

A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in *TGFBR1* or *TGFBR2*

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We report heterozygous mutations in the genes encoding either type I or type II transforming growth factor β receptor in ten families with a newly described human phenotype that includes widespread perturbations in cardiovascular, craniofacial, neurocognitive and skeletal development. Despite evidence that receptors derived from selected mutated alleles cannot support TGF β signal propagation, cells derived from individuals heterozygous with respect to these mutations did not show altered kinetics of the acute phase response to administered ligand. Furthermore, tissues derived from affected individuals showed increased expression of both collagen and connective tissue growth factor, as well as nuclear enrichment of phosphorylated Smad2, indicative of increased TGF β signaling. These data definitively implicate perturbation of TGF β signaling in many common human phenotypes, including craniosynostosis, cleft palate, arterial aneurysms, congenital heart disease and mental retardation, and suggest that comprehensive mechanistic insight will require consideration of both primary and compensatory events.

The TGF β s are a family of multipotential cytokines that influence diverse cellular fates including proliferation, migration, synthetic repertoire and death^{1,2}. Although targeted perturbations of TGF β signaling in model systems have demonstrated its essential role in diverse developmental and homeostatic processes, there are few examples of genetically defined human pathologic correlates³. In theory, this could reflect either a survival dependency on or extensive functional redundancy in TGF β -regulated programs. It has been proposed that heterozygous loss-of-function mutations in the gene

(*TGFBR2*) encoding type II transforming growth factor β receptor (T β RII) phenocopy Marfan syndrome (MFS)⁴, a connective tissue disorder caused by deficiency of the extracellular matrix protein fibrillin-1 (ref. 5). It was also proposed that heterozygous mutations in *TGFBR2* are clinically expressed as a decrease in TGF β signaling⁴ rather than an increase, as observed in certain tissues of mice deficient for fibrillin-1 (refs. 6,7).

We describe ten families with a new aortic aneurysm syndrome characterized by widely spaced eyes (hypertelorism), bifid uvula and/or cleft palate, and generalized arterial tortuosity with ascending aortic aneurysm and dissection (Figs. 1a and 2a and Table 1). This syndrome shows autosomal dominant inheritance and variable clinical expression (Figs. 1b and 2b). Other findings in multiple systems (Table 1 and Supplementary Note online) include craniosynostosis, structural brain abnormalities, mental retardation, congenital heart disease and aneurysms with dissection throughout the arterial tree.

We considered *TGFBR2* as a candidate gene because TGF β signaling has a prominent role in vascular and craniofacial development in mouse models^{8,9} and because conditional knockout of *TGFBR2* in neural crest cells causes cleft palate and calvaria defects¹⁰. We sequenced all exons of *TGFBR2* and identified heterozygous mutations in six of ten families (Fig. 1b). Five were missense mutations affecting evolutionarily conserved residues in the kinase domain of T β RII and related receptors (Y336N, A355P, G357W, R528H and R528C; Fig. 1b,c). R528H was previously reported as a somatic event in colon cancer and was shown to cause loss of function in a transient transfection assay¹¹. Three missense mutations were found in isolated cases and were not observed in the unaffected parents (Fig. 1b). The two other missense mutations segregated with the phenotype in small

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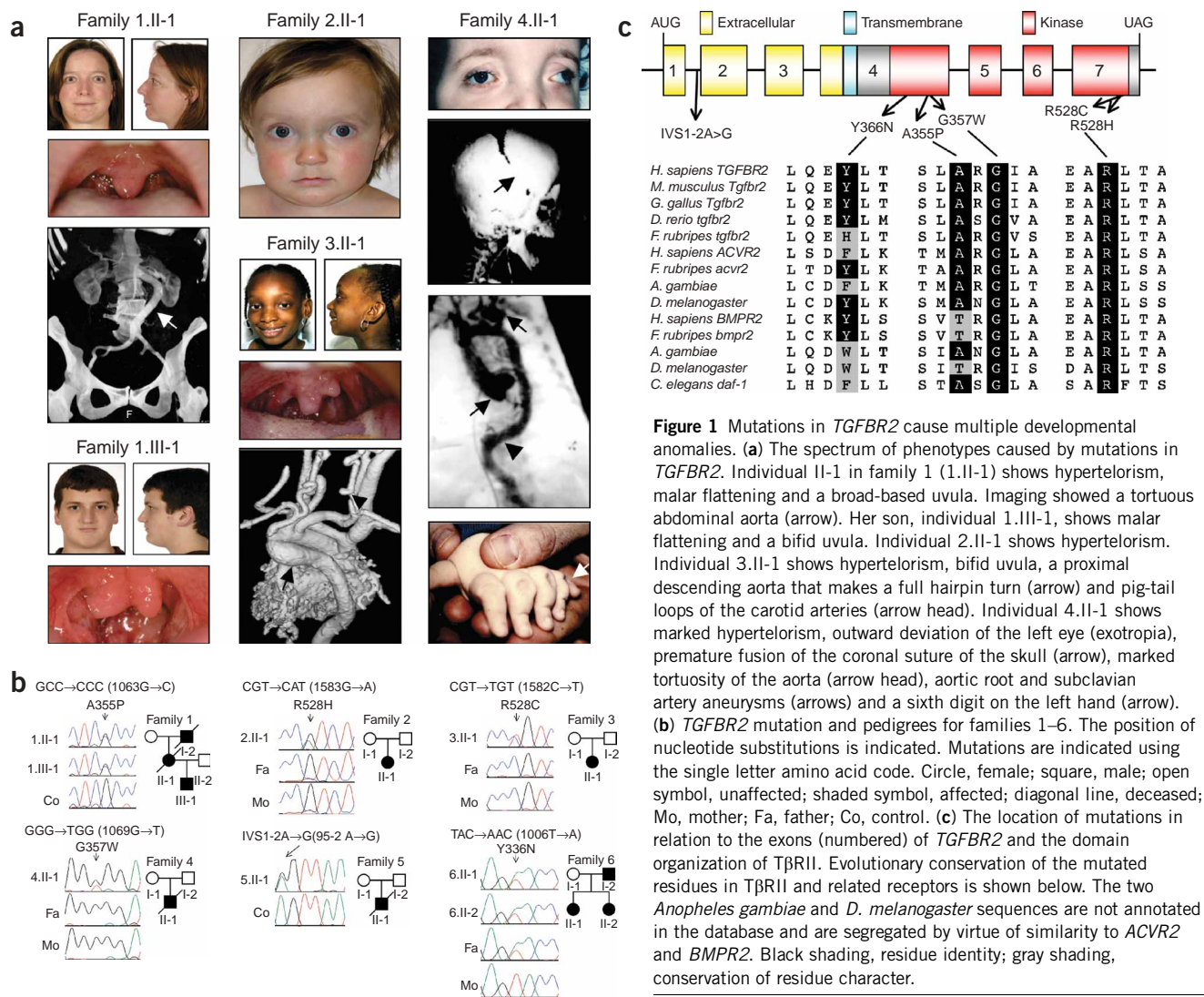


