SUPPLEMENTS

**Supplementary Figure 1.** Evaluation of Smad2 activation in aortic medial extracts.

(a) Quantification of Smad4 and Smad7 protein levels showing no difference in aneurysmal extracts compared with control. (b) Estimation of MAP Kinase activity by dot blot. No difference in MAPKinase phosphorylation was detected in aneurysmal and control aortic extracts. Phospho-MAPK levels are normalized to positive control intensity and quantification is presented in both a table and bar graphs (arbitrary units).

**Supplementary Figure 2.** Quantification of Smad2 and non Smad2 target expression in cultured aortic SMCs.

(a) Quantification of CTGF and α-actin mRNA levels in aortic tissue extracts. Increased CTGF mRNA levels were observed in aneurysmal extracts compared with controls (Marfan: 2.15±0.46; degenerative: 1.64±0.43; BAV: 1.71±0.59 vs control: 0.53±0.11; p<0.001) whereas no significant difference in α-actin mRNA levels were found (Marfan: 7.4±1.2; degenerative: 7.6±2.3; BAV: 9.1±1.3 vs control: 8.87±3.4). (b) Stimulation of control and aneurysmal SMCs by increasing concentrations of TGF-β1 (5-50 ng/mL; 24h) showed the constitutive overexpression of CTGF (mRNA) and the absence of response to TGF-β1 of aneurysmal SMCs (all etiologies included). This was not observed for α-actin mRNA expression.

**Supplementary Figure 3.** Dissociation between TGF-β receptor activity and Smad2 activation.

(a) Immunofluorescent staining performed with HA tag antibody on 293 cells transfected with HA-tagged wild-type, Q508Q or R537C TGFβR2 plasmids (green). Nuclear counterstaining:
dapi (blue). TGFβR2 is well expressed in 293 cells after transfection by WT or mutated TGFβR2 plasmid. HA-tagged TGFβRII expression was controlled by confocal microscopy. HA-tagged wild-type and mutated TGFβRII proteins were specifically localized at the plasma membrane (right panel, confocal imagery). (b) Transfection of HA-tagged wild-type, Q508Q or R537C TGFβR2 plasmids in 293 cells. After transfection, 293 cells are treated by TGF-β1 (5 ng/mL; 4h). Nuclear accumulation of pSmad2/3 is also observed in WT TGFβR2 transfected cells. Q508Q and R537C mutations abolish TGFβR2 activity and Smad2 activation and nuclear translocation. Scale bar: 50µm. (c) Quantification of TGFβR1 and TGFβR2 mRNA levels in aneurysmal and control SMCs showing no difference in either TGFβR1 (means ±s.e.m. TGFBR2: 0.52±0.46; FBN1: 0.73±0.7; degenerative: 0.6±0.2; BAV: 0.43±0.12 vs control: 0.55±0.5) or TGFβR2 (means ±s.e.m. TGFBR2: 0.65±0.18; FBN1: 0.74±0.45; degenerative: 0.57±0.46; BAV: 0.57±0.18 vs control: 0.69±0.4). (d) Incubation of aneurysmal fibroblasts (all etiologies included) with conditioned medium from aneurysmal SMCs (24h), followed by quantification of CTGF and Smad2 mRNA expression. No effect of the incubation was detected on Smad2 activation and expression. (d) Quantification of wild type and mutated TGFβR2 mRNA in SMCs with Q508Q and R537C mutations. 1524G→A (Q508Q) induces an abnormal splicing and the formation of a longer mRNA. 2 amplicons are amplified: wild type (152 bp) and mutated (175 bp). 1609C→T results in the amino acid substitution R537C creating a restriction site for BsmI enzyme. In all cases, mutated mRNA is equally represented compared with wild type mRNA (semi-quantification by densitometry).

Supplementary Figure 4.

(a) Incubation of aneurysmal fibroblasts with conditioned medium from corresponding aneurysmal SMCs (24h), followed by quantification of CTGF and Smad2 mRNA expression. No effect of the incubation is visualized on Smad2 activation (CTGF mRNA level) and
Smad2 expression. (b) Aneurysmal and control SMCs were treated with the transcription inhibitor 5,6-dichloro-1-ß-D-ribobenzimidazole (DRB: 60µM; 4-24h). A similar decrease in Smad2 mRNA levels was observed in aneurysmal and control SMCs extracts. These results indicate that there is no modification of Smad2 mRNA stability in TAA compared with control SMCs.

