Supplementary Information:

The thyroid hormones inhibit TGFβ signaling and attenuate fibrotic responses

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SUPPLEMENTARY MATERIALS AND METHODS

Plasmids. Luciferase reporter plasmids containing different SBE-bearing promoters (1-3), a construct containing a consensus TRE and expression vectors for WT and mutant TRα1 and TRβ1 (4,5), SMADs (6,7), and GST-fused SMADs (8-11) have been previously described. Some of these plasmids were obtained from Addgene (plasmids 12633, 14038, 14932 and 15706). SMAD3 mutants in which Ser423 and Ser425 were changed to Asp or Glu (S/D and S/E, respectively) were generated by VectorBuilder and confirmed by sequencing. GFP fusion constructs of native TRβ and the Lys184-Arg185 to Ala184-Ala185 mutant in the nuclear localization signal of the D domain were a gift from S-Y Chen and have been previously described (12).

Transient transfections and luciferase assays. All cell lines used were negative for mycoplasma contamination. Cells were obtained from the ATCC or were a gift and used previously in the laboratory and were not further authenticated. Cells were plated in 24-well plates in medium supplemented with 10 % thyroid hormone-stripped serum by treatment with AG1-X8 resin. Cells were transiently transfected with 300 ng of reporter plasmids and 50 ng of pRL-TK-Renilla (Promega) as a normalizer control, using Lipofectamine 2000 (InVitrogen) following the manufacturer's instructions. Cells were then treated with T3 (5 nM) for 36 h and with TGFβ (10 ng/ml) for the last 5 h. When appropriate, the reporter was cotransfected with expression vectors for wild-type or mutant TRs, SMAD3 (150 ng) and SMAD4 (200 ng), with SMAD7 (150 ng) or with the appropriate amount of empty noncoding vectors. Luciferase activity was measured using the Dual Luciferase Assay System (Promega). Experiments were performed in

triplicate and repeated at least three times. Data are mean ± s.d and are expressed as fold induction over the values obtained in the untreated cells.

Protein and mRNA analysis. Western analysis was performed as previously described (4,5), using the antibodies listed below in Table S1. Total RNA was extracted from tissues using RNeasy Mini Kit (Qiagen) and from cells using Trizol (Sigma) and mRNA levels were analyzed in technical triplicates by quantitative RT-PCR following specifications of SuperScript™ First-Strand Synthesis System (Invitrogen). Data analysis was done using the comparative CT method and data were corrected with GAPDH mRNA or 18S RNA levels. Primers used are listed below in Table S2.

GST pull-down assays. Recombinant GST-SMAD2, 3 and 4 as well as GST alone (GST-0) were synthesized, purified on glutathione Sepharose resin and analyzed by SDS-PAGE. ^{35}S-labeled TRα and TRβ either WT or mutated were generated with the TNT T7 Quick coupled in vitro transcription and translation and used in pull-down assays with 1 µg of GST or GST-fused proteins as described (5) in the presence and absence of 100 nM T3. Reactions were submitted to SDS-PAGE and autoradiography. Quantifications of the autoradiograms were performed using ImageJ software (http://rsb.info.nih.gov/ij/) and in the figures data are expressed as % of the inputs, that were run in parallel and represented 20% of the labeled receptor used in the reactions, after subtraction of the GST-0 values. Alternatively, p150 mm dishes of Cos-1 cells were transfected with 28 µg of Flag-TRβ vector and incubated with and without 50 nM T3 for 1h. Equal amounts of protein extracts were incubated with GST-SMADs and TRβ present in the complexes was analyzed by western blot with Flag antibody.

Coimmunoprecipitations. For immunoprecipitations, cells grown in p100 mm dishes were cotransfected with 12 μg of expression vectors for Flag-TRβ and 9 μg of SMAD4 with a HA-tag. The cells were either untreated or treated with T3 50 nM for 1h, harvested in lysis buffer and approximately 700μg of total cell extracts were immunoprecipitated with anti-Flag antibody or with control IgG at 4ºC overnight and for 1h with protein A/G Plus-Agarose (Santa Cruz). In an additional experiment, cytosolic and nuclear extracts were prepared with the NE-PER kit (Thermo scientific) and immunoprecipitated independently under the same conditions. For co-immunoprecipitation of endogenous proteins, cells were treated with 50 nM T3 and/or 10 ng/ml TGFβ for 1h and immunoprecipitated with SMAD2/3 antibodies. The immunocomplexes and proteins from whole cell extracts were resolved by SDS-PAGE, and analyzed by Western blot with SMAD2/3, SMAD4 or TRβ antibodies.

ChIP assays. Cells were plated in 150mm dishes and the next day they were washed and treated with 50 nM T3 and/or 10 ng/ml TGFβ for 1h, fixed and lysed following specifications of 17-295 Upstate kit, and sonicated in a *Bioruptor UCD-200TM* (Diagenode). In each immunoprecipitation approximately 3 x10^6 cells and the amount of antibodies listed in the Supporting Information were used. DNA was purified, precipitated and amplified with the primers listed in Table S2. When indicated, immunoprecipitated DNA was used for qRT-PCR amplification. Results were normalized and are presented as a fraction of the inputs.

