Human TGF-β1 deficiency causes severe inflammatory bowel disease and encephalopathy

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Transforming growth factor (TGF)-β1 (encoded by TGFB1) is the prototypic member of the TGF-β family of 33 proteins that orchestrate embryogenesis, development and tissue homeostasis1. Following its discovery, enormous interest and numerous controversies have emerged about the role of TGF-β in coordinating the balance of pro- and anti-oncogenic properties, pro- and anti-inflammatory effects, or pro- and anti-fibrinogenic characteristics. Here we describe three individuals from two pedigrees with biallelic loss-of-function mutations in the TGFB1 gene who presented with severe infantile inflammatory bowel disease (IBD) and central nervous system (CNS) disease associated with epilepsy, brain atrophy and posterior leukoencephalopathy. The proteins encoded by the mutated TGFB1 alleles were characterized by impaired secretion, function or stability of the TGF-β1-LAP complex, which is suggestive of perturbed bioavailability of TGF-β1. Our study shows that TGF-β1 has a critical and nonredundant role in the development and homeostasis of intestinal immunity and the CNS in humans.

TGF-β1 is translated as a precursor protein, which consists of an N-terminal signal peptide, the latency-associated peptide (LAP) and the C-terminal mature growth factor (TGF-β1). After proteolytic cleavage, LAP and TGF-β1 form the noncovalent small latent complex (SLC). The stabilization, secretion, deposition in the extracellular matrix and activation of SLCs are regulated by covalent association with latent TGF-β-binding proteins (LTBPs), resulting in formation of large latent complexes (LLCs). Multiple factors are known to control the release of active TGF-β1, for example, proteases, reactive oxygen species and integrins. Active TGF-β1 binds to a heterotetrameric transmembrane complex composed of TGF-β receptor type 1 (TGFBR1) and TGFBR2, which results in the phosphorylation of signal-transducing SMAD molecules and transcription of target genes2,9.

Dysfunction of TGF-β1 signaling has been implicated in several human diseases, including cancer, cardiovascular diseases, fibrosis, atherosclerosis and developmental defects. Heterozygous gain-of-function mutations in TGFB1 are associated with Camurati–Engelmann disease (CED), which is characterized by osteosclerotic lesions in the long bones and skull1. Increased TGF-β1-mediated signaling due to mutations in TGFB1R1 and TGFB2R2 has been documented in patients with Loeys–Dietz syndrome, which is characterized by connective tissue disorders and arterial aneurysms1. Here we report that biallelic loss-of-function mutations in TGFB1 result in very early-onset IBD and CNS dysfunction.

Patient 1 (also referred to as P1 or A.II-1), who was born to non-consanguineous parents from Malaysia (Fig. 1a), presented in the first months of life with bloody diarrhea and subsequently developed severe perianal abscesses and fistulae. Colonoscopy confirmed the diagnosis of chronic active pancolitis associated with diffuse erythema, superficial ulcerations and multiple pseudopolyps (Fig. 1b). Histology showed crypt abscesses and inflammatory infiltrations of the epithelium with mucosal ulcerations (Fig. 1b). In addition, P1 showed eosinophilic esophagitis and esophageal candidiasis. He was refractory to nutrition regimens and intensive conventional anti-inflammatory therapy, including mesalazine, steroids, azathioprine, methotrexate, infliximab, adalimumab and tacrolimus. At the age of 4 years, a total colectomy with ileostomy was performed. P1 also showed global developmental delay associated with impaired speech and cognitive dysfunction. Generalized skeletal muscle atrophy and muscular hypotonia were present, but neither pyramidal tract signs nor evidence for movement disorders were detected. Cranial magnetic resonance imaging (MRI) indicated global brain atrophy and posterior leukoencephalopathy (Fig. 1c). Electroencephalography (EEG) analysis showed a moderate global encephalopathic pattern lacking normal background activity and continuous mixed alpha and beta activity. No interictal

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epileptic discharges were recorded. Oligoclonal IgG bands and increased levels of IL-1β and IL-8 in the cerebrospinal fluid were suggestive of inflammatory processes.

