Supplemental Methods

Subjects, DNA amplification and DNA sequencing

We obtained written informed consent from all patients and available family members to participate in the clinical and genetic studies approved by the IRBs of the Johannes Gutenberg University and the University of Chicago. Genomic DNA from blood samples was isolated utilizing the QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany). The coding sequences of the candidate genes for TD as well as the promoter sequence of the *PAX8* gene were amplified by PCR (conditions and primer sequences for *TSHR*, *PAX8*, *TTF1*, *TTF2* and *NKX2.5* will be supplied upon request), purified enzymatically with *Exonuclease I* (New England Biolabs, Frankfurt, Germany) and *shrimp alkaline phosphatase* (GE Healthcare, München, Germany) and then sequenced using an automated sequencing system (3100 Avant Genetic Analyzer, Applied Biosystems, Darmstadt, Germany).

Constructs and mutagenesis:

The expression vector encoding murine Nkx2.5 was a gift from Dr. Paolo Macchia, Naples, Italy (1). The *NKX*2.5 gene mutation S265R was introduced utilizing the Quick Change Mutagenesis XL kit (Agilent Technologies, La Jolla, CA) according to the manufacturer's protocol using the following primers: S265R_F: 5'-CGCGGCCTGCAGAC-CCGGCTACAG-3'; S265R_R: 5'-GCTGTAGCCGGGTCTG-CAGGCCGCG-3'. To analyze whether the mutant NKX2.5 protein is localized correctly in the nucleus, we added a FLAG tag to the N-terminus of the cDNA sequence of the *WT NKX2.5* and the mutant expression vectors. The preparation of the reporter constructs *TG-luc* and *TPO-luc* has been described previously (2, 3).

To study the consequence of the *PAX8* promoter mutation a 1130 bp fragment containing sequences upstream of the ATG of the *PAX8* gene (-602 to +528) was amplified and cloned upstream of the *luciferase* gene of the pGL3 basic vector to generate *PAX8*proWT (a gift from Dr. Peter Kopp, Chicago, IL, USA). The *PAX8* promoter mutation at position -456 (*PAX8*proMUT) was introduced using again the Quick Change Mutagenesis XL kit (Agilent Technologies, La Jolla, CA) according to the manufacturer's recommendations with the primers *PAX8*_-456-F:5'-

CTTGCCTACAGCCCTGAAAGCAGGCTCTAC-3' and *PAX8*_-456_R:5'-GTAGAGCCTGCTTTCAGGGCTGTAGGCAAG-3', respectively. All constructs were verified by sequencing.

Cell culture and transfection

PCCL3 cells were a gift from Dr. Annette Altmann, Clinical Cooperation Unit, Nuclear Medicine, Heidelberg, Germany) and grown in Ham's F12 K medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (PAN Biotech GmbH, Aidenbach, Germany), 10 ng/ml somatostatin, 10 ng/ml Glys-His-Lys, 5µg/ml transferin, 10nM hydrocortisone, 10µg/ml insulin, and 10mU/ml TSH (Sigma-Aldrich, St. Louis, MO) at 37 C in a humidified chamber with 5% CO_2. Twelve hours before transfection, cells were seeded into 24 well plates. HeLa cells, derived from human cervical cancer, were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine, 4.5 g/liter D-glucose, 50 µg/ml gentamycin, and 10% fetal bovine serum. When the cells reached 70% confluence, transfection with SuperFect (Qiagen, Hilden, Germany) was carried out according to the manufacturer's instructions. Forty eight hours after transfection the cells were washed twice with ice cold PBS and lyzed in 150 µl passive lysis solution (Promega, Madison, WI). 10 µl of the protein extract was used for the dual luciferase assay (Promega, Madison, WI). The measurement of the *luciferase* and the *renilla luciferase* activities was performed according to the manufacturer's recommendations. Transfection studies were performed in sixplicates and repeated two times. Statistical significance of differences was analyzed by the Student's *t* test.

Immunofluorescence analysis

HeLa cells were cultured on glass cover slips in a 12 well plate. Cells were then transiently transfected with 400 ng plasmid DNA per well. Fortyeight hours after transfection cells were washed with PBS and fixed for 20 min at room temperature in 4% formalin (Fisher Scientific, Pittsburgh, PA). Afterwards the cells were permeabilized for 5 min with 0.2% TritonX100 in PBS. Blocking was performed with 1% goat serum for 30 min. The monoclonal Anti-FLAG M2 from Sigma (St. Louis, MO) was incubated for 30 min (dilution 1:1.000). The secondary antibody was Alexa Fluor568-

conjugated goat anti-mouse IgG (Invitrogen/Molecular Probes). Nuclei were counterstained with DAPI, and cover slips mounted using Prolong Gold Antifade (Invitrogen).

Electromobility shift assay (EMSA)

Recombinant proteins were generated using a coupled in vitro transcription/translation system (TNT®T7 Coupled Reticulocyte Lysate System; Promega, Madison, WI). Proteins were extracted from HeLa cells nuclei as described previously (4).

The *TPO* (5), *TG* (6), *DIO2* (7), and *NKE_2* (8) oligo sequences were synthesized as described previously and were 5'-end-labelled with IRD700 (Metabion, Martinsried, Germany).

The 10 µl binding reaction contained 1 µg of protein, 10-50 nM double stranded oligonucleotide, 150 ng poly (dI-dC), 500 ng salmon sperm DNA, 5mM DTT, 0.5% Tween20, 10 mM Tris, 50 mM NaCl, pH 7.5. The binding reaction was incubated for 30 min at room temperature. Afterwards 1 µl gel loading buffer (containing 650 mg/ml sucrose, 0.3% OrangeG, 10 µM Tris pH 7.5 and 10 µM EDTA pH 8.0) was added and the samples were loaded on a 4% native polyacrylamide gel (PAG). The PAG was supplemented with 50 mM Tris pH 7.5, 200 mM glycine, 2 mM EDTA. Towbin buffer (9) supplemented with 10 mM EDTA served as a running buffer. Thereafter the PAG were scanned and visualized using the Odyssey Model 9120 and the Odyssey v2.1 software (Li-Cor Biosciences, Bad Homburg, Germany).

To test the protein binding capacity of the WT and the mutant *PAX8* promoter the primers used for mutagenesis of *PAX8*proWT and *PAX8*proMUT were labelled with an infra red dye (IRD700) (Metabion, Martinsried, Germany) and incubated with proteins isolated from PCCL3 nuclei. The binding reaction contained 5 µg of protein in the same buffer as described above.

In silico analysis

To investigate whether the *PAX8* promoter mutation could affect binding of trans-acting factors to specific cis-regulatory elements we performed an *in silico* analysis of the WT and the mutant promoter

sequences utilizing the ModelInspector professional Release 5.4 May 2006 (Genomatrix, Munich, Germany).

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