Figure 1 Mutations in *TGFBR2* cause multiple developmental anomalies. **(a)** The spectrum of phenotypes caused by mutations in *TGFBR2*. Individual II-1 in family 1 (1.II-1) shows hypertelorism, malar flattening and a broad-based uvula. Imaging showed a tortuous abdominal aorta (arrow). Her son, individual 1.III-1, shows malar flattening and a bifid uvula. Individual 2.II-1 shows hypertelorism. Individual 3.II-1 shows hypertelorism, bifid uvula, a proximal descending aorta that makes a full hairpin turn (arrow) and pig-tail loops of the carotid arteries (arrow head). Individual 4.II-1 shows marked hypertelorism, outward deviation of the left eye (exotropia), premature fusion of the coronal suture of the skull (arrow), marked tortuosity of the aorta (arrow head), aortic root and subclavian artery aneurysms (arrows) and a sixth digit on the left hand (arrow). **(b)** *TGFBR2* mutation and pedigrees for families 1–6. The position of nucleotide substitutions is indicated. Mutations are indicated using the single letter amino acid code. Circle, female; square, male; open symbol, unaffected; shaded symbol, affected; diagonal line, deceased; Mo, mother; Fa, father; Co, control. **(c)** The location of mutations in relation to the exons (numbered) of *TGFBR2* and the domain organization of TβRII. Evolutionary conservation of the mutated residues in TβRII and related receptors is shown below. The two *Anopheles gambiae* and *D. melanogaster* sequences are not annotated in the database and are segregated by virtue of similarity to *ACVR2* and *BMPR2*. Black shading, residue identity; gray shading, conservation of residue character.

families and were absent in 100 control chromosomes (Fig. 1b and data not shown). The sixth mutation occurred in an individual with sporadic disease and affects the invariant A at the –2 position of the splice acceptor sequence in intron 1 (Fig. 1b). This mutation induces use of a cryptic splice acceptor in exon 2 that results in the in-frame skipping of nucleotides 95–112 (observed in four of nine subcloned and sequenced RT-PCR amplicons) and deletion of residues 32–37 in the extracellular domain of TβRII (Supplementary Fig. 1 online).

We did not find any mutations in *TGFBR2* in the four other families with a clinically indistinguishable phenotype (Table 1 and Fig. 2a). We sequenced all exons of *TGFBR2* and found a unique missense mutation in each family (families 7–10) that affected evolutionarily conserved residues in type I transforming growth factor β receptor (TβRI; Fig. 2b,c). Two missense mutations (M318R and D400G) occurred in the kinase domain, one mutation (T200I) occurred at the junction of the glycine-serine-rich domain and the kinase domain, and one mutation (R487P) occurred just past the kinase domain at the C terminus of TβRI (Fig. 2c). Substitution of the threonine at residue 200 (T200V or T200D) caused loss of TβRI function in a transient transfection assay¹². The mutations in families 7–9 occurred *de novo*, whereas mutation R487P segregated with disease in family

10 and was absent in 100 control chromosomes (Fig. 2b and data not shown).