P1 had a history of recurrent upper and lower respiratory tract infections and chronic cytomegalovirus (CMV) retinitis. Laboratory studies showed leukocytosis, thrombocytosis and hypochromic anemia. Serum levels of IgG (4.044 mg/dl; normal: 576–1,507) and IgE (2,665 IU/ml; normal: <90) were high, whereas IgA and IgM levels were within normal ranges. Immunophenotypic analysis of peripheral blood mononuclear cells (PBMCs) showed decreased proportions of activated memory regulatory T (T<sub>reg</sub>) cells, as well as CCR6<sup>+</sup>CXCR3<sup>+</sup> T helper 1 (T<sub>H1</sub>) and CCR6<sup>+</sup>CXCR3<sup>+</sup> T<sub>H17</sub> T cells (Fig. 1d). T cell activation following stimulation with anti-CD3 and anti-CD28, and T cell proliferation in response to specific antigens (diphtheria and tetanus toxoid), were reduced as compared to healthy donors and first-degree relatives (Fig. 1d). Mass cytometry (CyTOF) analysis of colonic lamina propria mononuclear cells showed a decreased frequency of CD45RO<sup>+</sup> and CD45RA<sup>+</sup>FOXP3<sup>+</sup>, CCR6<sup>+</sup>CXCR3<sup>+</sup>, CCR6<sup>+</sup>CXCR3<sup>+</sup> and CD103<sup>+</sup> T cells as compared to patients without IBD (control patients without (uninflamed) or with (inflamed) inflammation) and a patient with Crohn's disease (Fig. 1e and Supplementary Fig. 1). TGF-β1 exerts both stimulatory and inhibitory immunomodulatory effects; however, we cannot exclude the possibility that some of the clinical and immunological features we observed could have been influenced by infections or drug-associated immunosuppression. P1 is currently in stable clinical condition at the age of 11 years.

To elucidate the genetic etiology, we performed whole-exome sequencing and identified a compound heterozygous mutation in TGFβ1 (ENST00000221930.5) (c.[328C>T];[1159T>C], p.[Arg110Cys];[Cys387Arg]). Segregation of the sequence variant with the disease phenotype was confirmed by Sanger sequencing, which indicated that the heterozygous mutation located in the sequence encoding the LAP domain was inherited from the mother, whereas the mutation in the sequence encoding the mature TGF-β1 domain was inherited from the father (Fig. 2a and Supplementary Fig. 2).
In contrast to CED\textsuperscript{11}, radiographs of P1 were consistent with osteopenia (data not shown).

We also identified a homozygous missense mutation in \textit{TGFB1} (c.133C\textrightarrow{}T, p.Arg45Cys) in a second pedigree with two affected individuals (patients 2 and 3) born to consanguineous parents from Pakistan (Supplementary Fig. 3a,b). The p.Arg45Cys substitution is located in the LAP domain of the pro-pre-TGF-\(\beta\)-1 precursor (Fig. 2a). Patient 2 (also referred to as P2 or B.II-1) had a small head circumference (2.5th centile) and bloody diarrhea at 3 months of age. Neurological development reached a plateau at 9–10 months of age and subsequently regressed. At 19 months of age, P2 developed refractory complex partial and myoclonic seizures. EEG analysis showed a pathological pattern similar to that for hypsarrhythmia. Cerebral MRI showed volume loss, cortical atrophy and thinning of the corpus callosum. Between 19 and 20 months of age, P2 started to lose her abilities to communicate and became increasingly spastic. Despite optimization of her diet via a nasogastric tube, P2 failed to thrive and died at the age of 25 months while hospitalized for suspected septicemia.