**Supplementary Figure 5** DNA methylation on the Smad2 promoter.

(a) Methylation status of the CpG-Rich regions of Smad2 promoters. Diagrams showing the annotated CpG-rich areas on Smad2 genomic DNA on both sides of their respective Transcription Start Site (TSS) around the promoter regions, as defined by analysis with the MethylPrimer Express Software v 1.0. Inserts within each diagram show the parameters used to define the different CpG domains. The different TSS are indicated on the diagrams as well as the location of the different CpG domains. (b) Representative sequenced regions of Smad2 CpG2 and Smad2 CpG3 regions for unmethylated standard (line 1), methylated standard (line 2), control (line 3) and TAA samples (lines 4 and 5). Discriminative nucleotides are underlined in yellow and green respectively. The boxed regions within the sequences are indicative of their relative chromatogram.

**Supplementary Figure 6** Histone modifications on Smad2 promoter

(a) ChIP performed with specific H4K5/8/12/16ac antibody. No difference in H4 acetylation was found upstream to 1a TSS (left panel; TAA: 11.1±2.4 vs control: 14.6±4.1) and 1b TSS (right panel; TAA: 10.7±2 vs control: 14.8±5.5). Results are expressed as percentages of IP/INPUT. (b) ChIP performed with H3K9me2, H3K9me3 and H4K20me antibodies. No differences were observed upstream to 1a TSS; H3K9me3: TAA: 16.6±7.7 vs control:
13.1±1.9; H3K9me2: TAA: 19.7±7.1 vs control: 18.2±5.2; H4K20me: TAA: 17.1±7.6 vs control: 13.1±4.5). Results are expressed as percentages of IP/INPUT.

METHODS

Aortic tissue mRNA preparation
Powder of crushed aortic tissue was incubated in lysis buffer and filtrated. Total RNA from aneurymal and control tissue was extracted, using the EZNA kit (Omega Biotek, Norcross, Georgia), according to the manufacturer’s instructions. 0.2µg of RNA was reverse-transcribed using Taq Polymerase (Invitrogen, Carlsbad, California). Real-time PCR was performed in the LightCycler system with SYBR Green detection (Roche Applied Science, Basel, Switzerland) using 5ng of cDNA. mRNA levels were normalized to GAPDH mRNA. Respective primers are presented in Supplementary Table 1.

MAPKinase dot blot
A human phospho-MAPK array kit (R&D Systems, Minneapolis, Minnesota) was used to evaluate the phosphorylation of the principal MAP Kinases. Proteins were extracted from frozen samples of aneurysmal and control medial tissue in a hypotonic lysis buffer (50 mM TRIS [pH 8], 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, 5 mM EDTA) containing cocktails of protease inhibitors and serine/threonine and tyrosine phosphatase inhibitors (Sigma Aldrich, St. Louis, MO). The protein concentration was determined (Thermo Fisher Scientific, Rockford, Illinois) and 300µg of protein were used for each array. The human phospho-MAPK array is a nitrocellulose membrane where 21 anti-kinase antibodies have been spotted (plus positive and negative controls) in duplicate. Positive
controls are phosphorylated proteins, which are recognized by the anti-kinase antibodies. Binding assays were performed following the manufacturer’s instructions. The signal was detected using a chemiluminescence kit (Amersham). Positive immunoreactive bands were quantified by densitometry. Phospho-MAPK signals were normalized to positive control signals.

**TGFβR2 Transient Transfections**

293 cells are known to express very low levels of endogenous TGF-β receptors\(^5\). 293 cells were cultured in DMEM medium containing 10% fetal calf serum and antibiotics at 37°C in a 5% CO\(_2\) atmosphere. Exponentially growing cells were transfected, at 70% confluence, using Fugene (Roche), according to the manufacturer’s instructions with HA-tagged wild-type or mutated (Q508Q and R537C) TGFβR2 plasmids (1µg). 293 cells were incubated with TGF-β1 (5ng/mL) for 24h. Cells were fixed in 4% paraformaldehyde (20 min at room temperature), then blocked with 1% BSA for 1 hour and incubated with pSmad2/3 antibody (5µg/mL) for 1h at room temperature. Cells were counterstained with Dapi (0.1µg/mL).

**Confocal imagery**

293 cells cultured in DMEM medium were transfected, using Fugene (Roche Applied Science, Basel, Switzerland), according to the manufacturer’s instructions, with HA-tagged wild-type or mutated (Q508Q and R537C) TGFβR2 plasmids (1µg). Expression and localization of transfected TGFβR2 were verified using HA tag antibody (3µg/mL, Covance, Princeton, New Jersey), and visualized by confocal microscopy.