Histologic analysis showed loss of elastin content and disarrayed elastic fibers in the aortic media of individuals with classic MFS or mutations in *TGFBR2* compared with samples from age-matched controls (Fig. 3a). Ultrastructural analysis showed loss of intimate spatial association between elastin deposits and vascular smooth muscle cells (Fig. 3b). We observed these characteristics in young children and in the absence of inflammation, suggestive of a severe defect in elastogenesis rather than secondary elastic fiber destruction. In addition, we observed a marked excess of aortic wall collagen in individuals with MFS compared with age-matched controls; this collagen excess was accentuated in individuals with mutations in *TGFBR2* (Fig. 3a). As multiple collagens normally expressed in the aorta are derived from early-induced TGFβ target genes (including *COL1A1* and *COL3A1*), these data are consistent with increased (rather than decreased) TGFβ signaling¹³.

To assess the *in vivo* consequences of heterozygous mutations, which occur in the context of expression of wild-type TβRI or TβRII from the normal allele, we studied the acute-phase response of primary dermal fibroblasts to exogenous TGFβ1 by monitoring the activation

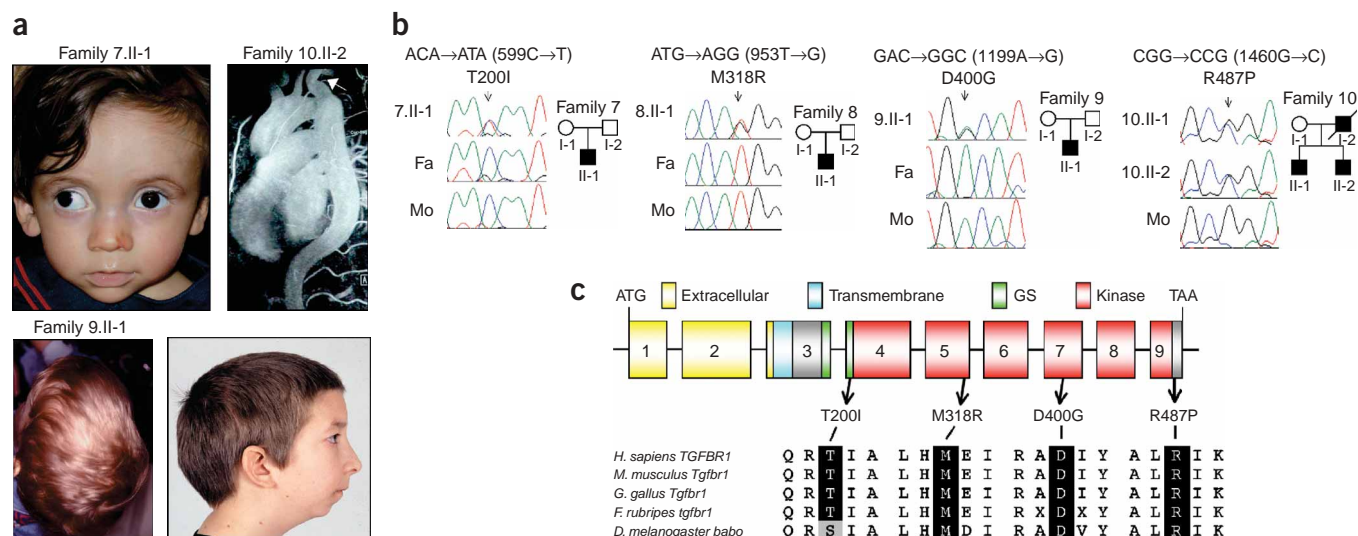


Figure 2 Mutations in *TGFBR1* cause multiple developmental anomalies. **(a)** The spectrum of phenotypes caused by mutations in *TGFBR1*. Individual 7.II-1 shows hypertelorism, exotropia and a small and receding chin (micrognathia and retrognathia, respectively). Individual 9.II-1 shows an abnormally shaped skull prior to surgical repair of craniosynostosis (left panel) and micrognathia and retrognathia (right panel). Magnetic resonance imaging of individual 10.II-2 shows marked tortuosity of the aorta and carotid arteries (arrow). **(b)** *TGFBR1* mutations and pedigrees for families 7–10. **(c)** The location of mutations in relation to the exons (numbered) of *TGFBR1* and the domain organization of TβRI. Evolutionary conservation of the mutated residues in TβRI and related receptors is shown below. GS, glycine-serine-rich domain.

(phosphorylation) status of Smad2, an essential mediator of TGFβ signaling. We exposed four fibroblast lines from affected individuals with mutations of TβRI or TβRII to recombinant TGFβ1 after a 24-h period of serum deprivation. These lines showed a normal rate and level of phosphorylation of Smad2 compared with three cell passage number-matched fibroblast lines from age-matched controls (Fig. 4a,b). There is direct (R528H) or indirect (T200I) evidence that two of the studied mutant receptors lack the ability to propagate TGFβ signal when expressed in a cell line naive for TβRII (refs. 11,12). These data show that acute responsiveness is preserved in cells heterozygous with respect to loss-of-function mutations of TGFβ receptors and effectively exclude the possibility that the phenotype of the affected individuals results from either potent dominant-negative interference or gain of function by representative mutant receptors.

