

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Moreno JC, Klootwijk W, van Toor H, et al. Mutations in the iodotyrosine deiodinase gene and hypothyroidism. *N Engl J Med* 2008;358:1811-8.

Supplementary Material

METHODS

DNA preparation and analysis. Genomic DNA was isolated from peripheral blood samples according to standard proteinase K digestion procedures. PCR amplification of the 6 exons of *DEHAL1* was performed (95° C 1 min, 60° C 1 min, 72° C 1 min, 38 cycles) in reactions containing 15 mM MgCl₂ and solution Q (Qiagen). PCR products were directly sequenced using BigDye terminator (Applied Biosystems) and analysed by capillary electrophoresis on an ABI 3100 sequencer (PerkinElmer). Primer sequences are available from the authors on request.

Subcloning of wild-type DEHAL1 cDNA and mutagenesis. The entire coding region of human *DEHAL1* was shuttled from the cloning vector pdNRLib (IMAGE clone ID: 4612526; GenBank accession ID: BC056253) into the expression vector pcDNA3.1 (Invitrogen) using *EcoRI* and *XhoI* restriction sites. This clone was subsequently mutated by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis kit, Stratagene) to restore one nucleotide variation (C at position 803 in BC056253 into a T present both in the genome and in AY259176, resulting in a Leu260Pro substitution) using the forward primer *P260Lfwd* 5'-CACATGAAAAGCTGCTGATGCTGCTCCCCG-3' and the corresponding reverse primer.

Mutations identified in patients were introduced in wild-type *DEHAL1* (Leu260) cDNA using the primers *R101Wfwd* 5'-TTATGAACTTCTCAATAAGAGATGGTCAGTCAGGTTTCATAAGTAATG-3', *F1105/6Lfwd* 5'-CAGGGTCAGTCAGGTTAAGTAATGAGCAAGTCCC-3' and *I116Tfwd* 5'-GTCCCAATGGAAGTCACTGATAATGTCATCAGAACG-3' with corresponding reverse primers, and verified by DNA sequencing. Primers to introduce the 3-bp deletion (Phe105-Ile106delinsLeu) were purified by polyacrylamide gel electrophoresis (PAGE) before use.

Five additional mutants were generated to introduce different amino acids at positions found mutated in patients. The Arg101Ala and Arg101His mutants were obtained using primers *R101Afwd* 5'-GAACTTCTCAATAAGCGAGCGTCAGTCAGGTTTCATAAGTAATGAGC-3' and *R101Hfwd* 5'-GAACTTCTCAATAAGAGGCATTCAGTCAGGTTTCATAAGTAATGAGC-3'; the Phe105Ala and Phe105Tyr mutants using *F105Afwd* 5'-CAATAAGAGACGGTCAGTCCGGGCCATAAGTAATGAGCAAGTCCC-3'

and *F105Yfwd* 5'-CAATAAGAGACGATCGGTCAGGTACATAAGTAATGAGCAAGTCCC-3'; and the Ile116Val mutant using *I116Vfwd* 5'-GAGCAAGTCCCAATGGAAGTCGTCGACAATGTCATCAGAAC GGC-3' with corresponding reverse primers.

Cell culture and transfection assays. Human embryonic kidney (HEK293) cells were grown in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal calf serum and 0.1 mM flavin mononucleotide (FMN, MP Biochemicals) in 6-well plates. At 50% confluency, cells were transfected with 1 µg of wild-type or mutant DEHAL1 plasmid or empty pcDNA3.1 plasmid, using Fugene6 (Roche). After 24 h incubation, cells were washed with 1 ml PBS and collected in 0.5 ml PED buffer (0.1 M phosphate, pH 7.2, 2 mM EDTA, 1 mM dithiothreitol) per well and kept on ice. Samples were sonicated twice for 10 sec using Soniprep150, frozen on dry ice-ethanol and stored at -80 °C. For experiments testing FMN-response, culture medium was supplemented with 0-2 mM FMN.

Iodotyrosine deiodinase assay. [¹²⁵I]MIT and [¹²⁵I]DIT were prepared by radioiodination of L-tyrosine (Sigma-Aldrich) and MIT (Sigma-Aldrich), respectively, with ¹²⁵I⁻ (specific activity: 15 Ci/mg, Amersham Biosciences) using the chloramine-T method¹ and purified by chromatography on cation-exchange resin (Dowex 50WX2-200, Sigma-Aldrich). HPLC analysis on a C18 reversed-phase column showed 96% and 93% purity of [¹²⁵I]MIT and [¹²⁵I]DIT, respectively, with trace amounts of ¹²⁵I⁻.