Immunofluorescence. Detection of SMAD2/3 was carried out in cells treated with 5 nM T3 and/or 10 ng/ml TGFβ. After incubation with primary and secondary antibodies, nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI), and samples

were mounted with *ProLong* (Molecular Probes). Cell images were captured with a Nikon E90i fluorescence microscopy with *NES Elements* software.

Animals and fibrosis models. All animal experiments were carried out in the animal facility of the Instituto de Investigaciones Biomédicas in compliance with the European Community Law (86/609/EEC) and the Spanish law (R.D. 1201/2005), with approval of the Ethics Committee of the Consejo Superior de Investigaciones Científicas. Mice were housed in pathogen-free condition, in a 12/12 h light/dark cycle with water and normal diet food available *ad libitum.* For the CCl4 model of liver injury and fibrosis 6-8 weeks old male C57BL/6J mice bred at the Instituto de Investigaciones Biomédicas were made hyperthyroid by adding thyroxine (7 ng/g of mice) and T3 (35 ng/g of mice) in the drinking water. These animals as well as their control littermates were injected intraperitoneally with 50µl/100g body weight CCl4 or with vehicle (olive oil) three times a week for 4 weeks. Thyroid hormone treatment was continued during the whole period and the animals were sacrificed 72h after the final CCl4 injection. To study acute injury, animals received a single injection of CCl4 or vehicle 48h before sacrifice. Livers obtained from untreated 18 month-old $TR\alpha1^{-/-}/TR\beta^{-/-}$ double KO mice and wild-type ($TR\alpha1^{+/+}/TR\beta^{+/+}$) animals with the same genetic background (13) were also used. For the bleomycin model of skin fibrosis, 100µl of 200µg/ml of bleomycin (Sigma) dissolved in PBS were injected subcutaneously into four different regions of the shaved dorsal skin of 3-months old CD1 control mice and of mice made hyperthyroid by treatment with thyroxine and T3 in the drinking water for the prior 7 days. Injections at the same sites were carried out 6 days/week for 24 days both in untreated and thyroid hormone-treated animals. Mice were euthanized 24h after the final injection. Serum was collected and livers and skin samples were either frozen to obtain RNA and proteins or

fixed with paraformaldehyde or ethanol and embedded in paraffin. In all experiments animals were randomly distributed among the different groups. Circulating T4 and T3 levels were measured by radioimmunoassay after extraction and purification of serum as previously described (14). High specific activity ^{125}I-T4 and ^{125}I-T3, and anti-T3 and anti-T4 antisera were kindly provided by M J Obregon.

AST activity. Serum AST activity was measured with the Reflotron GOT test (Roche), following the manufacturer´s instructions.

Histology, morphometric analysis and immunohistochemistry. Liver and skin tissues were fixed in 4% buffered formalin and embedded in paraffin wax. Deparaffinized tissue sections were stained with Hematoxylin, Masson´s trichrome or Picrosirius red using standard procedures. Liver fibrosis was quantified using ImageJ software (http://rsb.info.nih.gov/ij/), from Picrosirius red-stained sections by an examiner who was blinded to the experimental group of the mice. A red color threshold macro was used to measure the image Sirius red stained of collagen. Histological grading of fibrosis was performed using the Ishak modified histological activity index scoring system (15). The skin thickness was quantitated from dorsal skin sections stained with Masson's trichrome by measuring the areas of the dermal connective tissue and the hypodermis. Quantitations were made in at least 5 individual random fields from 4-6 mice of each experimental group. Immunohistochemistry of Collagen1 was performed on 4μm deparaffinized-rehydrated sections. Antigen retrieval was carried out with citrate buffer in microwave oven and endogenous peroxidase activity was inhibited with 3% H_2O_2. Samples were blocked and incubated overnight with the antibody, and signal was amplified with the ABC *Kit* (Vectastin). Slides were revealed with DAB

(Vector), counterstained with Hematoxylin and mounted with DePeX (Serva). All images were obtained using a high-resolution Leica DC200 digital camera mounted on an Olympus DMLB microscope. Picrosirius red stained skin slides were observed under polarized light using the Olympus DMBL light microscope equipped with a polarizer/analyzer set.

Statistical analysis. Statistics were calculated with the SPSS software. Shapiro-Wilk test was used to check a normal distribution and Levene test to assess the equality of variances. Statistical significance of data was determined by applying unpaired two-tailed Student *t*-test when two groups are compared, or ANOVA followed by the Bonferroni test for experiments with more than two experimental groups. Differences are presented in the figures by asterisks (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$). No previous calculations were used to determine sample size, which was chosen based on usual procedures in the field. The number of samples or animals is indicated in the corresponding figures legends. Results shown are means ± s.d or means± s.e.m, as specified.

