffin sections with xylene, and dehydrated and heated them for 20 min for antigen retrieval (pH 6; Dako). We then incubated sections in blocking solution (3% normal donkey serum, 0.1% BSA, 0.1% Triton X-100 in PBS) and subsequently incubated them with rabbit anti-pSmad2 antibody (1:500) (Cell signaling, #3101), donkey anti-rabbit secondary antibody conjugated to Alexa Flour 594 (1:600) (Invitrogen, A-21207) and mounted them with Prolong Gold antifade reagent with DAPI (Invitrogen). Fluorescent images from these sections were taken using a Zeiss microscope (Axiovision Software) using identical exposure times.

For lung histology and morphometry of 16-week-old mice, we stained parasagittal sections using a standard protocol for H&E staining. We used the mean
Biomechanical testing by three-point bending. We tested Crtap−/− and WT femurs by three-point bending using a span of 6 mm with an Instron 5848 device (Instron Inc., Norwood MA). All the femurs were tested wet at room temperature. We preloaded femurs to 1 N at a rate of 0.05 N s⁻¹ for 5 s. Following the preloading, we loaded the femurs to failure at a rate of 0.1 mm s⁻¹. Load and displacement data was captured at rate of 40 Hz by using BLUEHILL Software (Instron 5848). To determine the yield point, a region was identified after the preload and before the maximum load on the load-displacement curve. We separated this region into five segments, from which the fitted line of the segment with greatest slope was taken. Next, a 0.012-mm offset was implemented on the line. The point of intersection between the offset line and the load-displacement curve was the 0.012 offset yield point. This yield point corresponded more closely to a 0.2% offset strain, which is commonly chosen in the literature. The elastic region was identified as the region from the completion of the preload to the yield point. Postyield region was identified as the region from the yield point until the point at which the change in load exceeded −1 N, indicating failure. Elastic displacement was the displacement during which the specimen remained in the elastic region. Postyield displacement was the displacement during which specimen remained in the postyield region. Total displacement was calculated as the sum of elastic displacement and postyield displacement. Using the trapezoidal numerical integration method, we calculated energy to failure as the area under the load-displacement curve. We determined maximum load by finding the highest load value recorded by BLUEHILL before the specimen failed. To calculate stiffness, we applied the least-square fit method to the steepest region. Geometric data (diameter and moment of inertia) obtained from microCT analysis of the femoral midshaft were used to calculate the intrinsic material properties: ultimate strength, toughness to failure and elastic modulus.

Serum bone turnover markers. We quantified serum osteocalcin using the Mouse Osteocalcin EIA Kit from Biomedical Technologies Inc. and C-terminal cross-linked telopeptide of bone collagen (CTX) using the RatLaps EIA Kit from Immunodiagnostics Systems Ltd. Both analyses were performed according to the manufacturer’s protocols.

Collagen SDS-PAGE, mass spectrometry and cross-links analyses. For mass spectrometry, we prepared type I collagen from Crtap−/− and WT tibias. We defatted bone with chloroform/methanol (3:1 v/v) and demineralized it in 0.5 M EDTA, 0.05 M Tris-HCl, pH 7.5, all steps at 4 °C. We finely minced the bone samples and solubilized collagen by heat denaturation (90 °C) in SDS-PAGE sample buffer. Collagen α-chains were cut from SDS-PAGE gels and subjected to in-gel trypsin digestion. We performed electrospray mass spectrometry on the tryptic peptides using an LCQ Deca XP ion-trap mass spectrometer equipped with in-line liquid chromatography (ThermoFinnigan) using a C8 capillary column (300 µm × 150 mm; Grace Vydac 208 MS5.315) eluted at 4.5 µl per min. Sequest search software (ThermoFinnigan) was used for peptide identification using the NCBI protein database.

We quantified pyridinoline cross-links (hydroxylysyl pyridinoline and lysyl pyridinoline) by HPLC after hydrolyzing demineralized bone in 6 N HCl as described.

Surface plasmon resonance analysis. We performed surface plasmon resonance experiments using a BIAcore X instrument (GE Healthcare Bio-Sciences). We immobilized purified native mouse tendon type I collagen from three WT and three Crtap−/− mice (pooled for each genotype) on CMS sensor chips (GE Healthcare Bio-Sciences AB, BR100399) by amide coupling at a concentration of about 0.5 ng mm⁻² (500 RU) and 0.8 ng mm⁻² (800 RU), respectively. The experiments were conducted at a flow rate of 10 µl min⁻¹ and 20 °C in HBS-P buffer, pH 7.4, containing 150 mM NaCl and 0.005% surfactant P20. We then injected recombinant human decorin core protein (R&D systems) onto both type I CMS chips. We determined the concentration of the stock solution of human decorin by amino acid analysis. The binding response of decorin to WT and Crtap−/− mouse type I collagen was normalized by the amounts of immobilized type I collagen on the CMS sensor chips. We used three concentrations of decorin (3, 5 and 12 µM), and for each concentration the analysis was repeated three times. We performed this experiment twice with collagen isolated from different mice each time.

Statistical analyses. For comparisons between two groups, we used unpaired two-tailed Student’s t-tests. For comparisons between three groups, we performed one-way ANOVA if equal variance and normal distribution of groups were confirmed, followed by all pairwise multiple comparison using the Holm-Sidak method. If the equal variance or normal distribution test failed, we performed Kruskal-Wallis one-way ANOVA on ranks, followed by all pairwise multiple comparison using the Tukey’s test. A P value less than 0.05 was considered statistically significant for Student’s t-test, ANOVA and Kruskal-Wallis one-way ANOVA on ranks. For post hoc pairwise multiple comparisons, each P value was compared to a critical level depending on the rank of the P value and the total number of comparisons made to determine whether differences between groups are significant. We used Sigma Plot V11.0 (Systat Software Inc.) for statistical analyses. The effects of 1D11 on bone and lungs of OI mice were unknown at study start. To determine the initial sample size per group of mice, we calculated that to detect a minimal difference of 20% in bone mass (BV/TV) by MicroCT between 1D11- and control-treated OI mice with a 90% power, a group size of eight mice is required.

14. Daley, E. et al. Variable bone fragility associated with an Amish COL1A2 variant and a knock-in mouse model. J. Bone Miner. Res. 25, 247–261 (2010).
15. Liu, A.H. et al. Global analysis of Smad2/3-dependent TGF-β signaling in living mice reveals prominent tissue-specific responses to injury. J. Immunol. 175, 547–554 (2005).
16. Abe, M. et al. Variable bone fragility associated with an Amish COL1A2 variant and a knock-in mouse model. J. Bone Miner. Res. 25, 247–261 (2010).
17. Lin, A.H. et al. Global analysis of Smad2/3-dependent TGF-β signaling in living mice reveals prominent tissue-specific responses to injury. J. Immunol. 175, 547–554 (2005).
18. Abe, M. et al. Variable bone fragility associated with an Amish COL1A2 variant and a knock-in mouse model. J. Bone Miner. Res. 25, 247–261 (2010).