Incubations contained 10⁵ cpm [¹²⁵I]MIT or [¹²⁵I]DIT, 0.1 µM unlabeled MIT or DIT and 10-25 µg cell homogenate protein in a final volume of 0.1 ml PED buffer. Reactions were started by the addition of NADPH (final concentration 0.1 mM), and the mixtures were incubated for 60 min at 37°C. Reactions were stopped by the addition of 0.9 ml of 10% acetic acid on ice. Samples were applied to 2 ml Dowex 50WX2-200 columns, and successively eluted with 3x1 ml 10% acetic acid (containing eluted iodide), 1 ml water, and 4x1ml 1 M NH₄OH (containing iodotyrosines). Radioactivity was measured in each fraction in a NE1600 gamma counter (NE Technologies) and percentage deiodination was calculated. Protein concentrations in cell homogenates were measured by Bradford's assay; iodotyrosine deiodinase activity was corrected for the slight ¹²⁵I⁻ production in empty pcDNA3.1-transfected cell homogenates and expressed per mg protein. With [¹²⁵I]DIT as the substrate, calculation of deiodinase activity took into consideration that [¹²⁵I]MIT did not significantly accumulate during the reaction but was rapidly further deiodinated, as shown by HPLC (Supplementary Fig. 1).

DEHAL1 antibody production. A rabbit polyclonal antibody was raised against 2 synthetic peptides comprising the carboxy-terminal amino acid sequences Gly²⁶⁶-Lys²⁸⁰ and Pro²⁷⁵-Val²⁸⁹ of the DEHAL1 protein. Two rabbits were immunized with 4 injections of the combined antigens, and one of the antisera was purified by affinity chromatography using the Gly²⁶⁶-Lys²⁸⁰ peptide (Eurogentec).

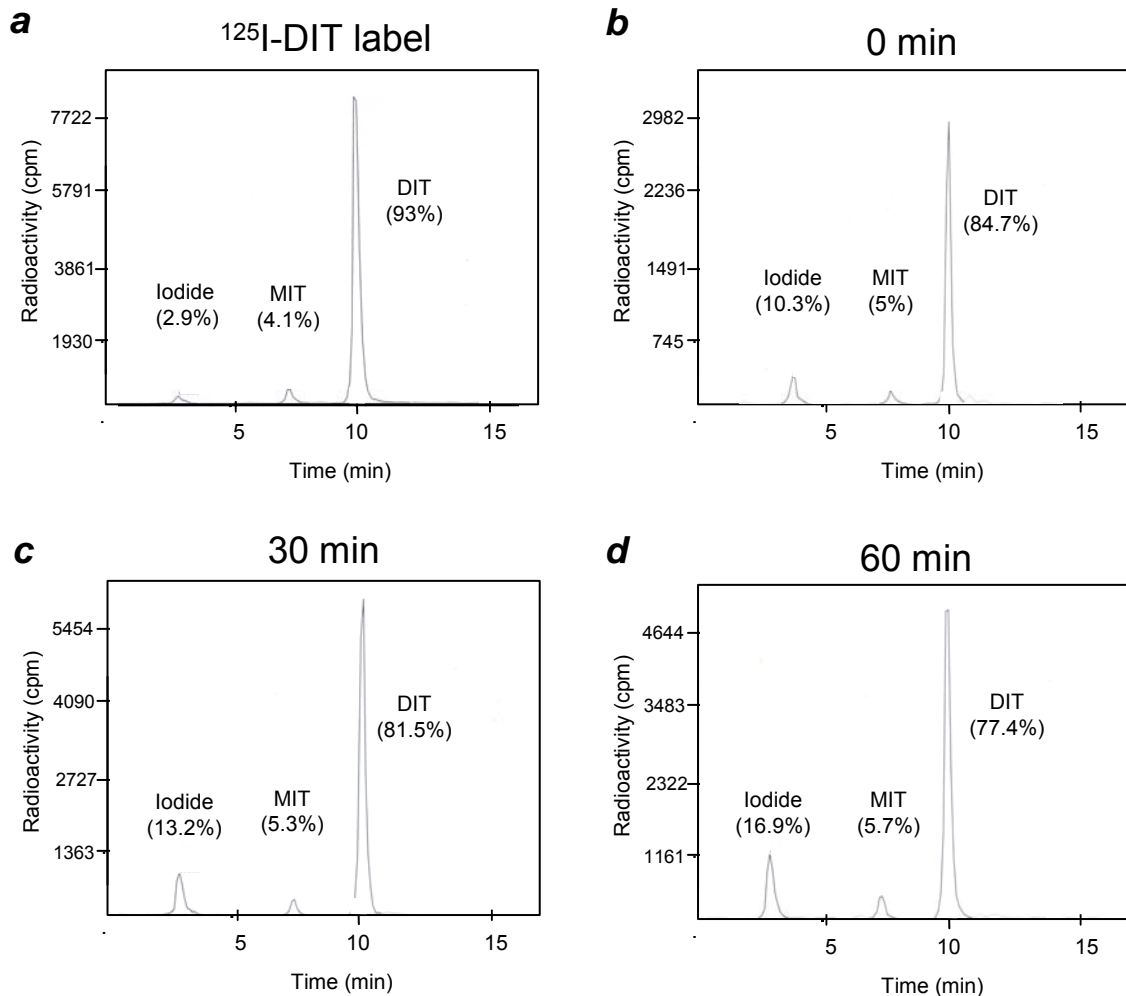
Western blots. Cell homogenate (10 µg protein) was subjected to SDS-PAGE (12%) according to Laemli's method and run for 1 h at 150 volts using the Mini-Protean 3 system (Bio-Rad). Gels were electroblotted to nitrocellulose filters, which were incubated overnight at 4 °C with anti-DEHAL1 antibody (1:500) in PBS containing 0.1% Tween-20, and 5% non-fat milk. After incubation with horseradish peroxidase-conjugated second antibody, immune complexes were visualized by incubation with a chemoluminescent substrate (Western Lightning, PerkinElmer) and autoradiography.