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Table S1

List of antibodies indicating the dilution for each use

Antibody	Dilution	Use	Catalog# and provider
Collagen I	1:100	Immuno-histochemistry	ab34710 (Abcam)
Erk2	1:20000	Western blot	sc-154 (Santa Cruz)
Flag	3µg/1: 2000	CoIP/Western blot	F1804(Sigma-Aldrich)
HA	1:2000	Western blot	1 867 432 (Roche)
H4Ac	5µg	ChIP	06-866 (Upstate)
Phospo-Smad2 (Ser465/467)	1:1000	Western blot	♯3101 (Cell Signaling)
Phospo-Smad3 (Ser423/425)	1:1000	Western blot	♯9520 (Cell Signaling)
Smad4	1:500	Western blot	sc-7966(Santa Cruz)
Smad4	5µg/1:500	ChIP/Western blot	sc-7154(Santa Cruz)
Smad2/3	5µg/4µg/ 1:200/1:500	CoIP/ChIP/Immuno-fluorescence/ Western blot	sc-8332(Santa Cruz)
TRα/β	5µg/1:1000	ChIP/Western blot	sc-737(Santa Cruz)
TRβ	1:1000	Western blot	sc-772(Santa Cruz)
TRβ	2µl/reaction	ChiP	From N.Buisine and L. Sachs [1]
α-SMA	1:2000	Western blot	A2547 (Sigma-Aldrich)
Tubulin	1:10000	Western blot	T5168 (Sigma-Aldrich)

[1] Rabbit polyclonal antibody for *Xenopus tropicalis* THRB1 was raised against bacterially expressed full-length protein after cloning in pET21 vector (EMD Millipore). Expression, production, and affinity purification were performed according to GeneCust

Table S2

Primer used for ChIP Assays

Primer	Sequence
Id1 -1300 forward	5'-GTTGACCCTTGGTCAGCAAC-3'
Id1 -1042 reverse	5'-GAGAAAGGAAAGGGAGGGG-3'
P15 -358 forward	5'-AATGTCTCCAGAGCGAAACC-3'
P15 -46 reverse	5'-AAGTCAGCTGTGCCTGAGGT-3'
Smad7 -679 forward	5'-CCCCAGCTCTTCCGATTT-3'
Smad7 -469 reverse	5'-GTCTAGACGGCCACGTGACGA-3'

Primers used for quantitative PCR

Primer	Sequence (F: forward; R: reverse)
Col1A1	F: 5'-CCTGAGTCAGCAGATTGAGAA-3'
	R: 5'-ACTGAACTTGACCGTACACCAGTACTCTCCGCTCTTCAA-3'
Col1A2	F: 5'-CCGTGCTTCTCAGAACATCA-3'
	R: 5'-CTTGCCCCATTCATTTGTCT-3'
Dio 1	F: 5'-GTTGAACTTTGGCAGTTGCAC-3'
	R: 5'-GGCTGTGGAGGCAAAGTCATC-3'
EGR1	F: 5'-TTTGCCTCCGTTCCACCTGC-3'
	R: 5'-TGCCAACTTGATGGTCATGCGC-3'
GAPDH	F: 5'-ACAGTCCATGCCATCACTGCC-3'
	R: 5'-GCCTGCTTCACCACCTTCTTG-3'
Id1	F: 5'-TGCAGCTGGAGCTGAACTCT-3'
	R: 5'-GACACAAGATGCGGTCGTC-3'
p15	F: 5'-GATCCCAACGCCGTCAAC-3'
	R: 5'-CCCTCTCGAGCTGCATCAT-3'
Smad7	F: 5'-TCCTGCTGTGCAAAGTGTTC-3'
	R: 5'-GAGGAAGGTACAGCGTCTGG-3'
TGFβ1	F: 5'-TACAGCAAGGTCCTTGCCCT-3'
	R: 5'-GCAGCACGGTGACGCC-3'
18S	F: 5'-TTCGAACGTCTGCCCTATCA-3'
	R: 5'-ATGGTAGGCACGGCGACTA-3'
α-SMA	F: 5'-CAGCGGGCATCCACGAA-3'
	R: 5'-GCCACCGATCCAGACAGA-3'

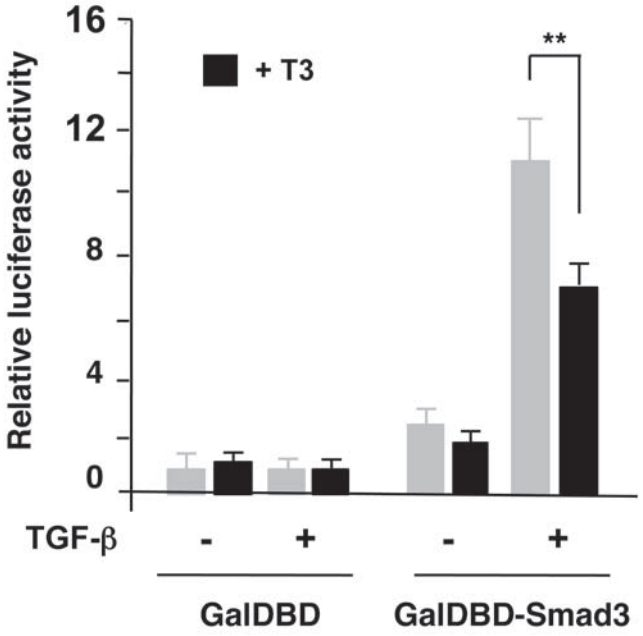

Fig. S1. T3 inhibits SMAD transcriptional activity. GH4C1 cells were transfected with 200 ng of UAS reporter plasmid and 100 ng of a GAL4-SMAD3 fusion construct or the GAL4 DBD alone. Luciferase activity was determined after incubation 5nM T3 for 36 h and/or with 10ng/ml TGFβ for the last 5 h. ** $p<0.01$ in ANOVA post-test between untreated and T3-treated cells.

