



**HAL**  
open science

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

Tina Di Palma, Emilia Zampella, Maria Grazia Filippone, Paolo Emidio  
Macchia, Carrie Ris-Stalpers, Monique de Vroede, Mariastella Zannini

► **To cite this version:**

Tina Di Palma, Emilia Zampella, Maria Grazia Filippone, Paolo Emidio Macchia, Carrie Ris-Stalpers, et al.. CHARACTERIZATION OF A NOVEL LOSS OF FUNCTION MUTATION OF PAX8 ASSOCIATED WITH CONGENITAL HYPOTHYROIDISM.. *Clinical Endocrinology*, 2010, 73 (6), pp.808. 10.1111/j.1365-2265.2010.03851.x . hal-00593442

**HAL Id: hal-00593442**

**<https://hal.science/hal-00593442>**

Submitted on 16 May 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Journal:	<i>Clinical Endocrinology</i>
Manuscript ID:	CEN-2010-000343.R2
Manuscript Type/Office:	1 Original Article - UK/Europe
Date Submitted by the Author:	13-Jul-2010
Complete List of Authors:	Di Palma, Tina; National Research Council, Institute of Experimental Endocrinology and Oncology "G. Salvatore" Zampella, Emilia; University of Naples Federico II, Dpt. of Molecular and Clinical Endocrinology and Oncology Filippone, Maria Grazia; University of Naples Federico II, Dpt. of Cellular and Molecular Biology and Pathology; National Research Council, Institute of Experimental Endocrinology and Oncology "G. Salvatore" Macchia, Paolo Emidio; University of Naples Federico II, Dpt. of Molecular and Clinical Endocrinology and Oncology Ris-Stalpers, Carrie; AMC, Laboratory of Pediatric Endocrinology de Vroede, Monique; University Medical Center, Division of Pediatric Endocrinology Zannini, Mariastella; National Research Council, Institute of Experimental Endocrinology and Oncology "G. Salvatore"
Key Words:	PAX8, congenital hypothyroidism, Thyroid, transcription factor, mutation

1  
2  
3 **CHARACTERIZATION OF A NOVEL LOSS OF FUNCTION MUTATION OF PAX8**  
4 **ASSOCIATED WITH CONGENITAL HYPOTHYROIDISM**

5 Tina Di Palma<sup>1</sup>, Emilia Zampella<sup>2</sup>, Maria Grazia Filippone<sup>1,3</sup>, Paolo Emidio Macchia<sup>2</sup>, Carrie Ris-  
6 Stalpers<sup>4</sup>, Monique de Vroede<sup>5</sup>, Mariastella Zannini<sup>1</sup>  
7  
8  
9

10  
11 <sup>1</sup>IEOS, Institute of Experimental Endocrinology and Oncology ‘G. Salvatore’ - National Research  
12 Council, Naples, Italy

13 <sup>2</sup>Dpt. of Molecular and Clinical Endocrinology and Oncology - University of Naples Federico II,  
14 Naples, Italy

15 <sup>3</sup>Dpt. of Cellular and Molecular Biology and Pathology – University of Naples Federico II,  
16 Naples, Italy

17 <sup>4</sup>Laboratory of Pediatric Endocrinology - AMC, Amsterdam, The Netherlands

18 <sup>5</sup>Division of Pediatric Endocrinology - University Medical Center, Utrecht, The Netherlands  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28

29 **Key words:** PAX8; congenital hypothyroidism; thyroid; transcription factor; mutation

30 **Word count:** 3005

31 **Short Title:** Novel PAX8 mutation in congenital hypothyroidism  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**Corresponding author:** Dr. Mariastella Zannini, Istituto di Endocrinologia e Oncologia  
Sperimentale “G. Salvatore” del CNR, via S. Pansini 5, 80131 Napoli - Italy; Fax: +39-081-  
2296674; E-mail: [s.zannini@ieos.cnr.it](mailto:s.zannini@ieos.cnr.it)

## 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 levels<sup>1</sup>. 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)<sup>2</sup>. About 5% of the TD cases are associated with mutations in the genes responsible for thyroid development and growth<sup>3</sup>, including genes that encode for TTF-1/NKX2.1<sup>4,5</sup>, FOXE1<sup>6,7</sup>, PAX8<sup>8,9</sup>, TSH receptor<sup>10</sup> and NKX2.5<sup>11</sup>.

