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**CHARACTERIZATION OF A NOVEL LOSS OF FUNCTION
MUTATION OF PAX8 ASSOCIATED WITH CONGENITAL
HYPOTHYROIDISM.**

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3 **CHARACTERIZATION OF A NOVEL LOSS OF FUNCTION MUTATION OF PAX8**
4 **ASSOCIATED WITH CONGENITAL HYPOTHYROIDISM**

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Summary

Background: Congenital hypothyroidism (CH) is a common endocrine disease that occurs in about 1:3000 newborns. In 80-85% of the cases, CH is presumably secondary to thyroid dysgenesis (TD), a defect in the organogenesis of the gland leading to an ectopic (30-45%), absent (agenesis, 35-40%) or hypoplastic (5%) thyroid gland. The pathogenesis of TD is still largely unknown. Most cases of TD are sporadic, although familial occurrences have occasionally been described. Recently, mutations in the PAX8 transcription factor have been identified in patients with TD.

Objective: Our aim was to identify and functionally characterize novel PAX8 mutations with autosomal dominant transmission responsible for TD.

Design: The *PAX8* gene was sequenced in a mother and child both suffering for CH due to thyroid hypoplasia. Subsequently, expression vectors encoding the mutated PAX8 were generated and the effects of the mutation on both the DNA binding capability and the transcriptional activity were evaluated.

Results: *PAX8* gene sequencing revealed an heterozygous mutation, that consists of the substitution of a histidine residue with a glutamine at position 55 of the PAX8 protein (H55Q). When tested in cotransfection experiments with a thyroglobulin promoter reporter construct, the mutant protein turned out to be still able to bind DNA in EMSA assays but transcriptionally inactive.

Conclusions: Our findings confirm the important role of PAX8 in normal thyroid development and support the evidence that in humans haploinsufficiency of PAX8 is associated to TD.

Introduction

Primary congenital hypothyroidism is a common endocrine disease in newborns, with an incidence of about 1:3000 live births and is characterized by elevated TSH levels at birth in response to reduced thyroid hormone levels¹. In 85% of the cases, CH is due to developmental anomalies of the thyroid gland (thyroid dysgenesis, TD), which result in a thyroid gland that is absent (thyroid agenesis or athyreosis), ectopically located (thyroid ectopy) or hypoplastic (thyroid hypoplasia)². About 5% of the TD cases are associated with mutations in the genes responsible for thyroid development and growth³, including genes that encode for TTF-1/NKX2.1^{4,5}, FOXE1^{6,7}, PAX8^{8,9}, TSH receptor¹⁰ and NKX2.5¹¹.

PAX8, like other members of the Pax family, recognizes DNA via a conserved 128-amino acid domain, the paired domain. The gene maps to human chromosome 2q12-14 and contains a 4-Kb transcription unit divided into 12 exons¹². PAX8 plays an important role in thyroid development, when the thyroid bud evaginates from the floor of the pharynx¹³ and it is required both for the morphogenesis of the thyroid gland⁹ and for the maintenance of the thyroid differentiated phenotype¹⁴. In the adult thyroid, PAX8 is essential for the transcriptional activation of the thyroperoxidase (TPO), thyroglobulin (Tg) and sodium/symporter (NIS) genes^{15,16}. Recently, it has been demonstrated that PAX8 and TTF-1/NKX2.1 biochemically associate and synergistically activate transcription from the TPO¹⁷ and Tg gene promoters^{18,19}. Homozygous Pax8 knockout mice die shortly after weaning, presumably from severe hypothyroidism due to thyroid hypoplasia, whereas heterozygotes were described unaffected⁹.