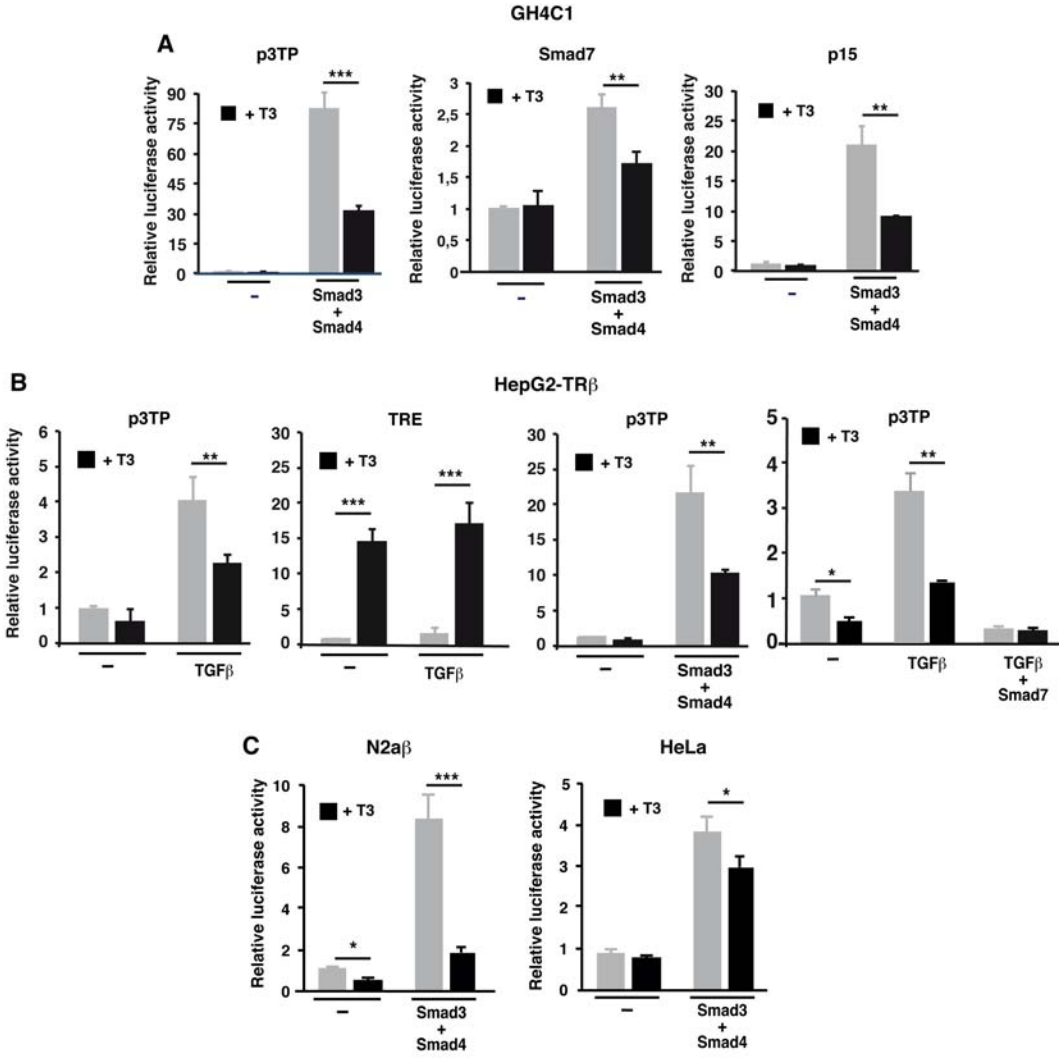

Fig. S2. T3 inhibits SMAD-dependent transcription irrespective of the promoter and the cell type. (A) GH4C1 cells were transfected p3TP-lux and with reporter constructs of the Smad7 or p15 promoters that also contain SBEs. The reporters were cotransfected with 150 ng of an expression vector for SMAD3, 200 ng of an expression vector of SMAD4 or an empty vector (-), and luciferase activity was determined after 36 h of incubation in control and T3-treated cells. (B) HepG2-TRβ cells were transfected with the p3TP-lux (left panel) or the TRE-containing promoter (second panel) and incubated with T3 and/or TGFβ. In the third panel cells were cotransfected with p3TP-lux and SMAD3 and SMAD4 expression vectors and incubated in the presence and absence of T3. In the right panel cells were transfected with the empty vector or a SMAD7 vector and treated with T3 and/or TGFβ as indicated. (C) N2aβ cells (also expressing TRβ in a stable manner) and HeLa cells were cotransfected with the p3TP-lux reporter construct and SMAD vectors. Luciferase activity was measured in untreated and T3-treated cells. Data are means±s.d (n=3). Statistically significant differences of ANOVA post-test between cells treated with and without T3 are indicated.