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 exons<sup>12</sup>. PAX8 plays an important role in thyroid development, when the thyroid bud evaginates from the floor of the pharynx<sup>13</sup> and it is required both for the morphogenesis of the thyroid gland<sup>9</sup> and for the maintenance of the thyroid differentiated phenotype<sup>14</sup>. In the adult thyroid, PAX8 is essential for the transcriptional activation of the thyroperoxidase (TPO), thyroglobulin (Tg) and sodium/symporter (NIS) genes<sup>15,16</sup>. Recently, it has been demonstrated that PAX8 and TTF-1/NKX2.1 biochemically associate and synergistically activate transcription from the TPO<sup>17</sup> and Tg gene promoters<sup>18,19</sup>. Homozygous Pax8 knockout mice die shortly after weaning, presumably from severe hypothyroidism due to thyroid hypoplasia, whereas heterozygotes were described unaffected<sup>9</sup>.

To date, eleven heterozygous inactivating mutations in the *PAX8* gene have been identified and characterized in patients with TD<sup>8,20,21</sup>. Only two of these mutations, the deletion in exon 7 (c.989-992delACCC) located outside the paired domain<sup>22</sup> and the p.S48F located within the paired domain<sup>23</sup>, show normal DNA binding affinity but are transcriptionally inactive. The remaining eight mutations already described are located within the paired domain of PAX8 and

1  
2  
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.  
5  
6

7  
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.  
14  
15  
16  
17  
18  
19

20 Our results strengthen the notion that PAX8 mutation contributes to the sporadic and familial  
21 cases of CH.  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## 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 T<sub>4</sub> 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 <sup>123</sup>I scintigraphy showed a hypoplastic gland in normal position. Since birth he is receiving substitutive L-T<sub>4</sub> 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 described<sup>8</sup>. 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-1<sup>24</sup>, pCMV5-H29a (also named PAX8-WT)<sup>12</sup>. 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 described<sup>25</sup>.

## 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  $3 \times 10^5$  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 described<sup>26</sup>. 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 described<sup>27</sup>, 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



1  
2  
3 membranes were blocked in 5% nonfat dry milk in Tris-buffered saline for 2 h before the addition  
4  
5 of the anti-PAX8 antibody (kindly provided by R. Di Lauro) for 1 h. The filters were washed  
6  
7 three times in Tris-buffered saline plus 0.5% Tween 20 before the addition of horseradish  
8  
9 peroxidase-conjugated secondary antibody for 45 min. Horseradish peroxidase was detected with  
10  
11 ECL (GE Healthcare, USA).  
12  
13

### 14 15 16 **EMSA**

17  
18 Double-stranded oligonucleotide C derived from the Tg promoter was labeled with [ $\gamma$ -<sup>32</sup>P] ATP  
19  
20 and T<sub>4</sub> polynucleotide kinase and used as probe as previously described<sup>16</sup>. The binding reactions  
21  
22 were incubated 30 min at room temperature in a binding buffer containing 10 mM HEPES (pH  
23  
24 7.9), 10% glycerol, 0.1 mM EDTA, 8 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 0.15 g/ml of poly  
25  
26 (dI-dC). DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel and  
27  
28 visualized by autoradiography.  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## 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 a heterozygous T- to - G transversion in exon 3 (Fig. 1B), resulting in the substitution of a histidine at position 55, within the  $\alpha$ -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 conformation<sup>28</sup> 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