To determine the chronic consequence of heterozygous mutations of TGFβ receptors, we assessed the amount of nuclear phosphorylated Smad2 in the aortic wall. We observed increased signal intensity for nuclear phosphorylated Smad2 in both individuals with classic MFS (MFS1) and two individuals with mutations in *TGFBR2* compared with control samples (Fig. 4c). Samples from affected individuals also showed increased expression of connective tissue growth factor (CTGF), which derives from a prototypical TGFβ-responsive gene with defined Smad-dependent promoter elements¹⁴ (Fig. 4d). Together with increased collagen expression (Fig. 3a), these data are suggestive of enhanced, rather than repressed, TGFβ signaling in the presence of heterozygous mutations of TβRII and in contexts relevant to the performance of cells during tissue development and homeostasis *in vivo*.

Some individuals with mutations in *TGFBR1* or *TGFBR2* have phenotypes that overlap somewhat with MFS, but none met the diagnostic criteria for MFS¹⁵. All affected individuals had manifestations in multiple organ systems that are not associated with MFS (Table 2). In these individuals, aneurysms tend to be particularly aggressive and to rupture at an early age (e.g., individuals 4.II-1 and

5.II-1) or be of a size not associated with high risk in MFS¹⁶ (e.g., individual 1.II-1). Therefore, from a management perspective, the distinction from MFS is neither ambiguous nor unimportant. Comprehensive mutation analysis of fibrillin-1 in 93 consecutive individuals referred with classic MFS identified 86 mutations¹⁷. We sequenced *TGFBR1* and *TGFBR2* in the other seven individuals and found no mutations (data not shown).

At the extreme of clinical severity, some individuals with mutations in *TGFBR1* or *TGFBR2* have phenotypes that overlap considerably with the Marfanoid craniosynostosis syndrome (called Shprintzen-Goldberg syndrome, SGS). SGS is not associated with cleft palate, arterial tortuosity or risk of aneurysm or dissection other than at the aortic root, and most affected individuals have no vascular pathology (Table 2)¹⁸. Sequencing of *TGFBR1* and *TGFBR2* in five individuals with classic SGS identified no mutations (data not shown). Nevertheless, given the extent of phenotypic overlap between SGS, MFS and selected individuals with mutations in either *TGFBR1* or *TGFBR2*, the pathogenesis of SGS probably also relates to alteration in TGFβ signaling.

Microfibrils composed of fibrillin-1 or fibrillin-2 bind the large latent complex of TGFβ, selected bone morphogenetic proteins (BMPs) and perhaps other members of the TGFβ superfamily^{19,20}. Excess TGFβ signaling correlates with both lung and heart valve pathology in fibrillin-1-deficient mice, and the phenotypes can be rescued by TGFβ antagonism *in vivo*^{6,7}. In contrast, there is synergism between fibrillin-2 and BMP7 deficiency in clinical expression of digit abnormalities²¹. The emerging view is that the fibrillins negatively regulate TGFβ activation but also positively regulate signaling by concentration of selected cytokines at the sites of intended function²². Thus, microfibrillar disorders such as MFS may represent the sum of diverse primary and compensatory events elicited by both positive and negative dysregulation of multiple cytokines.

Results of transient transfection studies in cells naive for TβRII indicated that the previously reported mutations in *TGFBR2* caused

loss of function and that the resultant MFS-like phenotype is caused by decreased TGF β signaling⁴. We reasoned that this experimental design failed to mimic the situation in affected individuals, where cells are expressing both functional and nonfunctional T β RII. The regulation of TGF β signaling is complex, with auto- and cross-regulation of ligands and their receptors^{2,23}. Such events might culminate in excess,

rather than decreased, TGF β superfamily signaling. This hypothesis was bolstered by the observation that transgenic mice expressing a dominant-negative (kinase domain-deleted) T β RII showed tissue fibrosis, upregulation of TGF β ligands, increased expression of TGF β -responsive genes and increased responsiveness to exogenous ligand^{24,25}. We now present evidence that a similar net effect can be