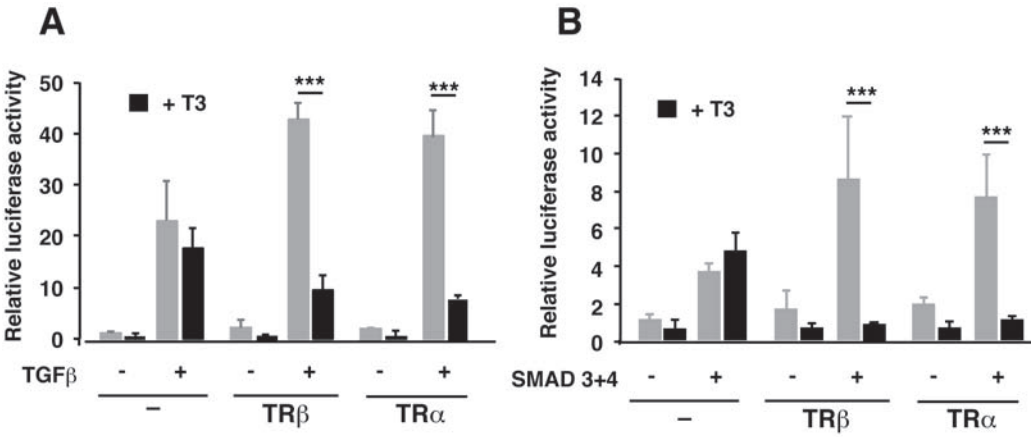

Fig. S3. Both TRα and TRβ can antagonize TFGβ/SMAD transcriptional activity. (A) Mv1Lu cells were cotransfected with the SBE-containing p3TP-lux plasmid and expression vectors for TRβ or TRα (25 ng) or with the same amount of a non-coding vector (-). Luciferase activity was determined in cells treated with T3 for 36 h and/or with TGFβ for 5h. (B) Mv1Lu cells were cotransfected with the reporter plasmids and expression vectors for SMAD3 (150 ng) and SMAD4 (200 ng) or with the empty vector and reporter activity was measured in untreated cells and in cells treated with T3 for 36 h. Asterisks denote post-test statistically significant differences between cells treated with and without T3.

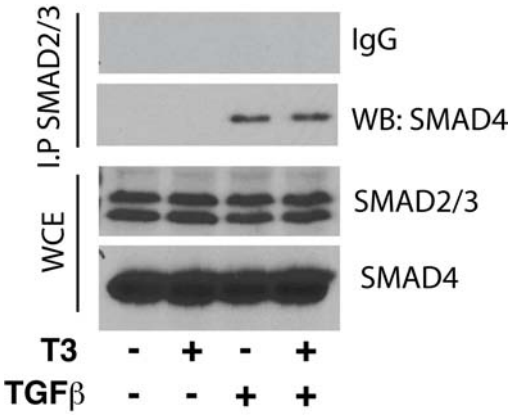

Fig. S4. T3 does not disrupt the interaction of SMAD2/3 with the coregulatory SMAD. GH4C1 cells were treated with 5nM T3 and/or with 10ng/ml TGFβ for 1h. Cell extracts (1.7 mg) were immunoprecipitated with SMAD2/3 antibody or control IgG and SMAD4 was detected by Western blot. SMAD2/3 and SMAD4 levels in 70 µg of the whole cells extract (WCE) are also shown.