1  
2  
3 contacts with DNA or not. Specifically, if the H55 residue is not involved in contacts with DNA  
4  
5 we expected that the PAX8-H55A mutant did not show any impairment in DNA binding. Indeed,  
6  
7 our results of the EMSA assays clearly indicated that the PAX8-H55A mutant is able to bind to  
8  
9 the PAX8 binding site contained in the oligonucleotide C and shows no significant differences  
10  
11 when compared to wild-type PAX8 and PAX8-H55Q (Fig. 2C, 2D). The same results were  
12  
13 obtained when *in vitro* translated proteins (Fig. 2C) or total extract of HeLa cells transiently  
14  
15 transfected with either the wild-type or the mutated expression vectors (Fig. 2D) were employed  
16  
17 in EMSA assays. Western blotting of wild-type and mutated proteins produced *in vitro* or  
18  
19 transiently expressed in HeLa cells revealed no difference in the synthesis efficiency, indicating  
20  
21 that the amino acid substitution does not cause destabilization of the mutant proteins (Fig. 2C,  
22  
23 2D).  
24  
25  
26  
27  
28

### 29 **Transcriptional activity of PAX8-H55Q mutant**

30  
31 To evaluate the functional relevance of the H55Q mutation, we relied on the ability of PAX8 to  
32  
33 activate transcription from the thyroglobulin (Tg) promoter<sup>16</sup> and we investigated the ability of  
34  
35 the PAX8-H55Q mutant to activate transcription of a reporter gene under the control of the Tg  
36  
37 promoter. HeLa cells were transiently transfected with expression vectors encoding wild-type  
38  
39 PAX8 or PAX8 mutants, together with a reporter construct in which the minimal region of the Tg  
40  
41 promoter is subcloned upstream of the CAT gene (pTACAT3<sup>25</sup>). In agreement with previous  
42  
43 results<sup>16, 18</sup>, wild-type PAX8 is able to stimulate transcription from the Tg promoter up to 7-fold,  
44  
45 whereas PAX8-H55Q or PAX8-H55A show a significantly reduced Tg reporter activation (Fig.  
46  
47 3A). These results led us to the conclusion that the PAX8-H55Q mutant has a significant reduced  
48  
49 ability to activate transcription despite the fact that it has no impairment in DNA binding activity.  
50  
51 Recently, it has been demonstrated that a physical interaction between PAX8 and the  
52  
53 transcription factor TTF-1/NKX2.1 underlies the synergistic effect of both factors on Tg promoter  
54  
55 activation<sup>18</sup>. To test whether the mutant PAX8-H55Q and TTF-1/NKX2.1 could synergistically  
56  
57  
58  
59  
60

1  
2  
3 stimulate transcription from the Tg promoter, we transiently co-transfected in HeLa cells the  
4  
5 reporter construct TgCAT together with the expression vectors encoding wild-type PAX8, PAX8-  
6  
7 H55Q, PAX8-H55A and TTF-1/NKX2.1 separately or in combination. Our data show that  
8  
9 although the activity of the PAX8-H55Q mutant is significantly lower than the activity of wild-  
10  
11 type PAX8, it can still display a robust synergism with TTF-1/NKX2.1 in the transcriptional  
12  
13 activation of the Tg promoter (Fig. 3B). A similar behavior was observed with the PAX8-H55A  
14  
15 mutant (Fig. 3B).  
16  
17

18 These results indicate that the H55Q mutation retains the ability to functionally interact with  
19  
20 TTF-1/NKX2.1.  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## 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 mutation<sup>23</sup> 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<sup>29, 30</sup>, histidine 55 of PAX8 is located in the  $\alpha$ -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* genes<sup>29</sup>. 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 DNA<sup>31</sup>. 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

1  
2  
3 mutation affects only the transactivation property of PAX8. Hence, the H55Q mutation  
4 represents, together with the previously reported S48F mutation<sup>23</sup>, a new type of PAX8 mutations  
5 responsible for congenital hypothyroidism.  
6  
7