DIT radioimmunoassay. Serum DIT concentrations were determined using a rabbit anti-DIT antibody in a slightly modified two-step radioimmunoassay.² Briefly, 100 µl purified [¹²⁵I]DIT (2x10⁴ cpm) was added to 100 µl patient serum, 100 µl DIT-antibody (final dilution 1:200) and 700 µl barbital buffer, and incubated overnight at room temperature. Antibody-bound [¹²⁵I]DIT was precipitated by incubation with 100 µl suspension of goat anti-rabbit antibody coupled to cellulose (Sac-cel, IDS). After centrifugation, pellets were washed and counted in the NE1600 gamma counter.

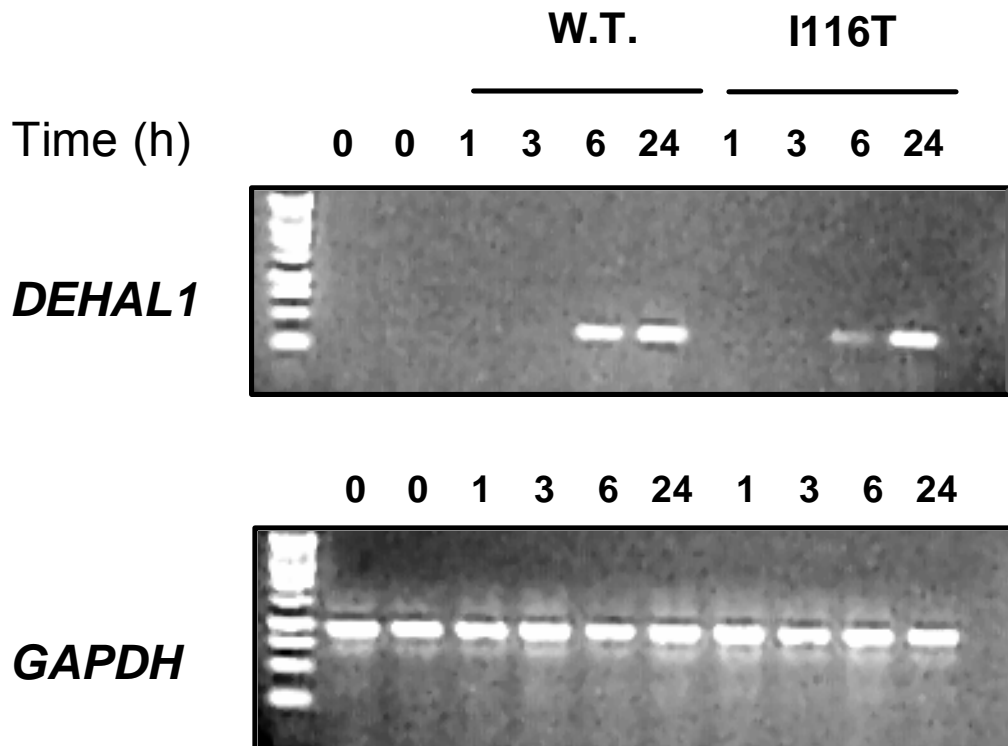
Descriptive data analysis. Data represent the mean ± standard deviation of values obtained in 3 or more independent experiments, performed in duplicate.