Patient 3 (also referred to as P3 or B.II-4) had microcephaly at birth (head circumference <2.5th centile). At 3 months of age, he presented with failure to thrive and bloody diarrhea. Colonoscopy and histology showed chronic active inflammation with abscesses and crypt branching (Supplementary Fig. 3c). Psychomotor regression, muscular hypotonia and hypertonia were diagnosed at 12 months of age. At 25 months of age, P3 had complex partial seizures and a hynsarrhythmia-like EEG pattern. Cerebral MRI examination showed gross cortical atrophy, delayed myelination and marked thinning of the corpus callosum (Supplementary Fig. 3d). He developed spasticity and contractures and lost visual and social contact, as well as the ability to perform voluntary movements. Moreover, P3 had fungal dermatitis, scabies skin infection and an episode of severe varicella infection. An influenza A viral infection triggered respiratory failure and subsequently regressed. At the age of 39 months, Immunophenotyping of PBMCs showed normal numbers and distributions for T cells, B cells and NK cells but a reduced ability for CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells to proliferate after stimulation with anti-CD3 (data not shown).

We studied the structural consequences of the amino acid substitutions in the mutant TGF-\(\beta\)-1 proteins by analyzing the crystal structure of latent TGF-\(\beta\)-1 (Protein Data Bank (PDB) accession 3RJR)\textsuperscript{11}. The substitutions we identified may perturb the interaction of TGF-\(\beta\)-1 with the pro-domain or with the TGF-\(\beta\)-1 cyanine knot (Fig. 2b). Arg110 maps to a region denoted as the ‘fastener’, with the pro-domain or with the TGF-\(\beta\)-3RJR)\textsuperscript{13}. The substitutions we identified may perturb the interaction of latent TGF-\(\beta\)-1 (Protein Data Bank (PDB) accession 1P5Z) with anti-CD3 (data not shown).

To validate the predicted consequences of the mutations on the biosynthesis and function of the TGF-\(\beta\)-1–LAP complex, we used heterologous HEK293T cells that were transduced with lentiviral particles encoding wild-type (WT) TGF-\(\beta\)-1 or mutant TGF-\(\beta\)-1 variants. The CED-causing TGF-\(\beta\)-1 variant Arg218Cys was used as a control\textsuperscript{11}. Immunoblotting of cell lysates confirmed stable expression of TGF-\(\beta\)-1–LAP homodimers in cells that had been transduced with lentivirus expressing either WT or mutant TGF-\(\beta\)-1 (Fig. 2d). Latent and mature TGF-\(\beta\)-1 could be detected in conditioned medium from HEK293T cells overexpressing the TGF-\(\beta\)-1 Arg45Cys, Arg110Cys or Arg218Cys variant. The TGF-\(\beta\)-1 variants Arg45Cys and Arg110Cys showed reduced levels of secreted TGF-\(\beta\)-1. In contrast, the Cys387Arg mutant could not be detected in supernatants, suggesting that abrogation of the disulfide bond prevented proper assembly and secretion. Correspondingly, ELISAs showed that (i) only the Arg218Cys variant was detected in the mature form under cell culture conditions (without acidification with HCl), indicating a gain of function for this mutant\textsuperscript{11}, (ii) mature TGF-\(\beta\)-1 was released from the SLC after HCl treatment in the cases of the Arg45Cys and Arg110Cys variants, although at lower levels than for WT TGF-\(\beta\)-1, and (iii) secretion of the Cys387Arg variant was completely abrogated (Fig. 2e). To analyze downstream effects on TGF-\(\beta\)-1-mediated signaling, we examined conditioned medium in HEK293T cells expressing a SMAD-sensitive luciferase reporter. Cells expressing the Arg45Cys and Arg110Cys variants exhibited reduced luciferase activity as compared to the activity with WT TGF-\(\beta\)-1, whereas no activity could be detected for the Cys387Arg mutant (Fig. 2f). To assess the stability of WT and mutant SLCs, we monitored re-association of LAP and TGF-\(\beta\)-1 over time. The SLCs in supernatants from HEK293T cells were destabilized by HCl treatment and subsequently allowed to reassemble after neutralization with NaOH. In contrast to WT TGF-\(\beta\)-1, all of the mutants showed compromised re-association capacity, suggesting reduced stability of the SLC (Fig. 2g). To assess TGF-\(\beta\)-1 signaling in mucosal tissue, we performed CyTOF analysis on colonic biopsies from P1. As compared to patients without IBD (uninflamed and inflamed controls), the mean expression values of phosphorylated SMAD2 and SMAD3 (p-SMAD2/3) were reduced in lamina propria mononuclear CD4\textsuperscript{+} and CD19\textsuperscript{+} cells from P1 (Fig. 2h), whereas TGF-\(\beta\)-1–independent STAT6 phosphorylation was normal (Supplementary Fig. 4). Reduced levels of phosphorylated SMAD2/3 were also seen in CD4\textsuperscript{+} and CD19\textsuperscript{+} cells from an unrelated patient with Crohn’s disease, confirming impaired SMAD3 activity in the mucosal tissue of patients with IBD\textsuperscript{14}. Taken together, all of the newly identified mutated TGFB1 alleles seem to have deleterious consequences with respect to TGF-\(\beta\)-1 complex formation, secretion and/or bioavailability for signal transduction, as well as direct effects on downstream SMAD2/3 signaling in vivo.