8  
9  
10 PAX8 is a major gene in the regulation of the thyroid differentiated phenotype<sup>14</sup> and plays an  
11 important role in the morphogenesis of thyroid gland<sup>9</sup>. To date, all PAX8 gene mutations reported  
12 are located within the paired domain, except for one mutation c.989-992delACCC<sup>22</sup>, located in  
13 exon 7 outside the paired domain that cause a frameshift with a premature stop codon after codon  
14 277. The PAX8/277del protein retains the DNA binding ability but it is not capable of activating  
15 transcription, thus indicating that the C-terminal region is essential for PAX8 transcriptional  
16 activity. Recently, it has been demonstrated that PAX8 activity can be modulated by the  
17 interaction with other proteins, forming complexes on regulatory regions of target genes<sup>18, 27, 32</sup>.  
18  
19 Therefore, our findings suggest that the PAX8-H55Q mutant does not efficiently activate  
20 transcription because it does not allow the recruitment of other factors or coactivators to the target  
21 promoters. Furthermore, it has been demonstrated that PAX8 cooperates with the transcription  
22 factor TTF-1/NKX2.1<sup>17, 19</sup>, and our laboratory specifically demonstrated that PAX8 and TTF-  
23 1/NKX2.1 form a functional heterocomplex responsible for the synergistic transcriptional  
24 activation of the *Tg* promoter in differentiated thyroid cells<sup>18</sup>. The relevance of this interaction has  
25 been further confirmed by the analysis of the *in vivo* mouse model bearing a partial deficiency of  
26 *Ttf1* and *Pax8* genes. The double-hetero-zygous null mice show thyroid dysgenesis, a phenotype  
27 that is completely absent in either of the single heterozygous mice<sup>33</sup>. Our results demonstrate that  
28 the PAX8-H55Q mutant is still able to synergize with TTF-1/NKX2.1 on the *Tg* promoter, as  
29 indicated by the significantly higher stimulation of the reporter gene expression in cells  
30 expressing both PAX8-H55Q and TTF-1/NKX2.1, suggesting that the H55 residue is not  
31 essential for the functional interaction with TTF-1/NKX2.1. Therefore, it will be of interest to  
32 further investigate the effect of this novel mutation with respect to the already described  
33 interactions that PAX8 makes with other partners.  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
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<sup>8, 34</sup>. 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 gland<sup>9</sup>. Haploinsufficiency, monoallelic  
9 expression, imprinting, dominant negative properties<sup>8, 34</sup> and stochastic expression of the PAX  
10 alleles, as noted for *Pax5*<sup>35</sup>, 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.  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24

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.  
28  
29  
30  
31  
32

### 33 **Acknowledgments**

34 We would like to thank Federico Fogolari for very helpful discussions and suggestions. We would  
35 also like to thank the Service of Molecular Biology (SBM) of the Stazione Zoologica A. Dohrn of  
36 Naples for their technical assistance. This work was supported by grants from the Associazione  
37 Italiana per la Ricerca sul Cancro (AIRC) and from the Italian Ministry of Education, University  
38 and Research (MIUR-PRIN 2007) to MZ. The work was also partially supported by a grant of the  
39 Italian Ministry of Education, University and Research (PRIN 2007) to PEM.  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50