References

1. Hussain AA, Jona JA, Yamada A, Dittert LW. Chloramine-T in radiolabeling techniques. II. A nondestructive method for radiolabeling biomolecules by halogenation. *Anal Biochem* 1995;224:221-6.
2. Meinhold H, Beckert A, Wenzel KV. Circulating diiodotyrosine: studies of its serum concentration, source and turnover using radioimmunoassay after immunoextraction. *J Clin Endocrinol Metab* 1981;53:1171-8.



Supplementary Figure 1. HPLC profiles of (a) [^{125}I]DIT label and of homogenates of HEK293 cells transfected with wild-type DEHAL1 cDNA after incubation with the [^{125}I]DIT label for 0 (b), 30 (c) or 60 (d) min. at 37°C. To study the dynamics of the MIT generated from DIT by DEHAL1, mixtures of 2×10^5 cpm [^{125}I]DIT, 0.1 μM unlabeled DIT and 10-25 μg cell homogenate protein were incubated for 0, 30 and 60 min at 37 C in a final volume of 0.1 ml PED buffer. Reaction mixtures were extracted with equal volumes of methanol, spun down and 100 μl supernatant were added to 100 μl of 0.02 M ammonium acetate (pH 4.0). 100 μl of the latter mix (5×10^4 cpm) was analyzed by HPLC on a C18 reversed-phase column eluted with a gradient of 5-40% acetonitrile in 0.02 M ammonium acetate (pH 4.0). The [^{125}I]DIT label used was analysed by HPLC in parallel. As shown, the percentage radioactivity corresponding to [^{125}I]MIT does not change significantly during the assay (5.0, 5.3 and 5.7% after 0, 30 and 60 min incubation), and mainly corresponds to the MIT already present in the [^{125}I]DIT label used (4.1%). This suggests that deiodination of MIT to tyrosine by DEHAL1 is fast compared with its production from DIT.



Supplementary Figure 2. *Semi-quantitative RT-PCR of DEHAL1 and GAPDH.* To study the mRNA abundance of wild-type DEHAL1 and the Ile116Thr mutant, HEK293 cells were transfected with the corresponding plasmids and grown in culture to 90% confluence in 6-well plates. Total RNA was isolated from 10^6 cells using the High Pure RNA Isolation Kit (Roche Diagnostics). RNA concentrations were measured by spectrophotometry (NanoDrop B1123) and 500 ng RNA was used for cDNA synthesis using Taqman RT reagents (Roche Diagnostics). Two μ l cDNA were used for semi-quantitative RT-PCR of DEHAL1 and GAPDH at standard conditions for 27 cycles amplification. Primers used were DEHAL1ex2.1fwd 5'-GAGCAAGTCCCAATGGAAGT-3' and DEHAL1ex4.1rev 5'-CACAAGCGATGGAAACTG-3', GAPDHfwd 5'-AAGGTGAAGGTCGGAGTCAAC-3' and GAPDHrev 5'-TTGTCATACCAGGAAATGAGC-3'. 24 h after transfection, mRNA levels of wild-type DEHAL1 and the Ile116Thr mutant are not significantly different.

Exon	Primer	Primer sequences
1	1F	5'-TGA TCT TTG CTC CTT TGC AA-3'
	1R	5'-CAT AAG CCC TGC CAT TGA CT-3'
2	2F	5'-CGGTCACCTTATGACCAAGG-3'
	2R	5'-TTCTGGCACTCACTCAGCAT-3'
3	3F	5'-TGC TTG GAC TAC AGG GAT GA-3'
	3R	5'-ATG GTG GTG GTG GTG ATT TT-3'
4	4F	5'-TGG GCA AAT ATT ATC CTG AATC-3'
	4R	5'-TCC AAA TGT CCC TGA ATT GAA-3'
5	5F	5'-CTG AAG GAG CTG GCA TTG AT-3'
	5R	5'-CAG CAA ACT CAA TGG CAA AG-3'
6	6F	5'-GAA GGT CCC CAG GTA AGA GG-3'
	6R	5'-GCC AAG AGA CCC AAG AGG AG-3'

Suppl Table 1. Intronic primer sequences for amplification of DEHAL1 coding region.