To date, eleven heterozygous inactivating mutations in the *PAX8* gene have been identified and characterized in patients with TD^{8,20,21}. Only two of these mutations, the deletion in exon 7 (c.989-992delACCC) located outside the paired domain²² and the p.S48F located within the paired domain²³, show normal DNA binding affinity but are transcriptionally inactive. The remaining eight mutations already described are located within the paired domain of PAX8 and

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3 result in a severe reduction of DNA binding. The autosomal dominant transmission is well
4 established with variable degrees in the severity of hypothyroidism.
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8 In the present study, we report the identification and functional characterization of a novel *PAX8*
9 mutation in a mother and child with CH due to thyroid hypoplasia. The mutation results in the
10 substitution of histidine for glutamine at codon 55 of the PAX8 protein. Although the mutated
11 residue is located within the paired domain, the mutated protein does not display an impairment
12 in the DNA binding activity, but is unable to activate transcription from known PAX8 target
13 promoters.
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20 Our results strengthen the notion that PAX8 mutation contributes to the sporadic and familial
21 cases of CH.
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Patients and Methods

Case reports

Two members of a nonconsanguineous family were diagnosed with hypothyroidism (Fig. 1A). Patient II-1 was diagnosed as hypothyroidism at birth in the frame of a neonatal screening, displaying a TSH level of 200 mU/ml (normal range: 1-10), and a low free T₄ of 13 pmol/l (normal range: 20-30). The patient had no detectable TG antibodies and his serum TG was 160 ug/l (normal, 20-70). Thyroid ¹²³I scintigraphy showed a hypoplastic gland in normal position. Since birth he is receiving substitutive L-T₄ treatment. His physical and psychomotor development are normal.

Patient I-1, 24-year-old woman, is the mother of patient II-1. During the CH screening she was diagnosed as athyreotic, but a recent ultrasonography showed a hypoplastic thyroid (right lobe: 0,4 x 1,4 x 1,3 cm; left: 0,9 x 0,5 x 1,5 cm.) (Fig. 1A). She was treated since birth, and had normal development in terms of growth and intelligence. Ultrasonography of the kidneys did not reveal any structural abnormalities in the renal system. Her family history is unknown, and unfortunately the other members of the family did not agree to provide DNA for the genetic studies.

DNA sequencing

All the coding region of the PAX8 gene was amplified as previously described⁸. PCR products were then purified with Antarctic Phosphatase-Exonuclease I (New England BioLabs, USA) at the following conditions: 37°C for 15 min and 80°C for 15 min respectively for enzymes activation and inactivation, and, then, directly sequenced bi-directionally with a 377 ABI sequencer with the same primers used for PCR amplification.

Plasmids

The plasmids were previously described and were as follows: CMV-TTF-1²⁴, pCMV5-H29a (also named PAX8-WT)¹². The H55Q and H55A mutations were introduced by site-directed mutagenesis (QuikChange II-E Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA). All constructs were confirmed by sequencing. The reporter plasmid TgCAT, originally named pTACAT-3, used in transient transfection experiments was previously described²⁵.

Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Euroclone, Pero, MI, Italy). For transient transfection experiments, cells were plated at 3×10^5 cells/60-mm tissue culture dish 5 to 8 h prior to transfection. Transfections were carried out with the FuGENE6 (Roche Diagnostics, Basel, CH) according to the manufacturer's directions. The DNA/FuGENE ratio was 1:2 in all experiments. The plasmid CMV-LUC was used as internal control in transfection assays. Cells extracts were prepared after 48 h to determine either the levels of CAT protein with a CAT enzyme-linked immunosorbent assay kit (Roche Diagnostics, Basel CH) or LUC activities by Luciferase assays as previously described²⁶. Transfection experiments were done in duplicate and repeated at least three times. CAT activity values on the graph are the means of all experiments (\pm) S.D. Statistical analysis uses two-tailed Student's *t* test to obtain the *P* value associated with the observed fold of activation differences.