### 51 **Competing interests/financial disclosure**

52 The authors have nothing to disclose  
53  
54  
55  
56  
57  
58  
59  
60

## References

- 1 Vassart, G. & Dumont, J.E. (2005) Thyroid dysgenesis: multigenic or epigenetic ... or both? *Endocrinology* 146, 5035-5037.
- 2 Fisher, D.A. & Klein, A.H. (1981) Thyroid development and disorders of thyroid function in the newborn. *N Engl J Med* 304, 702-712.
- 3 De Felice, M. & Di Lauro, R. (2004) Thyroid development and its disorders: genetics and molecular mechanisms. *Endocr Rev* 25, 722-746.
- 4 Ferrara, A.M., De Michele, G., Salvatore, E., Di Maio, L., Zampella, E., Capuano, S., Del Prete, G., Rossi, G., Fenzi, G., Filla, A. & Macchia, P.E. (2008) A novel NKX2.1 mutation in a family with hypothyroidism and benign hereditary chorea. *Thyroid* 18, 1005-1009.
- 5 Moya, C.M., Perez de Nanclares, G., Castano, L., Potau, N., Bilbao, J.R., Carrascosa, A., Bargada, M., Coya, R., Martul, P., Vicens-Calvet, E. & Santisteban, P. (2006) Functional study of a novel single deletion in the TITF1/NKX2.1 homeobox gene that produces congenital hypothyroidism and benign chorea but not pulmonary distress. *J Clin Endocrinol Metab* 91, 1832-1841.
- 6 Baris, I., Arisoy, A.E., Smith, A., Agostini, M., Mitchell, C.S., Park, S.M., Halefoglu, A.M., Zengin, E., Chatterjee, V.K. & Battaloglu, E. (2006) A novel missense mutation in human TTF-2 (FKHL15) gene associated with congenital hypothyroidism but not athyreosis. *J Clin Endocrinol Metab* 91, 4183-4187.
- 7 De Felice, M., Ovitt, C., Biffali, E., Rodriguez-Mallon, A., Arra, C., Anastassiadis, K., Macchia, P.E., Mattei, M.G., Mariano, A., Scholer, H., Macchia, V. & Di Lauro, R. (1998) A mouse model for hereditary thyroid dysgenesis and cleft palate. *Nat Genet* 19, 395-398.
- 8 Macchia, P.E., Lapi, P., Krude, H., Pirro, M.T., Missero, C., Chiovato, L., Souabni, A., Baserga, M., Tassi, V., Pinchera, A., Fenzi, G., Gruters, A., Busslinger, M. & Di Lauro, R. (1998)



1  
2  
3 PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. *Nat*  
4  
5 *Genet* 19, 83-86.

6  
7  
8 9 Mansouri, A., Chowdhury, K. & Gruss, P. (1998) Follicular cells of the thyroid gland  
9  
10 require Pax8 gene function. *Nat Genet* 19, 87-90.

11  
12 10 Corvilain, B., Van Sande, J., Dumont, J.E. & Vassart, G. (2001) Somatic and germline  
13  
14 mutations of the TSH receptor and thyroid diseases. *Clin Endocrinol (Oxf)* 55, 143-158.

15  
16 11 Dentice, M., Cordeddu, V., Rosica, A., Ferrara, A.M., Santarpia, L., Salvatore, D.,  
17  
18 Chiovato, L., Perri, A., Moschini, L., Fazzini, C., Olivieri, A., Costa, P., Stoppioni, V., Baserga,  
19  
20 M., De Felice, M., Sorcini, M., Fenzi, G., Di Lauro, R., Tartaglia, M. & Macchia, P.E. (2006)  
21  
22 Missense mutation in the transcription factor NKX2-5: a novel molecular event in the  
23  
24 pathogenesis of thyroid dysgenesis. *J Clin Endocrinol Metab* 91, 1428-1433.

25  
26 12 Poleev, A., Wendler, F., Fickenscher, H., Zannini, M.S., Yaginuma, K., Abbott, C. &  
27  
28 Plachov, D. (1995) Distinct functional properties of three human paired-box-protein, PAX8,  
29  
30 isoforms generated by alternative splicing in thyroid, kidney and Wilms' tumors. *Eur J Biochem*  
31  
32 228, 899-911.

33  
34 13 Plachov, D., Chowdhury, K., Walther, C., Simon, D., Guenet, J.L. & Gruss, P. (1990)  
35  
36 Pax8, a murine paired box gene expressed in the developing excretory system and thyroid gland.  
37  
38 *Development* 110, 643-651.

39  
40 14 Pasca di Magliano, M., Di Lauro, R. & Zannini, M. (2000) Pax8 has a key role in thyroid  
41  
42 cell differentiation. *Proc Natl Acad Sci U S A* 97, 13144-13149.

43  
44 15 Ohno, M., Zannini, M., Levy, O., Carrasco, N. & di Lauro, R. (1999) The paired-domain  
45  
46 transcription factor Pax8 binds to the upstream enhancer of the rat sodium/iodide symporter gene  
47  
48 and participates in both thyroid-specific and cyclic-AMP-dependent transcription. *Mol Cell Biol*  
49  
50 19, 2051-2060.