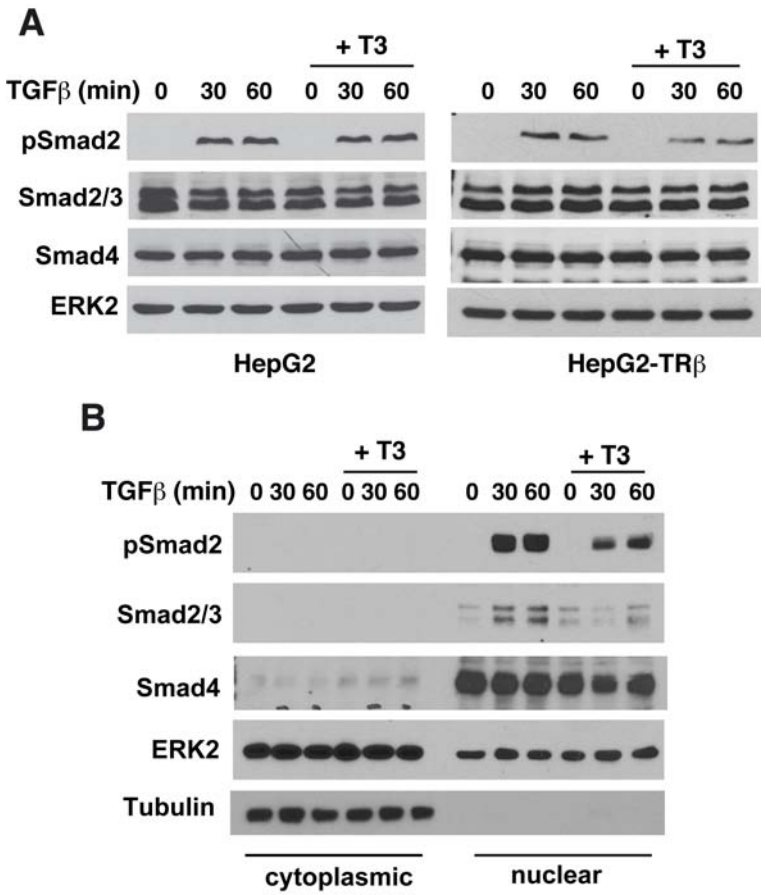

Fig. S5. T3 inhibits SMAD2 phosphorylation and nuclear accumulation in HepG2-TRβ cells. (A) Parental hepatocarcinoma HepG2 cells, as well as HepG2-TRβ cells expressing TRβ in a stable manner were incubated with 5nM T3 for 36h and/or with 10ng/ml TGFβ for the times indicated. Western blot analysis of total cell extracts was performed with phospho-SMAD2 (pSMAD2), SMAD2/3 and SMAD4 antibodies. ERK2 was used as a loading control. (B) Cytoplasmic and nuclear extracts of HepG2-TRβ cells were prepared after incubation with T3 for 36 h and/or with TGFβ for 0, 30 or 60 min. Proteins (15µg) were used in western blots with the same antibodies. Tubulin was used as a cytoplasmic marker.

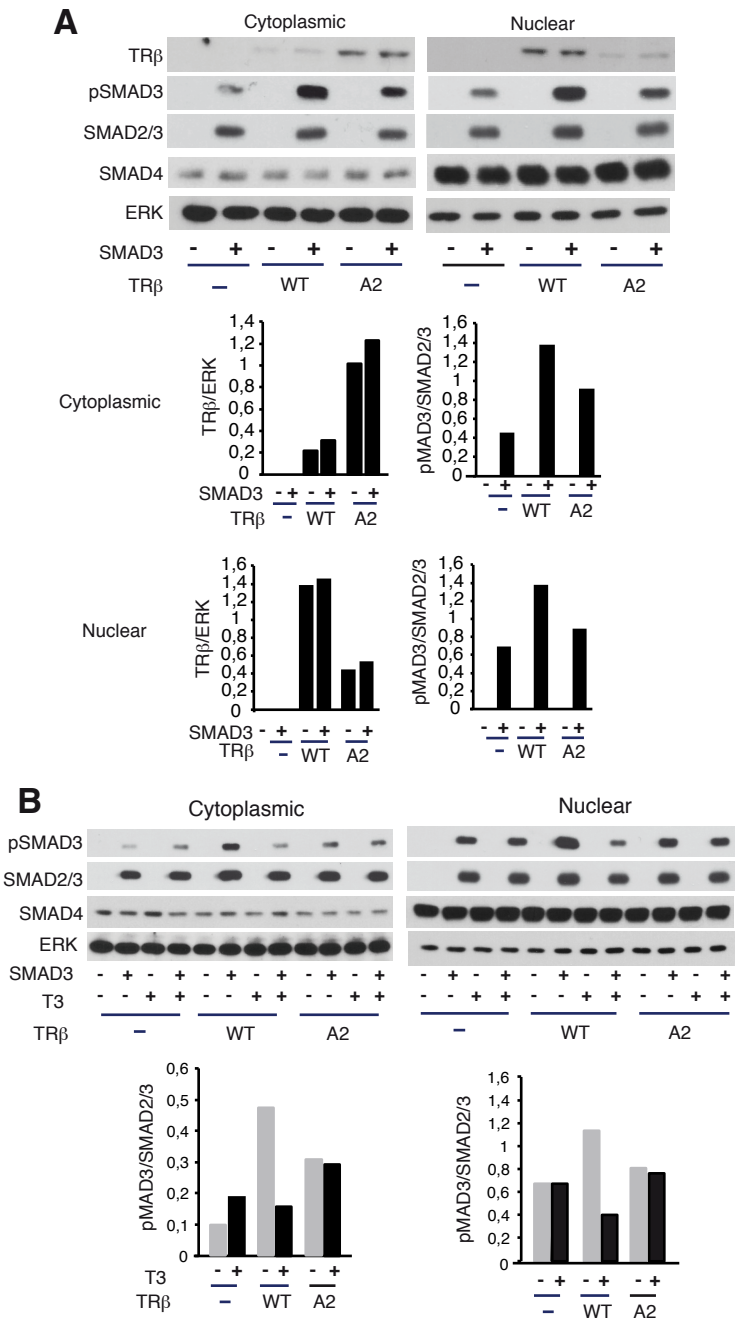

Fig. S6. TR nuclear translocation is required for T3-dependent inhibition of SMAD phosphorylation. (A) Western blot analysis of TRβ, phospho SMAD3 (pSMAD3), SMAD2/3, SMAD4 and ERK in cytoplasmic and nuclear extracts of Cos-1 cells transfected with expression vectors for SMAD3, wild-type TRβ or the 2A TRβ mutant in the nuclear localization signal. Quantifications of the TRβ/ERK and pSMAD3/total SMAD2/3 ratios in both compartments are shown in the lower panels. (B) pSMAD3, SMAD2/3, SMAD4 and ERK levels in cytoplasmic and nuclear extracts of Cos-1 cells transfected with expression vectors for SMAD3 and WT or 2A TRβ and treated in the presence and absence of 5nM T3 for 36h. Quantifications are shown in the lower panels.