Table 1 Clinical data of individuals with mutations in *TGFB1* and *TGFB2*

Family	<i>TGFB2</i>										<i>TGFB1</i>					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Individual	I-2	II-1	III-1	II-1	II-1	II-1	II-1	I-2	II-1	II-2	II-1	II-1	II-1	I-2	II-1	II-2
Age	29 y*	37 y*	17 y	18 mo	6 y	9 y*	3 y*	36 y	10 y	6 y	15 mo	17 mo	10 y	27 y*	25 y	22 y
Sex	M	F	M	F	F	M	M	M	F	F	M	M	M	M	M	M
Craniofacial																
Hypertelorism		+	-	+	+	+	+	+	+	+	+	+	+		+	+
Cleft palate	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-
Bifid/broad uvula		+	+	+	+	+		+	+	+	+	-	+			
Malar hypoplasia		+	+	-	+	+		+	+	+	+	-	+		+	+
Retrognathia		-	-	-	-	+	-	+	+	+	-	+	+		+	+
Craniosynostosis		-	-	-	-	+	+				-	+	+		-	-
Exotropia		-	-	-	-	+	+	+	+	+	+	+			-	-
Proptosis		+	-	-	-	+	+	+	+	+	-	-			-	-
Blue sclerae		-	-	+	-	+		+	+	+	+	+	+		-	-
Cardiovascular																
Aortic root aneurysm	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Patent ductus arteriosus	+	-	-	+	-	+	-	+	+	-	+	-	-			+
Arterial tortuosity		+	+	+	+	+	+	+	+		+	+	+		+	+
Bicuspid aortic valve		-	+	-	-	+	-	-	-	-	-	-	-		-	-
Bicuspid pulmonary valve		-	-	-	-	+					-	-			-	-
Mitral valve prolapse		-	+	-	+	-	+	-	-	-	-	+	-		-	-
Pulmonary artery aneurysm		-	-	+	+	+	+	-	+	-	+	+			+	+
Descending aortic aneurysm	+	+		-	+		-	-	-	-	-				-	-
Ductal aneurysm	-	-	+	-	+		-	-	+	-	-	-	-			-
Subclavian artery aneurysm	-	-	-	-	+		-								+	
Superior mesenteric artery aneurysm	-	-	-	-	+		-								-	-
Cerebral aneurysm	-	-	-	-	+	+	-	-	-	-						
Atrial septal defect	-	-	+	-	+	+	-	-	-	-	-		-		-	+
Skeletal																
Dolichostenomelia	-	-	-	-	-	+	-	-	-	-	+	-			+	+
Arachnodactyly	-	-	+	+	+	+	-	+	+	+	-	+	+		-	-
Pectus deformity	-	+	-	-	+	+	+	+	+	+	+	-	+		+	-
Camptodactyly	-	-	+	+	+	-	-	-	+	-	+	+	+		-	-
Scoliosis	+	+	-	-	-	+	+	+	+	+	+	-	+		+	+
Postaxial polydactyly	-	-	-	-	+	-	-	-	-	-	-	-	-		-	-
Talipes equinovarus	-	-	-	-	+	-	+	+	-	-	+	-	-		-	-
Joint laxity	-	+	+	-	+	+	+	+	+	+	+	+	+		+	+
Skin																
Velvety texture	-	-	-	-	+		-	+	+	-	-	-			+	+
Translucency	-	-	+	-	-	+	+	+	+	-	-	-			+	+
Nervous system																
Chiari malformation	-	-	-	-	+	+					-	-			-	-
Hydrocephalus	-	-	-	-	+	+	-	-	-	-	-	-			-	-
Developmental delay	-	-	-	-	+	+	-	-	-	-	-	+	-		-	-
Dural ectasia					+		-									

+, present; -, absent; blank, unknown; *, deceased.

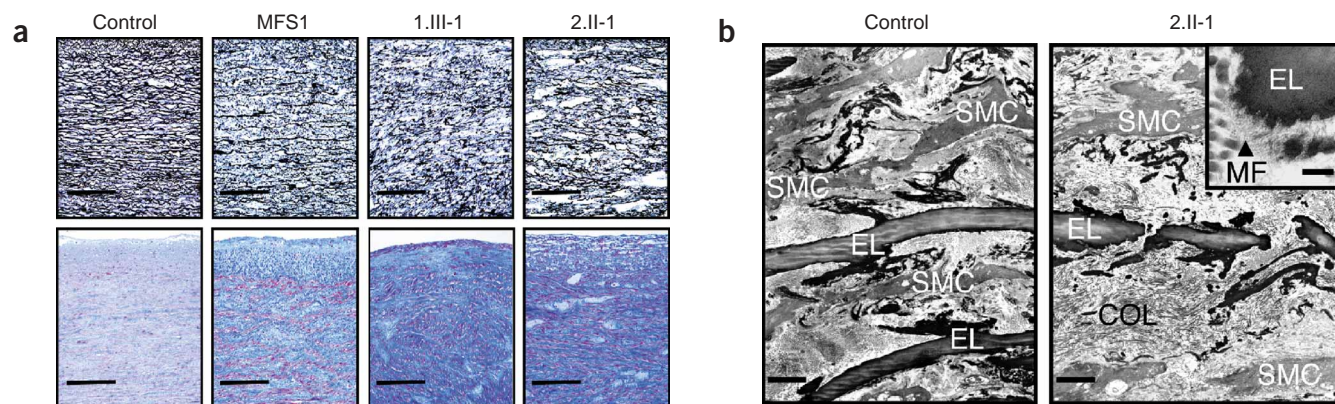


Figure 3 Histology and ultrastructure of the aortic wall. **(a)** Verhoeff–Van Gieson (elastin) stain (top) shows disorganization of the elastic matrix of the medial layer of the aorta in an individual with MFS (MFS1) and in two individuals (1.III-1 and 2.II-1) with mutations in *TGFBR2*. Mason's trichrome stain (bottom) shows increased collagen deposition (blue) in samples from all three individuals. Results shown are representative of analysis of three controls and two MFS samples. Scale bars, 100 μ m. **(b)** Low- and high-power (inset) ultrastructural images showing loss of elastic fiber architecture in the aortic media of individual 2.II-1 (compared with a normal control). Note the lack of association between elastin deposits with smooth muscle cells in the affected individual. The spatial association between elastin and microfibrils (arrowhead) is preserved (inset). SMC, smooth muscle cell; COL, collagen; EL, elastin deposit; MF, microfibrils. Low-power scale bars, 2.5 μ m; inset scale bar, 0.25 μ m.