The role of TGF-\(\beta\) signaling in human disease has been controversial. Although increased TGF-\(\beta\) activity has been linked to cancer, fibrosis and progressive diaphyseal dysplasia, decreased TGF-\(\beta\) activity has been associated with early tumorigenesis, vascular dysplasia, developmental defects and atherosclerosis\textsuperscript{15}. Our studies highlight a nonredundant role of TGF-\(\beta\) in controlling intestinal immune homeostasis and CNS function, whereas other organ systems apparently were not affected. These findings are reminiscent of those in patients with IL-10 or IL-10 receptor deficiency who present predominantly with infantile IBD\textsuperscript{15,16}, even though IL-10 is known to mediate pleiotropic stimulatory and suppressive functions in the immune system.

The role of TGF-\(\beta\)-1 in immunity has previously been documented in experimental models. Mice that have a constitutive disruption of Tgb\textsuperscript{15} or a T cell–specific deletion of Tgb1\textsuperscript{19} or that express dominant-negative Tgfbfr2\textsuperscript{20} develop a lethal wasting syndrome, including severe colitis. In patients with Crohn’s disease, intestinal tissue or mucosal T cells are characterized by increased activation of SMAD7, an inhibitor of TGF-\(\beta\)-1 signaling\textsuperscript{18}. Treatment with SMAD7–specific antisense oligonucleotides holds promise to alleviate colitis in mice\textsuperscript{21} and in patients\textsuperscript{16} by restoring TGF-\(\beta\)-1 signaling.

The role of TGF-\(\beta\)-1 in the brain is less well understood. Brionne et al. have reported that lack of TGF-\(\beta\)-1 expression in mice
results in neuronal cell death and microgliosis. Tissue-specific deletion of Tgfb1 in the mouse CNS prevents lethal hyperinflammation but leads to progressive defects in synaptic plasticity and loss of microglia. Decreased plasma levels of TGF-β1 and reduced neuronal expression of TGFBR2 have been documented in patients with Alzheimer’s disease. Genetic polymorphisms altering TGF-β1 expression have been associated with increased risk for conversion of mild cognitive impairment in patients with Alzheimer’s disease.

Our studies suggest that TGF-β1 may have a neuroprotective role, but the mechanisms remain unknown. Human TGF-β1 deficiency is a life-threatening disease, yet clinical management remains challenging. In view of the documented role of TGF-β1 in T cells, allogeneic hematopoietic stem cell transplantation might be considered to alleviate intestinal inflammation. We opted not to pursue this approach given the severe neurological comorbidities. Substitution with recombinant TGF-β1 may represent an alternative.
alternative experimental approach, but currently no such product is available for therapeutic use, and controlling tissue- and context-specific bioavailability of TGF-β is challenging, in particular in the CNS.