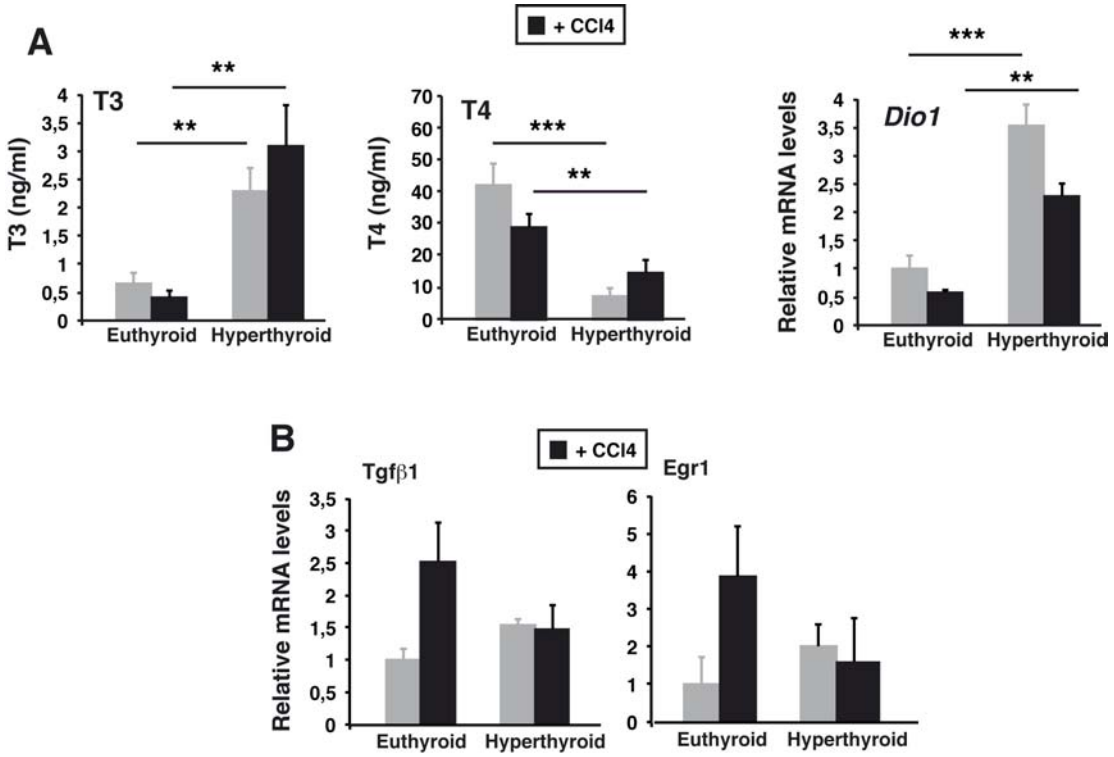

Fig. S7. Thyroid hormone treatment reduces expression of genes induced in acute liver injury. (A) Circulating levels of T3 and T4 and hepatic levels of Dio1 mRNA (means±s.e.m) in mice treated chronically with thyroid hormones and with CCl4 48 h before sacrifice. Results are means±s.e.m. (B) Tgfβ1 and Egr-1 mRNA levels in the same groups. Transcript levels (means±s.e.m) are expressed relative to the values obtained in the euthyroid untreated animals. Significances between euthyroid and hyperthyroid mice are indicated.

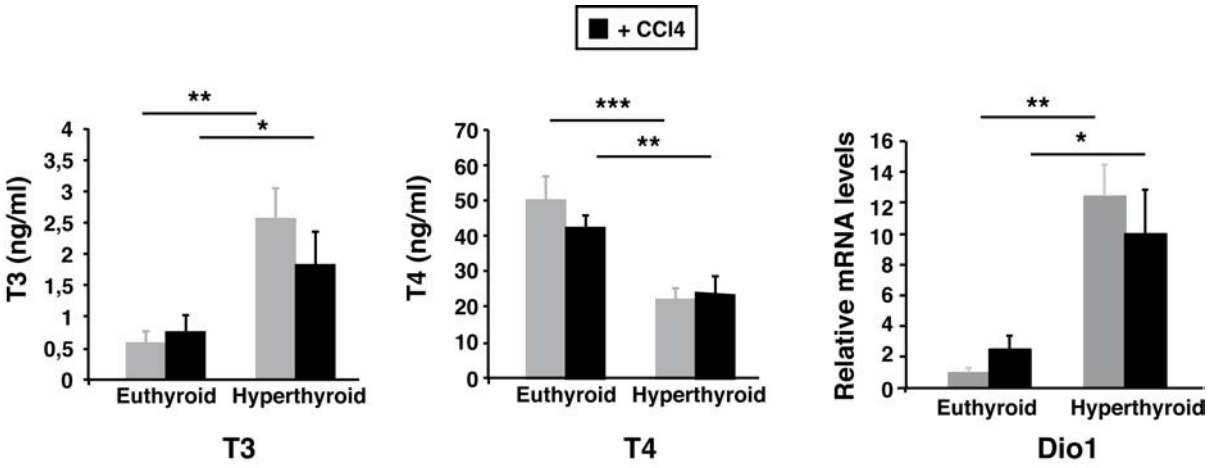

Fig. S8. Bleomycin treatment did not alter thyroid hormone levels. Circulating levels of T3 and T4, measured by radioimmunoassay, in mice treated with thyroid hormones and bleomycin. Hepatic Dio1 mRNA levels (means±s.e.m) in the different groups were used to demonstrate hyperthyroidism. Significances between euthyroid and hyperthyroid mice are indicated.