**Quantification of wild type and mutated forms of TGFβR2 by PCR**

cDNA from patients with mutations in *TGFBR2* were used: 1524G→A resulting in an abnormal splicing causing a premature stop codon (Q508Q) and 1609C→T resulting in the
amino acid substitution R537C. To quantify wild type and mutated cDNA, special primers were designed (Supplemental Table 1). With the mutation Q508Q, 2 forms were amplified using specific primers: wild type amplicon (152bp) and mutated amplicon (175bp). PCR products were separated on a 2% agarose gel. The mutation R537C induces appearance of a restriction site for BsmI enzyme. A digestion by BsmI is performed followed by a migration in a 1% agarose gel. PCR amplifications are semi-quantified using densitometry.

**DRB Stimulation**

Aneurysmal and control SMCs were treated with the transcription inhibitor, 5,6-dichloro-1-β-D-ribobenzimidazole (DRB). After incubation in free-serum medium (24h), SMCs were treated with DRB 60µM for various times (4, 6, 8 and 24h)⁶,⁷.

**DNA methylation analysis by Bisulfite DNA sequencing**

Genomic DNA was isolated from crushed frozen tissue using a tissue DNA kit (Qiagen) from at least 5 samples per group (control and TAA). Bisulfite modification was performed using the EZ DNA methylation-Direct kit (Zymo Research) following the manufacturer’s instructions. CpG methylated and unmethylated genomic Jurkat DNAs from New England Biolabs were used as standards. Converted DNA was used fresh or stored at -20°C. The conversion rate was checked to be 100% (data not shown). For methylation analysis, regions between -2000 to +2000 on both sides of the Transcription Start Site (TSS) of the Smad2 promoters were investigated using the MethylPrimer Express Software v 1.0 from Applied Biosystems. 5 and 6 CpG-rich regions were defined for Smad2 and Smad3 promoters respectively (Supplementary Table 2). The methylation status around the TSS within the Smad2 and Smad3 promoters was estimated performing BSP sequencing (sequencer: 3730 xl Applied Biosystem, sequence alignment: Sequencher 4.0 Genes Codes). DNA samples
(500ng) were bisulfite modified and amplified using specific primers for each of the CpG-rich domains for Smad2 promoters (Smad2 CpG 2 and 3 [38 and 29 potential methylation sites respectively]). PCR conditions were 95°C for 10mn then 40 cycles (95°C: 45’; Tm: 45’; 72°C: 45’) followed by 5mn at 72°C. PCR products were checked on 2% agarose gel and were forward and reverse sequenced with their respective primers according to the manufacturer’s instructions (Applied Biosystem).
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Supplementary figure 1

(a) Western blot analysis of Smad4 and Smad7.

(b) Western blot analysis of various kinases under Control and TAA conditions. The table shows the arbitrary units for each condition.

| Kinase  | Control | TAA      |
|---------|---------|----------|
| p-ERK1  | 3766±1026 | 3864±1226 |
| p-ERK2  | 3516±1200 | 4155±1328 |
| p-p38α  | 1474±423  | 1522±465  |
| p-p38β  | 1375±441  | 1389±436  |
| p-JNK1  | 1272±276  | 1352±410  |
| p-JNK2  | 1372±440  | 1408±465  |
Supplementary figure 2

(a) CTGF mRNA and α-actin mRNA levels in different groups.

(b) Effect of TGF-β1 on CTGF and α-actin mRNA expression in control and aneurysm groups.
Supplementary figure 3

a

HA-TGFβR2

Merge

Dapi

b

c

d

WT

WT TGF-B1

Q561Q TGF-B1

Q561Q TGF-B1

R537C TGF-B1

R537C TGF-B1

pSmad2/3 dapi merge

TGFβR2/GapDH

TGFβR2/GapDH

Cycles: 23 26 29 32 35 38

Control

Q561Q

Bsm1

Control R537C
Supplementary figure 4

(a)

(b)