In conclusion, our study demonstrates a nonredundant role of TGF-β-mediated signaling for intestinal immune homeostasis and neurological development in humans.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41588-018-0063-6.

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Author contributions

D.K. and C.K. designed and directed the study, managed recruitment of study participants, obtained clinical samples, supervised B.M. and interpreted the data; B.M. conducted and analyzed functional assays on heterologous cellular models; D.M., E.F. and P.S. supervised T.B. and E.M.S., initiated genetic analysis and drafted the clinical report of P2 and P3, and provided critical revision of the manuscript; T.B. acquired and interpreted genetic data from P2 and P3; R.C. conducted immunophenotypic analysis of PBMCs; T.M. and A.S.L. performed functional immunological assays; S.M.W. performed CyTOF analysis; L.K. supervised S.M.W. and analyzed the CyTOF results; S.H. performed the bioinformatics analysis of sequencing data; K.-P.H. performed structural analysis of protein variants encoded by the identified TGFBR1 mutations; W.S.L., I.B., F.H., F.B., E.M.S. and B.S.B. cared for the patients, collected patient samples and drafted clinical reports; C.W. examined histology; H.H.U. provided clinical information and a specimen from a patient with CED; A.M.M. and S.B.S. screened local cohorts of patients with very early-onset inflammatory bowel disease for mutations in TGFBR1 and were instrumental in the interpretation of the human data; C.K. provided laboratory resources; and D.K. and C.K. wrote the manuscript with help from B.M. The manuscript was reviewed and approved by all co-authors.

Competing interests

The authors declare no competing interests.

Additional information

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Methods
Patients. Patients were originally identified by the Departments of Pediatrics at the Faculty of Medicine, University Malaya, Kuala Lumpur, Malaysia (family A) or Oslo University Hospital, Norway (family B), and they were referred for further studies to the Dr. von Hauner Children’s Hospital at the Ludwig-Maximilians-Universität (LMU) München, Germany. Peripheral blood samples and biopsies from patients and their unaffected first-degree relatives and from healthy volunteers for genetic and functional experiments, as well as photographs of patients for publication, were obtained upon written consent. The investigation was performed in accordance with current ethical and legal frameworks, and the study protocols were approved by the Institutional Review Boards at the LMU (#66-14) and by the Health South-East Regional Ethics Committee, Norway.

Whole-exome sequencing. Genomic DNA from patients and parents was isolated using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Segregation of identified mutations in TGFBI was confirmed in available family members in families A and B by DNA Sanger sequencing. Primer sequences are listed in Supplementary Table 1. Sanger sequencing was done in house on a Hitachi 3130x genetic analyzer or by GATC Biotech, Konstanz, Germany. The sequence reads were analyzed using the DNASTAR Lasergene software.

DNA sequencing. Genomic DNA from patients, parents and healthy siblings was isolated using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. After enrichment for all coding exons using the SureSelect Human All Exon Kit (Agilent Technologies), sequencing was performed on an Illumina Genome Analyzer II (family A) or Illumina HiSeq 2000 (family B). Short paired sequence reads were mapped to the human reference genome GRCh37 with BWA. Genome Analysis Tool Kit (GATK) was used to analyze the WES data, and functional annotation was performed with snpEff and Variant Effect Predictor (VEP) using Ensembl release 85 (family A) or 71 (family B). WES data were filtered and analyzed using an in-house SQL database (family A) or FILTUS v.0.99-934 (family B). Rare variants were distinguished by incorporating frequency information from the 1000 Genomes Project, NHBLI GO Exome Sequencing Project (ESP), EVS (http://evs.gs.washington.edu/EVS/) and for HAAC (https://www.ncbi.nlm.nih.gov/books/NBK155699/). Effects of filtered variants were predicted with a multitude of software, including snpEff, VEP, SIFT and PolyPhen-2. The remaining variants were compiled and filtered for rare homozygous and compound heterozygous mutations following a pattern of autosomal recessive inheritance.