Protein production and Western blot analysis

Wild-type and mutated PAX8 cDNA constructs were transcribed and translated using the TnT Quick Coupled Transcription/Translation System from Promega Corporation. The level of transcription/translation products and total extract of transfected Hela, prepared as previously described²⁷, were assessed by Western blot. For these analysis, proteins were separated by SDS-10% PAGE, gel were blotted onto Immobilon P (Millipore, Bredford, MA, USA) for 2 h and the

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3 membranes were blocked in 5% nonfat dry milk in Tris-buffered saline for 2 h before the addition
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5 of the anti-PAX8 antibody (kindly provided by R. Di Lauro) for 1 h. The filters were washed
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7 three times in Tris-buffered saline plus 0.5% Tween 20 before the addition of horseradish
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9 peroxidase-conjugated secondary antibody for 45 min. Horseradish peroxidase was detected with
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11 ECL (GE Healthcare, USA).
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14 15 16 **EMSA**

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18 Double-stranded oligonucleotide C derived from the Tg promoter was labeled with [γ -³²P] ATP
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20 and T₄ polynucleotide kinase and used as probe as previously described¹⁶. The binding reactions
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22 were incubated 30 min at room temperature in a binding buffer containing 10 mM HEPES (pH
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24 7.9), 10% glycerol, 0.1 mM EDTA, 8 mM MgCl₂, 1 mM dithiothreitol, and 0.15 g/ml of poly
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26 (dI-dC). DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel and
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28 visualized by autoradiography.
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Results

Sequencing of PAX8

In both affected members of the family (I-1 and II-1 in Fig. 1A) sequence analysis of the *PAX8* coding region gene revealed an heterozygous T- to - G transversion in exon 3 (Fig. 1B), resulting in the substitution of a histidine at position 55, within the α -helix 3 of the PAX8 paired domain, with a glutamine residue (PAX8-H55Q) (Fig. 1B-C).

DNA-binding properties of the PAX8-H55Q mutant

To evaluate whether the PAX8-H55Q mutation produces a defect in the DNA binding ability of the PAX8 protein we carried out EMSA analysis with *in vitro* translated PAX8 and PAX8-H55Q proteins. As shown in Fig. 2A, the PAX8-H55Q mutant is able to bind DNA similarly to wild-type PAX8 when challenged with the oligo C derived from the Tg promoter and containing a well-known PAX8 binding site. Equivalent amounts of each protein were used in the EMSA assay reactions as confirmed by the Western blot analysis (Fig. 2A). The same DNA binding ability was observed when total protein extracts of HeLa cells transiently transfected with expression vectors encoding either the wild-type or the mutated PAX8 protein were employed in the EMSA assay (Fig. 2B). Also in this case, the amounts of each total extract used in the binding reactions were normalized by Western blot (Fig. 2B).

Therefore, our data indicate that the mutation of the histidine residue within the paired domain does not affect the ability of PAX8 to recognize its binding site on DNA, suggesting that the H55 residue is not involved in contacts with DNA. To further confirm these results we have generated an additional mutant, named PAX8-H55A in which the histidine residue is substituted by an alanine. The alanine is the substitution residue of choice that does not alter the main-chain conformation²⁸ and it has been proven to be unable to make contact with DNA. Consequently, we generated this mutation to clarify whether the H55 residue of PAX8 is directly involved in

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3 contacts with DNA or not. Specifically, if the H55 residue is not involved in contacts with DNA
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5 we expected that the PAX8-H55A mutant did not show any impairment in DNA binding. Indeed,
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7 our results of the EMSA assays clearly indicated that the PAX8-H55A mutant is able to bind to
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9 the PAX8 binding site contained in the oligonucleotide C and shows no significant differences
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11 when compared to wild-type PAX8 and PAX8-H55Q (Fig. 2C, 2D). The same results were
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13 obtained when *in vitro* translated proteins (Fig. 2C) or total extract of HeLa cells transiently
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15 transfected with either the wild-type or the mutated expression vectors (Fig. 2D) were employed
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17 in EMSA assays. Western blotting of wild-type and mutated proteins produced *in vitro* or
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19 transiently expressed in HeLa cells revealed no difference in the synthesis efficiency, indicating
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21 that the amino acid substitution does not cause destabilization of the mutant proteins (Fig. 2C,
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23 2D).
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29 **Transcriptional activity of PAX8-H55Q mutant**