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Control SMCs

TAA SMCs
Supplementary figure 5
Supplementary figure 6

a

1a TSS

H4K5/8/12/16ac
Control IP INPUT TAA IP INPUT

H4K5/8/12/16ac - % IP/INPUT
Control TAA

1b TSS

H4K5/8/12/16ac
Control IP INPUT TAA IP INPUT

H4K5/8/12/16ac - % IP/INPUT
Control TAA

b

Control
Aneurysm

H3K9me2 H3K9me3 H4K20me
INPUT H3K9me2 H3K9me3 H4K20me

H3K9me2, % IP/INPUT
Control TAA

H3K9me3, % IP/INPUT
Control TAA

H4K20me, % IP/INPUT
Control TAA
# Supplementary Table 1

| Access number | Gene        | Sequence                  | Application | Length (bp) |
|---------------|-------------|---------------------------|-------------|-------------|
| NM_005901     | Smad2       | forward 5'-CCCTGCAACAGTGTGTAAAAT-3' | qPCR        | 195         |
|               |             | reverse 5'-GTACTTGTGCCCTGCTGCTTC-3' |             |             |
| NM_005902     | Smad3       | forward 5'-AGACACATCGGAGGAGGCTG-3' | qPCR        | 187         |
|               |             | reverse 5'-CTCCTGCTGCTGCTTCTG-3' |             |             |
| NM_005359     | Smad4       | forward 5'-AACATTGGATGGAGGCTCTCA-3' | qPCR        | 332         |
|               |             | reverse 5'-TTGGTGATGGATGATGT-3' |             |             |
| NM_001901     | CTGF        | forward 5'-CCGGGTTCACACTGACACGG-3' | qPCR        | 337         |
|               |             | reverse 5'-CGGGACAGTTGTAATGCGAGC-3' |             |             |
| NM_001613     | α-actin     | forward 5'-GGGCGCTGCTGCTGCTGCT-3' | qPCR        | 157         |
|               |             | reverse 5'-GACTGCGCCTGCTGCTGCT-3' |             |             |
| NM_003242     | TGFβR2      | forward 5'-GGGCGCTGCTGCTGCTGCT-3' | qPCR        | 386         |
|               |             | reverse 5'-GAGCTATTGTCTGCTGCTGCT-3' |             |             |
| NM_004612     | TGFβR1      | forward 5'-GGGCGCTGCTGCTGCTGCT-3' | qPCR        | 307         |
|               |             | reverse 5'-GACTGCGCCTGCTGCTGCT-3' |             |             |
| NM_003242     | GAPDH       | forward 5'-GACACTGCTGCTGCTGCTGCT-3' | PCR         | 152/175     |
|               |             | reverse 5'-GGGCGCTGCTGCTGCTGCT-3' |             |             |
| NM_003242     | TGFβR2 Q508Q| forward 5'-GACACTGCTGCTGCTGCTGCT-3' | PCR         | 716         |
|               |             | reverse 5'-GGGCGCTGCTGCTGCTGCT-3' |             |             |
| NM_005901     | Smad2 1a TSS| forward 5'-GGAGAGAGACGCTGCTGCTGCT-3' | PCR         | 211/298     |
|               |             | reverse 5'-GGGCGCTGCTGCTGCTGCT-3' |             |             |
| NM_005901     | Smad2 1b TSS| forward 5'-GGAGAGAGACGCTGCTGCTGCT-3' | PCR         | 301         |
|               |             | reverse 5'-GGGCGCTGCTGCTGCTGCT-3' |             |             |
| NT_010966.14  | Smad2 1a TSS| forward 5'-GGGCGCTGCTGCTGCTGCTGCT-3' | ChIP        | 194         |
|               |             | reverse 5'-GGGCGCTGCTGCTGCTGCT-3' |             |             |
| NT_010966.14  | Smad2 1b TSS| forward 5'-GGGCGCTGCTGCTGCTGCTGCT-3' | ChIP        | 226         |
|               |             | reverse 5'-GGGCGCTGCTGCTGCTGCT-3' |             |             |
| NT_010966.14  | Smad2 CpG2  | forward 5'-GGGCGCTGCTGCTGCTGCTGCT-3' | Bisulfite sequencing | 266         |
|               |             | reverse 5'-GGGCGCTGCTGCTGCTGCT-3' |             |             |
| NT_010966.14  | Smad2 CpG3  | forward 5'-GGGCGCTGCTGCTGCTGCTGCT-3' | Bisulfite sequencing | 211         |
|               |             | reverse 5'-GGGCGCTGCTGCTGCTGCT-3' |             |             |
| NT_010966.17  | Smad3 CpG1  | forward 5'-GGGCGCTGCTGCTGCTGCTGCT-3' | Bisulfite sequencing | 282         |
|               |             | reverse 5'-GGGCGCTGCTGCTGCTGCT-3' |             |             |
### Supplementary Table 2

Localization of CpG islands in Smad2 and Smad3 promoters

| Smad2 | CpG | length (bp) | C+G % | CpG % |
|-------|-----|-------------|-------|-------|
| 1     | 400 | 51.8        | 4.5   |
| 2     | 400 | 73.5        | 14.3  |
| 3     | 400 | 80.0        | 15.5  |
| 4     | 400 | 77.5        | 11.5  |
| 5     | 350 | 70.8        | 9.9   |

| Smad3 | CpG | length (bp) | C+G % | CpG % |
|-------|-----|-------------|-------|-------|
| 1     | 400 | 54.7        | 4.5   |
| 2     | 450 | 62.7        | 4.0   |
| 3     | 450 | 77.1        | 13.8  |
| 4     | 450 | 74.4        | 14.0  |
| 5     | 450 | 69.6        | 8.0   |
| 6     | 450 | 61.3        | 7.1   |