DNA sequencing. Genomic DNA from patients, parents and healthy siblings was isolated using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Segregation of identified mutations in TGFBI was confirmed in available family members in families A and B by DNA Sanger sequencing. Primer sequences are listed in Supplementary Table 1. Sanger sequencing was done in house on a Hitachi 3130x genetic analyzer or by GATC Biotech, Konstanz, Germany. The sequence reads were analyzed using the DNASTAR Lasergene software.

Electroencephalography and magnetic resonance imaging. 24-channel EEG recordings using Xilek hardware and software equipment (Natus NBA, Excel-Tech Corp.) was performed using standard adjustments (0.5 Hz-low frequency filter, 70-Hz high-frequency filter, resistance 5–10 kΩ). MRI of the brain was obtained using a 3-Tesla high-resolution scanner (1.0- to 1.5-mm slices, T1 (longitudinal relaxation time) with and without gadolinium contrast enhancement, T2 (transverse relaxation time) and fluid attenuated inversion recovery techniques) in axial, sagittal and coronal planes (Philips Ingenia).

Structural analysis of TGF-β1 mutants. Structural visualization and modeling of the amino acids encoded by the identified TGFBI mutations was performed with PyMol (Schrödinger, LLC).

Construction of expression vectors, cell culture, transfection and lentiviral transduction. Human WT TGFBI was amplified from a Mammalian Gene Collection (MGC) sequence-verified cDNA clone (cat. no. MH6278-202757887, accession: BC022242, Dharmacon GE Healthcare). Mutations in TGFBI (encoding Arg45Cys, Arg110Cys, Arg218Cys or Cys387Arg) were introduced by site-directed mutagenesis using a 3-Tesla high-resolution scanner (1.0- to 1.5-mm slices, T1 (longitudinal relaxation time) with and without gadolinium contrast enhancement, T2 (transverse relaxation time) and fluid attenuated inversion recovery techniques) in axial, sagittal and coronal planes (Philips Ingenia).

FACS and immunophenotyping. For immunophenotypic analysis, blood samples were washed with PBS and stained with monoclonal antibodies, as indicated in Supplementary Table 2. Red blood cells were lysed by 1× BD FACS Lysing Solution (BD Biosciences) according to the manufacturer's instructions. The samples were acquired using a LSRFortessa Flow Cytometer (BD Bioscience), and data were analyzed using FlowJo Software (TreeStar). Gating strategies are shown in Supplementary Fig. 5.

CYTOF analysis. Colonie tissue was digested overnight on a shaker at 37 °C in complete RPMI medium (Gibco, Life Technologies) with 2 μl of collagenase and 2 μl of Dnase per 10 ml of medium. Undigested material was filtered out using a 10-μm filter. Single cells were resuspended in CYTOF staining buffer, and 1 × 10^6 to 2 × 10^6 cells/sample were prepared for CYTOF analysis according to the Fluidigm protocol. Briefly, cells were stained with 0.1% viability dye, washed, blocked with Fc Block and incubated with the cocktail of metal-coupled antibodies specific for surface molecules for 30 min. Next, cells were fixed in 1.6% formaldehyde and treated with isopropanol for detection by the phospho-specific antibodies or were permeabilized with the FOXP3/Transcription Factor Staining Buffer Set (ebioscience) for staining with a cocktail of intracellular antibodies. Cells were then re-fixed in 1.6% formaldehyde and stained with iP- DNA intercalator solution (Fluidigm). Finally, cells were resuspended in containing a 1:10 dilution of EQ beads and run on a Helios CYTOF machine, Fluidigm, at the Harvard Medical School (HMS) CYTOF Core. Antibodies used for CYTOF analysis are summarized in Supplementary Table 3. Antibodies not previously conjugated from Fluidigm were conjugated at the HMS CYTOF core. Data were analyzed using the Premium CyTOFAN cloud-based software. Gating strategies are shown in Supplementary Fig. 6.