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31 To evaluate the functional relevance of the H55Q mutation, we relied on the ability of PAX8 to
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33 activate transcription from the thyroglobulin (Tg) promoter¹⁶ and we investigated the ability of
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35 the PAX8-H55Q mutant to activate transcription of a reporter gene under the control of the Tg
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37 promoter. HeLa cells were transiently transfected with expression vectors encoding wild-type
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39 PAX8 or PAX8 mutants, together with a reporter construct in which the minimal region of the Tg
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41 promoter is subcloned upstream of the CAT gene (pTACAT3²⁵). In agreement with previous
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43 results^{16, 18}, wild-type PAX8 is able to stimulate transcription from the Tg promoter up to 7-fold,
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45 whereas PAX8-H55Q or PAX8-H55A show a significantly reduced Tg reporter activation (Fig.
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47 3A). These results led us to the conclusion that the PAX8-H55Q mutant has a significant reduced
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49 ability to activate transcription despite the fact that it has no impairment in DNA binding activity.
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51 Recently, it has been demonstrated that a physical interaction between PAX8 and the
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53 transcription factor TTF-1/NKX2.1 underlies the synergistic effect of both factors on Tg promoter
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55 activation¹⁸. To test whether the mutant PAX8-H55Q and TTF-1/NKX2.1 could synergistically
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3 stimulate transcription from the Tg promoter, we transiently co-transfected in HeLa cells the
4 reporter construct TgCAT together with the expression vectors encoding wild-type PAX8, PAX8-
5 H55Q, PAX8-H55A and TTF-1/NKX2.1 separately or in combination. Our data show that
6 although the activity of the PAX8-H55Q mutant is significantly lower than the activity of wild-
7 type PAX8, it can still display a robust synergism with TTF-1/NKX2.1 in the transcriptional
8 activation of the Tg promoter (Fig. 3B). A similar behavior was observed with the PAX8-H55A
9 mutant (Fig. 3B).

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12 These results indicate that the H55Q mutation retains the ability to functionally interact with
13 TTF-1/NKX2.1.
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Discussion

We have identified and functionally characterized a novel loss-of-function mutation in the transcription factor PAX8, dominantly inherited in two members of a family with severe thyroid hypoplasia. The mutation, a T→G transversion in exon 3 of the *PAX8* gene, results in the substitution of a histidine at position 55 by a glutamine residue (H55Q). Although the H55Q mutation is located within the paired domain, we demonstrated that it does not alter the DNA binding ability of the mutant however it modifies the functional properties of the protein. Our data are in agreement with the results previously reported by Grasberg et al. for the PAX8-S48F mutation²³ showing that the mutation, even if located within the paired domain, abrogates the transactivation property of PAX8 but not its DNA binding ability.

Based on the crystal structure models of paired domain-DNA complexes^{29, 30}, histidine 55 of PAX8 is located in the α -helix 3 of the paired domain and is predicted to make contacts with the DNA backbone. However, this aminoacid represents a variant residue, being not conserved among the *PAX* genes²⁹. It is worth noting that the structure of PAX8 bound to DNA has not been determined yet, therefore any assessment on the PAX8-DNA complex has been made by comparison with other PAX homologues bound to DNA³¹. Our hypothesis is that the H55Q mutation within helix 3 of the paired domain is not involved in contacts with the DNA but might affect the folding and the conformation of the C-terminal region of PAX8, possibly of domains of the protein known to be crucial for the transcriptional activity because involved in physical interactions with other coactivators and/or the basal transcriptional machinery. This hypothesis is supported by the observation that also the PAX8-H55A mutation that we have generated retains the ability to bind DNA but is not able to efficiently activate transcription, as it happens with PAX8-H55Q. Because the alanine residue has a small side chain, the substitution of the histidine residue by alanine should severely interfere with the DNA recognition and consequently show an impairment in DNA binding of PAX8-H55A. However, our results demonstrated that such a