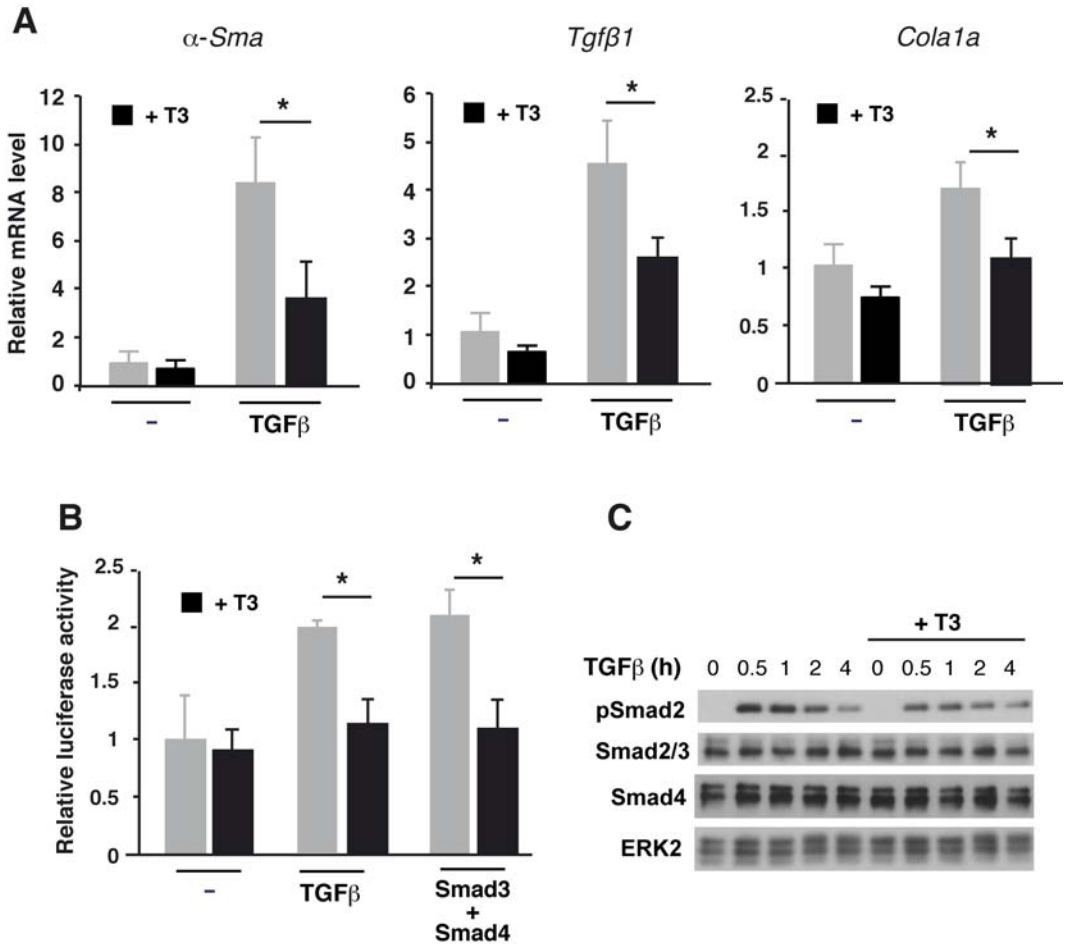

Fig. S9. T3 antagonizes fibroblasts activation by TGFβ. (A) Fibroblasts obtained from 13.5 p.c embryos were cultured in the presence and absence of 5nM T3 for 36 h and 10ng/ml TGFβ for the last 24h. α-Sma, Tgfβ1 and Cola1a mRNA levels (means±s.d) were determined and are expressed relative to the values obtained in the untreated cells. Significance of ANOVA post-test (n=3) of cells treated in the absence and presence of T3 is indicated. (B) Cells were cotransfected with the p3TP-lux plasmid and expression vectors for SMAD3 (150 ng) and SMAD4 (200 ng) or with the empty vector (-). Reporter activity was measured in untreated cells and in cells treated with T3 for 36 h and/or with TGFβ for 5h, as indicated. Statistically significant differences between cells treated with and without T3 are shown. (C) Cellular extracts from fibroblasts incubated with T3 for 36h and/or with TGFβ for the times indicated were subjected to Western blot with phospho-SMAD2 (pSMAD2), SMAD2/3 and SMAD4 antibodies. ERK2 was used as a loading control.