Protein blot analysis and ELISA. To study TGF-β1–LAP biosynthesis and secretion, cell lysates and supernatants of HEK293T cells overexpressing WT and mutant TGF-β1 were analyzed by immunoblotting and ELISA following standard protocols. Briefly, 0.5 × 10^6 HEK293T cells and their derivatives were cultured in 1 ml of FBS-containing DMEM. After incubation for 12 h, cell lysates or supernatants were fractionated under reducing conditions by SDS–PAGE. Proteins were blotted onto polyvinylidene difluoride membranes using the Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in 5% skim milk before staining. Antibodies used for detection are indicated in Supplementary Table 4. Membranes were developed using a chemiluminescent substrate (Thermo Fisher Scientific). Images were captured using a ChemiDoc XRS+ System (Bio-Rad). Uncropped immunobots are shown in Supplementary Fig. 7.

TGF-β1 levels in serum samples and cellular supernatants were measured by using the Human TGF-β1 ELISA DuoSet (DY240, R&D Systems) according to the manufacturer's instructions. To release the mature TGF-β1 from latent complexes, conditioned medium was treated with 1 N HCl for 10 min, followed by neutralization with a solution containing 1.2 N NaOH and 0.5 M HEPES. Supernatants were analyzed in duplicate by using a Synergy H1 microplate reader (BioTek Instruments).

TGF-β1-sensitive firefly luciferase reporter assays. A lentiviral TGF-β1–sensitive firefly luciferase reporter plasmid was designed by insertion of the SMAD response elements (CAGA)38 into the pGreenFire1-mCMV vector (#TR010PA-1-SBI, Biocat) to avoid the potential influence of TGF-β1 contained in FBS. Conditioned medium from cultured cells was harvested after 12 h. To measure TGF-β1–mediated SMAD signaling activity of the identified mutants, 0.5 × 10^6 HEK293T cells encoding the firefly luciferase reporter were plated in 0.5 ml of serum-supplemented DMEM in each well of a 48-well plate. After 4–6 h of incubation, cells were rinsed with PBS, and medium was replaced with 1 ml of serum-free MEM. Conditioned medium was added to the reporter cell line in both the native and HCl-activated forms. Stimulated reporter cells were incubated for 12 h at 37 °C and subsequently lysed and assayed for firefly luciferase activity using the Firefly and Renilla Dual Luciferase Assay Kit (#30005, Biotium, USA) according to the manufacturer’s instructions. Briefly, 45 μl of lysate from samples was transferred in duplicate to a 96-well luminometry plate (NUNC) and mixed with 80 μl of firefly working solution. Luminescence signals were measured for a period of 10 s.

TGF-β1–LAP re-association assays. To examine the stability of latent complexes, HEK293T cells expressing the TGFBI mutants and their association of TGF-β1 and LAP after complex disruption in a time-dependent manner, as described previously1. To release the mature TGF-β1 from latent complexes, conditioned medium from transduced HEK293T cells was acidified with 1 M HCl for 10 min at room temperature and neutralized with 1.2 M NaOH and 0.5 M HEPES. After neutralization, samples were incubated at 37 °C for 5, 30, 60, 120 and 240 min, and levels of free TGF-β1 were analyzed by ELISA. These were plotted by applying a one-phase exponential decay data transformation using GraphPad Prism software (GraphPad Software).
Statistical analysis. Statistical evaluation of experimental data was performed using Prism version 6 (GraphPad Software). No method of randomization or blinding was used, and no samples were excluded from analysis. Data in Fig. 2e,f are reported as box-and-whisker plots, with the median (center line), upper and lower quartiles (box limits) and quartile range (whiskers) indicated. Data in Fig. 2g are means ± s.e.m. To analyze quantitative datasets, either a two-tailed unpaired t test with Welch’s correction to account for unequal variances (Fig. 2e,f) or two-way repeated-measures ANOVA with Dunnett’s correction for multiple comparisons (Fig. 2g) was performed. All tests were two-tailed, and P values <0.05 were considered to be statistically significant. Sample numbers are referred to as n unless indicated otherwise. Gaussian distribution of the data was confirmed by D’Agostino and Pearson’s omnibus normality test. No statistical method was used to predetermine sample size for analyses.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. The identified TGFβ1 mutations have been submitted to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) with accessions SCV000678250 [c.328C>T], SCV000678251 [c.1159T>C] and SCV000622112 [c.133C>T]. Information on the raw whole-exome sequencing data supporting the findings of this study are available from the corresponding author upon request. These data will not be publicly available as they contain information that could compromise research participant privacy.