seen in tissues functionally haploinsufficient for $T\beta$ RII or $T\beta$ RI. Candidate effectors of the enhanced signaling include upregulation of other ligands or receptors in the superfamily. Analogy can be drawn to *Ltbp4*^{-/-} mice in which decreased secretion and activation of

TGF β 1 correlated with increased production of TGF β 2, TGF β 3 and BMP4 and increased expression from multiple BMP4-responsive genes²⁶. Therefore, as with fibrillin-1 and MFS, the predominant consequence of heterozygous mutations in the TGF β receptors on

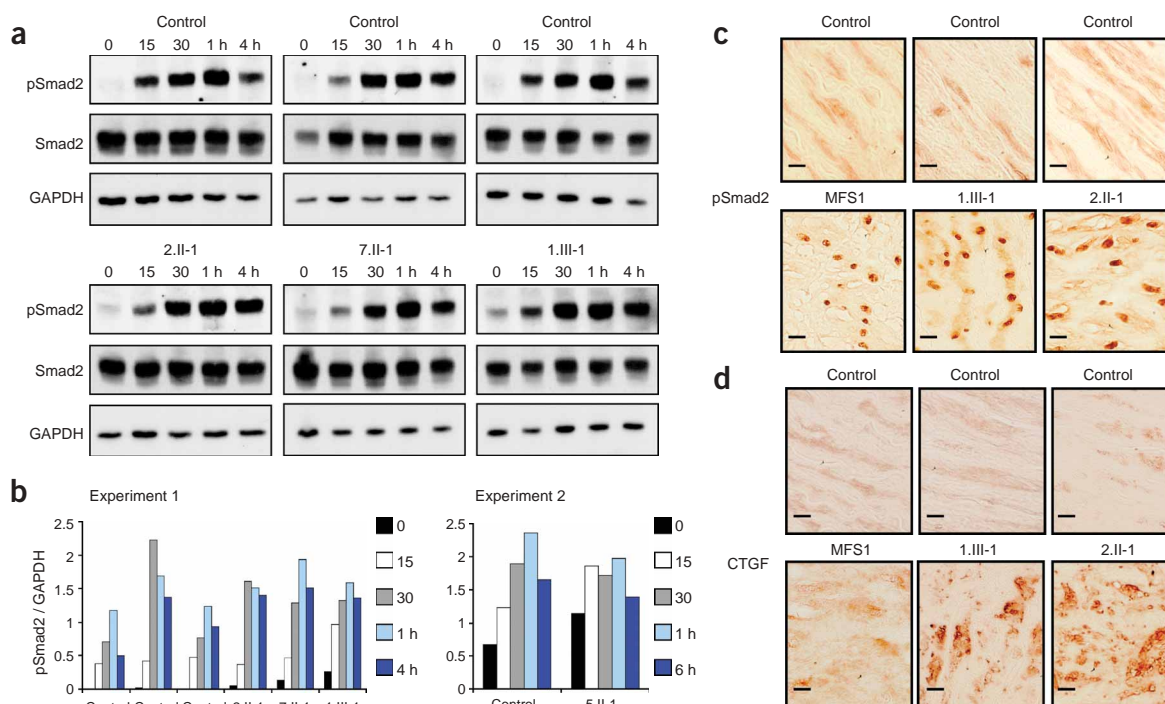


Figure 4 Assessment of TGF β signaling in cells and tissues from affected individuals. **(a,b)** Assessment of the rate of phosphorylation of Smad2 (pSmad2) in fibroblasts from control and affected individuals at 0 min, 15 min, 30 min, 1 h and 4 h after addition of exogenous TGF β 1. The immunoblots **(a)** correspond to the chart for experiment 1 **(b)**. In total, cell passage number-matched fibroblasts from four affected individuals and three independent age-matched controls were analyzed. Results of the independent experiments cannot be combined owing to minor variation in signal intensity and film exposure that precludes direct comparison of phosphorylated Smad2–GAPDH ratios between experiments. **(c,d)** Immunostaining for phosphorylated Smad2 and CTGF in the aortic media of three control individuals, an individual with classic MFS (MFS1) and individuals 1.III-1 and 2.II-1 with mutations in *TGFBR2*. Note the increased intensity of nuclear phosphorylated Smad2 **(c)** and CTGF **(d)** staining in samples from the three affected individuals, indicative of increased TGF β signaling *in vivo*. Scale bars, 10 μ m.