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3 mutation affects only the transactivation property of PAX8. Hence, the H55Q mutation
4 represents, together with the previously reported S48F mutation²³, a new type of PAX8 mutations
5 responsible for congenital hypothyroidism.
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9 PAX8 is a major gene in the regulation of the thyroid differentiated phenotype¹⁴ and plays an
10 important role in the morphogenesis of thyroid gland⁹. To date, all PAX8 gene mutations reported
11 are located within the paired domain, except for one mutation c.989-992delACCC²², located in
12 exon 7 outside the paired domain that cause a frameshift with a premature stop codon after codon
13 277. The PAX8/277del protein retains the DNA binding ability but it is not capable of activating
14 transcription, thus indicating that the C-terminal region is essential for PAX8 transcriptional
15 activity. Recently, it has been demonstrated that PAX8 activity can be modulated by the
16 interaction with other proteins, forming complexes on regulatory regions of target genes^{18, 27, 32}.
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18 Therefore, our findings suggest that the PAX8-H55Q mutant does not efficiently activate
19 transcription because it does not allow the recruitment of other factors or coactivators to the target
20 promoters. Furthermore, it has been demonstrated that PAX8 cooperates with the transcription
21 factor TTF-1/NKX2.1^{17, 19}, and our laboratory specifically demonstrated that PAX8 and TTF-
22 1/NKX2.1 form a functional heterocomplex responsible for the synergistic transcriptional
23 activation of the *Tg* promoter in differentiated thyroid cells¹⁸. The relevance of this interaction has
24 been further confirmed by the analysis of the *in vivo* mouse model bearing a partial deficiency of
25 *Ttf1* and *Pax8* genes. The double-hetero-zygous null mice show thyroid dysgenesis, a phenotype
26 that is completely absent in either of the single heterozygous mice³³. Our results demonstrate that
27 the PAX8-H55Q mutant is still able to synergize with TTF-1/NKX2.1 on the *Tg* promoter, as
28 indicated by the significantly higher stimulation of the reporter gene expression in cells
29 expressing both PAX8-H55Q and TTF-1/NKX2.1, suggesting that the H55 residue is not
30 essential for the functional interaction with TTF-1/NKX2.1. Therefore, it will be of interest to
31 further investigate the effect of this novel mutation with respect to the already described
32 interactions that PAX8 makes with other partners.
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3 The mutation here reported in the child was inherited from his mother, in line with the dominant
4 model of inheritance proposed for the cases of familial thyroid dysgenesis due to PAX8
5 mutations^{8, 34}. The present case, as the others previously published, demonstrates that loss-of-
6 function mutations of PAX8 are symptomatic in heterozygotes in humans, whereas only
7 homozygous mice for a targeted disruption of *Pax8* display severe hypothyroidism, characterized
8 by absence or dramatic reduction of the thyroid gland⁹. Haploinsufficiency, monoallelic
9 expression, imprinting, dominant negative properties^{8, 34} and stochastic expression of the PAX
10 alleles, as noted for *Pax5*³⁵, are the molecular mechanism that could lead to disturbed thyroid
11 development in humans. The patients reported here carry the H55Q mutation and both present CH
12 with thyroid hypoplasia.
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25 In conclusion, the novel PAX8-H55Q mutation identified in patients with CH and thyroid
26 hypoplasia confirms the role of PAX8 in normal thyroid development and might be helpful to
27 better understand the role of PAX8 in the pathogenesis of TD.
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51 **Competing interests/financial disclosure**

52 The authors have nothing to disclose
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FIGURE LEGENDS

Figure 1. Identification of a novel PAX8 mutation. A, Pedigree of the family presenting with CH associated with the H55Q mutation of PAX8 in the heterozygous state. B, Chromatogram of part of exon 3 of patients showing a heterozygous T→G transversion at nucleotide 165 of the paired domain PAX8 (c.165T>G). This mutation replaces a histidine residue by glutamine (H55Q). C, Schematic representation of the paired domain (PD) and the position of the H55Q mutation in α -helix 3 (α 3). The octapeptide (OP) and the residual paired-type homeodomain (HD) are shown. Also, the previously identified PAX8 mutations are indicated by asterisks.