References
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### Experimental design

1. **Sample size**
   - Describe how sample size was determined.
   - No statistical method was used to predetermine sample size for analyses.

2. **Data exclusions**
   - Describe any data exclusions.
   - No samples were excluded from analysis.

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced.
   - In vitro experimental data (Western Blot, Luciferase assay and ELISA) were reliably reproduced. For each assay at least 3 independent experiments have been performed. Exact numbers of experiments/samples are indicated in the corresponding figure legends. FACS-based immunophenotyping was performed twice, if primary patient material was accessible. The CyTOF experiment on patient’s intestinal biopsies was performed once.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - Sample randomization was not necessary. Group allocation was not relevant for this study. Covariates were not specifically controlled. This study emerged with the first patients presenting with an ultrarare disease. Heterozygous parents and unrelated healthy individuals served as controls. In addition, molecular studies of TGFB1 variants used wildtype TGFB1 as control.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - Investigators were not blinded to group allocation.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

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- The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. \(p\) values) given as exact values whenever possible and with confidence intervals noted
- A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

7. Software

Policy information about availability of computer code

Describe the software used to analyze the data in this study.

- GraphPad Prism 6 (GraphPad Software, USA)
- DNASTAR Lasergene 10 (DNASTAR, USA)
- FlowJo 9 (TreeStar, USA)
- Xltek EEG 1.3 (Natus DBA, Excel-Tech Corp., Canada)
- Philips Ingenia 3T (Philips Ingenia®, Netherlands)
- Image Lab 5 (Bio-Rad, Germany)
- CyTOBANK Premium 6 cloud-based software (Cytobank Inc., USA)
- FILTUS 0.99-934
- VEP v85
- SIFT build132
- PolyPhen v2
- Genome Analysis Tool Kit 3.6
- PyMol 2 (Schrödinger, LLC)

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The Nature Methods guidance for providing algorithms and software for publication may be useful for any submission.

8. Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All unique materials, except primary patients’ biospecimens, used are readily available from the authors or from standard commercial sources, as specified in the Supplementary Information.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All antibodies used for this study were obtained from commercial sources and validated by its manufacturer as stated online.

Western Blot:
- β-Actin-HRP C4 sc-47778 Santa Cruz
  https://www.scbt.com/scbt/de/product/beta-actin-antibody-c4
- rabbit-IgG-HRP 7074S Cell Signaling
  https://www.cellsignal.de/products/secondary-antibodies/anti-rabbit-igg-hrp-linked-antibody/7074
- TGF-β1 56E4 3709 Cell Signaling
  https://www.cellsignal.com/products/primary-antibodies/tgf-b-56e4-rabbit-mab/3709
- Immunophenotyping antibodies (all from BD Biosciences)
  References available online https://wwwbdbiosciencescom/us/p/ followed by catalogue number e.g. https://wwwbdbiosciencescom/us/p/560620
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. HEK293T cells (ATCC, USA)
   b. Describe the method of cell line authentication used. Cell lines have been thoroughly tested and authenticated by ATCC.
   c. Report whether the cell lines were tested for mycoplasma contamination. HEK293T cells were routinely tested negative for Mycoplasma contaminations.
   d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No commonly misidentified cell lines were used.

11. Description of research animals
   No animals were used in this study.

12. Description of human research participants
   The study involved 3 human research participants (2 unrelated families) presenting with rare forms of very early onset inflammatory bowel disease and neurological development delay, family members, and healthy donor controls. The demographic and clinical data are described in the Manuscript text, “Results and Discussion” section. There are no specific covariate-relevant population characteristics of the human research participants.