Table 2 Clinical characteristics of individuals and comparison to MFS and SGS

	This report	MFS*	SGS ¹⁸
Hypertelorism	13/14 (93%)	Not associated	14/15
Cleft palate/bifid uvula	11/11 (100%)	Not associated	1/15
Aortic root aneurysm	16/16 (100%)	Typical	2/15
Arterial tortuosity	11/11 (100%)	Not associated	Not associated
Aneurysm of other vessels	12/13 (92%)	Rare	Not associated
Craniosynostosis	4/11 (36%)	Not associated	Typical
Malar hypoplasia	11/13 (85%)	Associated	15/15
Blue sclerae	8/13 (62%)	Not associated	Not associated
Ectopia lentis	0/16 (0%)	Typical	1/15
Arachnodactyly	8/14 (57%)	Typical	14/15
Dolichostenomelia	4/14 (29%)	Typical	Typical
Pectus deformity	9/14 (64%)	Typical	15/15
Scoliosis	10/14 (71%)	Typical	5/15
Talipes equinovarus	4/14 (29%)	Not associated	6/15
Camptodactyly	6/14 (43%)	Associated	8/15
Joint laxity	12/14 (86%)	Typical	8/15
Patent ductus arteriosus	7/13 (54%)	Not associated	Not associated
Atrial septal defect	4/13 (31%)	Not associated	0/15
Chiari type I	2/10 (20%)	Not associated	2/2
Developmental delay	3/14 (21%)	Not associated	15/15
Hydrocephalus	2/13 (15%)	Not associated	6/15

*Our experience.

cytokine signaling and disease pathogenesis is difficult to predict and may vary in a context- and genotype-specific manner. These issues will be best addressed using TGF β receptor-deficient animal models.

In summary, our data identify the crucial contribution of TGF β signaling to diverse developmental and homeostatic processes and provide definitive proof of its role in the pathogenesis of many common human phenotypes. They also show that isolated consideration of the obvious proximal consequence of a given perturbation may be inadequate to achieve comprehensive mechanistic insight. Rather, many alterations in the complex processes of TGF β secretion, localization, activation and signaling probably have diverse consequences for the microenvironments in which morphogenetic events take place.

METHODS

Subjects and clinical evaluation. This study was approved by the Institutional Review Board of the Johns Hopkins University School of Medicine. We obtained informed consent from all subjects involved in the study and specific consent for the publication of photographs that show identity. Detailed clinical descriptions of the families are provided in **Supplementary Note** online.

Mutation analysis. We amplified genomic DNA by PCR using primers in the flanking introns of *TGFBR1* and *TGFBR2* (primer sequences available on request). We carried out sequence analysis using an Applied Biosystems automated DNA sequencer and protocols provided by the manufacturer. For individuals with seemingly sporadic disease, we sequenced both parents' DNA, when available. We used paternity testing to confirm the association between *de novo* mutation and sporadic disease. All mutations showing familial segregation were not found in a panel of at least 100 control chromosomes. We carried out family and population screening by virtue of the loss of a restriction site for *RsaI* or *AccI* for mutation Y366N or R487P, respectively. For mutation A355P, we carried out screening by allele-specific PCR using primers (sequences available on request) that allowed specific amplification of the mutant sequence from genomic DNA using an annealing temperature of 56 °C.

Cell culture. We derived fibroblast cultures from skin biopsies on the forearm. We cultured cells in minimal essential medium with 15% fetal bovine serum in

the presence of antibiotics and passaged them at confluence. Cells from affected individuals and controls were matched for early passage number (<5). We assessed TGF β responsiveness by adding recombinant TGF β 1 (R&D Systems; 5 ng ml⁻¹) to the media of cells that had been deprived of serum for 24 h. We collected protein immediately, 15 min, 30 min, 1 h and 4 or 6 h after addition of TGF β 1.

Western-blot analysis. We carried out western-blot analysis as previously described⁶. For studies examining the phosphorylation status of effectors of TGF β signaling, we extracted protein in mammalian protein extraction (M-Per) buffer (Pierce) after adding the protein phosphatase inhibitor set in accordance with the manufacturer's instructions (Upstate). Antibodies used in this study were directed against GAPDH (Santa Cruz Biotechnology), Smad2 and pSmad2 (Cell Signaling Technology). We repeated these analyses on multiple lysates with comparable results. We quantified immunoblot signals using the Quantity One package of analysis software (Bio-Rad).

Light and electron microscopy. We obtained aortic samples from the sinuses of Valsalva of affected individuals at the time of surgery. We obtained control samples from young individuals (<22 years) after death from a cause unrelated to aortic disease. We stained aortic sections with Verhoeff–Van Gieson (for elastin) and Mason's trichrome (for collagen) stains. We analyzed immunohistochemistry using antibodies directed against phosphorylated Smad2 and CTGF (Cell Signaling Technology and Abcam, respectively) and previously described methods⁶. We carried out electron microscopy as previously described⁶.