Figure 2. DNA-binding of the PAX8-WT and PAX8 mutant proteins. The DNA-binding properties of PAX8-WT, PAX8-H55Q and PAX8-H55A mutants were tested on a PAX8 response element, oligonucleotide C. EMSA were performed with *in vitro*-translated WT, H55Q and H55A mutant PAX8 proteins (A, C), and protein extract prepared from HeLa cells transiently transfected with PAX8-WT, PAX8-H55Q and PAX8-H55A (B, D). PAX8-H55Q mutant (A, lane 3; B, lane 4; C, lane 3; D, lane 4) and PAX8-H55A mutant (C, lane 4; D, lane 5) are able to form a low-mobility complex with the oligo C as the PAX8-WT (A, lane 2; B, lane 3; C, lane 2; D, lane 3). No retarded band is present in the extract prepared from non-transfected HeLa cells used as a negative control (B and D, lane 2). The analysis by Western blotting of PAX8-WT, PAX8-H55Q and PAX8-H55A proteins showed that equivalent amounts of each protein were produced.

Figure 3. Functional analysis of PAX8-H55Q. A, HeLa cells were transiently transfected with the reporter plasmid TgCAT (2 μ g), the expression vector encoding PAX8-WT (100 ng), the PAX8-H55Q mutant (100 ng) and the PAX8-H55A mutant (100ng). The protein levels of PAX8-WT, PAX8-H55Q and PAX8-H55A after transfection were measured by immunoblot and are shown.

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3 B, HeLa cells were transiently transfected with the reporter plasmid TgCAT (2 μ g) with or
4 without the expression vectors encoding for TTF-1 (50 ng), PAX8-WT (100 ng), PAX8-H55Q
5 mutant (100 ng), and PAX8-H55A mutant (100 ng). Folds of activation are considered as ratio
6 between values obtained with and without cotransfection of the expression vectors. CMV-LUC as
7 added as internal reference and all data were normalized to the ratio CAT/LUC activity. Results
8 are means \pm SD from three independent experiments, each performed in triplicate transfection.
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10 Statistical analysis has been performed by means of an unpaired two-tailed Student's *t* test to
11 obtain the *P* value associated with the observed fold of activation differences (*P* < 0,1 and *P* <
12 0,05, respectively).
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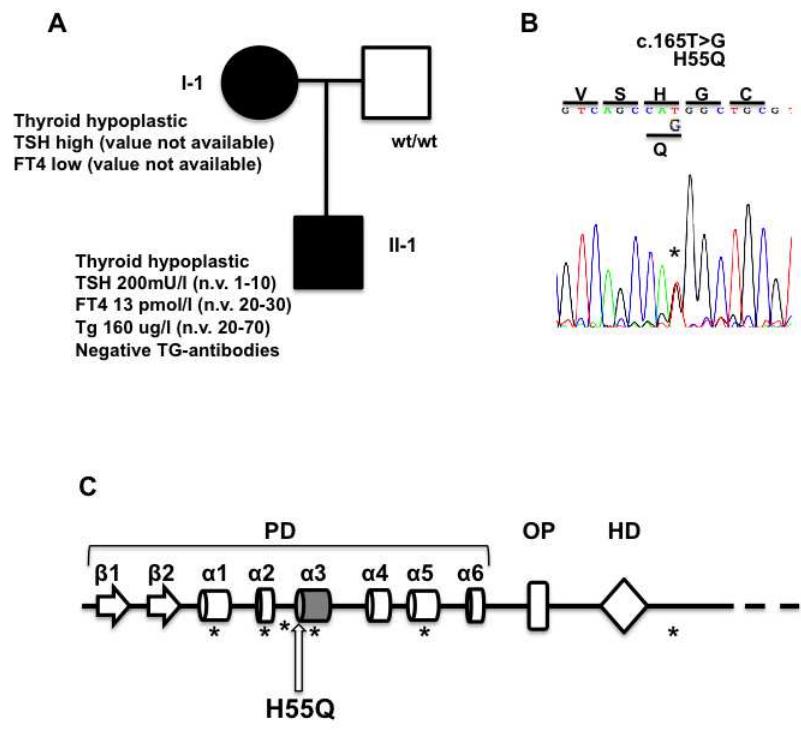


Fig. 1

264x352mm (72 x 72 DPI)

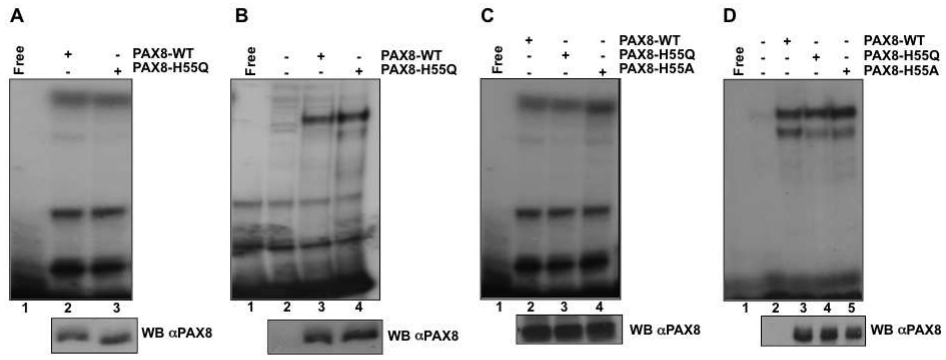


Figure 2

352x264mm (72 x 72 DPI)

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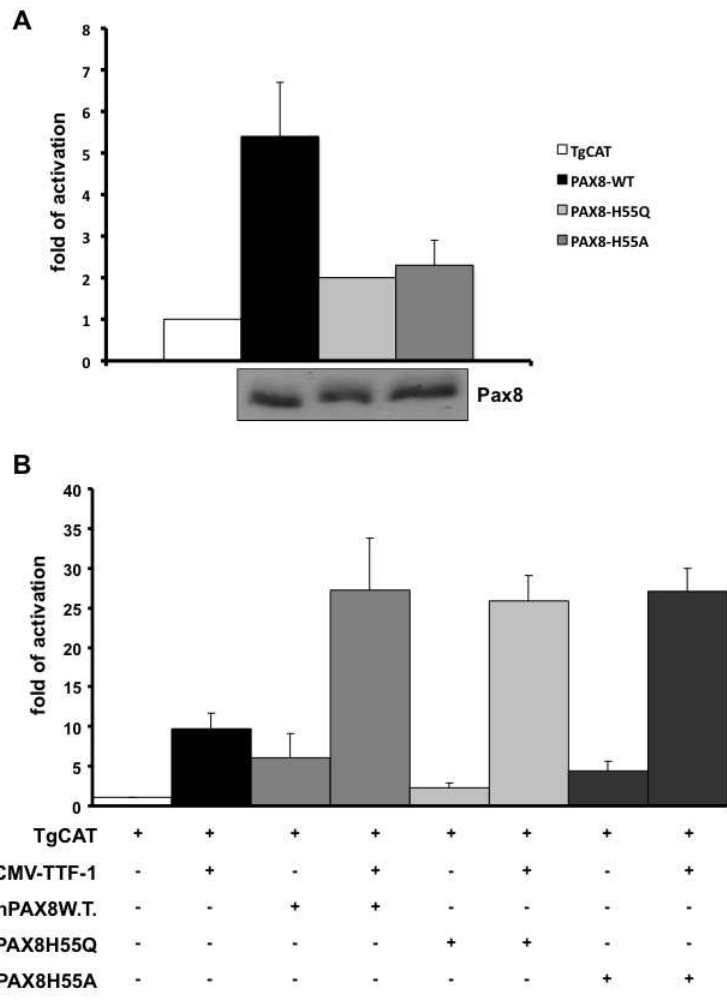


Fig. 3

264x352mm (72 x 72 DPI)