GenBank accession numbers. *TGFBR2* cDNA, NM_003242; *TGFBR2* coding region, NT_022517; *TGFBR1* cDNA, NM_004612; *TGFBR1* coding region, NT_086754.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank the families for their interest and cooperation; S. Kahler, C. Scott and H. Collmann for referring affected individuals; M. Awad for assistance in preparing the figures; and D. Devos and J. Bauer for radiological support. This work was supported by the National Marfan Foundation, the William Smilow Center for Marfan Syndrome Research, the Howard Hughes Medical Institute, the Robert Wood Johnson Foundation, the Dana and Albert Broccoli Center for Aortic Diseases, the Bijzonder Onderzoeksfonds of Ghent University, the Institute for the Promotion of Innovation by Science and Technology in Flanders, the National Institutes of Health and the Fund for Scientific Research-Flanders.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 15 October; accepted 27 December 2004

Published online at <http://www.nature.com/naturegenetics/>

- Annes, J.P., Munger, J.S. & Rifkin, D.B. Making sense of latent TGF β activation. *J. Cell Sci.* **116**, 217–224 (2003).
- ten Dijke, P. & Hill, C.S. New insights into TGF- β -Smad signalling. *Trends Biochem. Sci.* **29**, 265–273 (2004).
- Cohen, M.M. Jr. TGF β /Smad signaling system and its pathologic correlates. *Am. J. Med. Genet.* **116A**, 1–10 (2003).
- Mizuguchi, T. *et al.* Heterozygous *TGFBR2* mutations in Marfan syndrome. *Nat. Genet.* **36**, 855–860 (2004).
- Dietz, H.C. *et al.* Marfan syndrome caused by a recurrent *de novo* missense mutation in the fibrillin gene. *Nature* **352**, 337–339 (1991).
- Neptune, E.R. *et al.* Dysregulation of TGF- β activation contributes to pathogenesis in Marfan syndrome. *Nat. Genet.* **33**, 407–411 (2003).
- Ng, C.M. *et al.* TGF β -dependent pathogenesis of mitral valve prolapse in a mouse model of Marfan syndrome. *J. Clin. Invest.* **114**, 1586–1592 (2004).
- Azhar, M. *et al.* Transforming growth factor β in cardiovascular development and function. *Cytokine Growth Factor Rev.* **14**, 391–407 (2003).
- Sanford, L.P. *et al.* TGF β 2 knockout mice have multiple developmental defects that are non-overlapping with other TGF β 2 knockout phenotypes. *Development* **124**, 2659–2670 (1997).
- Ito, Y. *et al.* Conditional inactivation of *Tgfb2* in cranial neural crest causes cleft palate and calvaria defects. *Development* **130**, 5269–5280 (2003).
- Grady, W.M. *et al.* Mutational inactivation of transforming growth factor β receptor type II in microsatellite stable colon cancers. *Cancer Res.* **59**, 320–324 (1999).

12. Wieser, R., Wrana, J.L. & Massague, J. GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. *EMBO J.* **14**, 2199–2208 (1995).
13. Verrecchia, F., Chu, M.L. & Mauviel, A. Identification of novel TGF-beta/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. *J. Biol. Chem.* **276**, 17058–17062 (2001).
14. Leask, A. *et al.* The control of *ccn2* (*ctgf*) gene expression in normal and scleroderma fibroblasts. *Mol. Pathol.* **54**, 180–183 (2001).
15. De Paepe, A., Devereux, R.B., Dietz, H.C., Hennekam, R.C. & Pyeritz, R.E. Revised diagnostic criteria for the Marfan syndrome. *Am. J. Med. Genet.* **62**, 417–426 (1996).
16. Kouchoukos, N.T. & Dougenis, D. Surgery of the thoracic aorta. *N. Engl. J. Med.* **336**, 1876–188 (1997).
17. Loeys, B. *et al.* Comprehensive molecular screening of the FBN1 gene favors locus homogeneity of classical Marfan syndrome. *Hum. Mutat.* **24**, 140–146 (2004).
18. Greally, M.T. *et al.* Shprintzen-Goldberg syndrome: a clinical analysis. *Am. J. Med. Genet.* **76**, 202–212 (1998).
19. Charbonneau, N.L., Ono, R.N., Corson, G.M., Keene, D.R. & Sakai, L.Y. Fine tuning of growth factor signals depends on fibrillin microfibril networks. *Birth Defects Res. Part C Embryo Today* **72**, 37–50 (2004).
20. Isogai, Z. *et al.* Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein. *J. Biol. Chem.* **278**, 2750–2757 (2003).
21. Arteaga-Solis, E. *et al.* Regulation of limb patterning by extracellular microfibrils. *J. Cell. Biol.* **154**, 275–281 (2001).
22. Kaartinen, V. & Warburton, D. Fibrillin controls TGF-beta activation. *Nat. Genet.* **33**, 331–332 (2003).
23. de Caestecker, M. The transforming growth factor-beta superfamily of receptors. *Cytokine Growth Factor Rev.* **15**, 1–11 (2004).
24. Bottinger, E.P. *et al.* Expression of a dominant-negative mutant TGF-beta type II receptor in transgenic mice reveals essential roles for TGF-beta in regulation of growth and differentiation in the exocrine pancreas. *EMBO J.* **16**, 2621–2633 (1997).
25. Denton, C.P. *et al.* Fibroblast-specific expression of a kinase-deficient type II transforming growth factor beta (TGFbeta) receptor leads to paradoxical activation of TGFbeta signaling pathways with fibrosis in transgenic mice. *J. Biol. Chem.* **278**, 25109–25119 (2003).
26. Koli, K. *et al.* Disruption of LTBP-4 function reduces TGF-(beta) activation and enhances BMP-4 signaling in the lung. *J. Cell. Biol.* **167**, 123–133 (2004).