51  
52 16 Zannini, M., Francis-Lang, H., Plachov, D. & Di Lauro, R. (1992) Pax-8, a paired  
53  
54 domain-containing protein, binds to a sequence overlapping the recognition site of a  
55  
56  
57  
58  
59  
60

1  
2  
3 homeodomain and activates transcription from two thyroid-specific promoters. *Mol Cell Biol* 12,  
4 4230-4241.

5  
6  
7 17 Miccadei, S., De Leo, R., Zammarchi, E., Natali, P.G. & Civitareale, D. (2002) The  
8 synergistic activity of thyroid transcription factor 1 and Pax 8 relies on the promoter/enhancer  
9 interplay. *Mol Endocrinol* 16, 837-846.

10  
11  
12 18 Di Palma, T., Nitsch, R., Mascia, A., Nitsch, L., Di Lauro, R. & Zannini, M. (2003) The  
13 paired domain-containing factor Pax8 and the homeodomain-containing factor TTF-1 directly  
14 interact and synergistically activate transcription. *J Biol Chem* 278, 3395-3402.

15  
16  
17 19 Espinoza, C.R., Schmitt, T.L. & Loos, U. (2001) Thyroid transcription factor 1 and Pax8  
18 synergistically activate the promoter of the human thyroglobulin gene. *J Mol Endocrinol* 27, 59-  
19 67.

20  
21  
22 20 Al Taji, E., Biebermann, H., Limanova, Z., Hnikova, O., Zikmund, J., Dame, C., Gruters,  
23 A., Lebl, J. & Krude, H. (2007) Screening for mutations in transcription factors in a Czech cohort  
24 of 170 patients with congenital and early-onset hypothyroidism: identification of a novel PAX8  
25 mutation in dominantly inherited early-onset non-autoimmune hypothyroidism. *Eur J Endocrinol*  
26 156, 521-529.

27  
28  
29 21 Narumi, S., Muroya, K., Asakura, Y., Adachi, M. & Hasegawa, T. (2010) Transcription  
30 factor mutations and congenital hypothyroidism: systematic genetic screening of a population-  
31 based cohort of Japanese patients. *J Clin Endocrinol Metab* 95, 1981-1985.

32  
33  
34 22 de Sanctis, L., Corrias, A., Romagnolo, D., Di Palma, T., Biava, A., Borgarello, G.,  
35 Gianino, P., Silvestro, L., Zannini, M. & Dianzani, I. (2004) Familial PAX8 small deletion  
36 (c.989\_992delACCC) associated with extreme phenotype variability. *J Clin Endocrinol Metab*  
37 89, 5669-5674.

38  
39  
40 23 Grasberger, H., Ringkananont, U., Lefrancois, P., Abramowicz, M., Vassart, G. &  
41 Refetoff, S. (2005) Thyroid transcription factor 1 rescues PAX8/p300 synergism impaired by a  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52

1  
2  
3 natural PAX8 paired domain mutation with dominant negative activity. *Mol Endocrinol* 19, 1779-  
4  
5 1791.

6  
7  
8 24 Bohinski, R.J., Di Lauro, R. & Whitsett, J.A. (1994) The lung-specific surfactant protein  
9  
10 B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3,  
11  
12 indicating common factors for organ-specific gene expression along the foregut axis. *Mol Cell*  
13  
14 *Biol* 14, 5671-5681.

15  
16  
17 25 Sinclair, A.J., Lonigro, R., Civitareale, D., Ghibelli, L. & Di Lauro, R. (1990) The tissue-  
18  
19 specific expression of the thyroglobulin gene requires interaction between thyroid-specific and  
20  
21 ubiquitous factors. *Eur J Biochem* 193, 311-318.

22  
23 26 de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R. & Subramani, S. (1987) Firefly  
24  
25 luciferase gene: structure and expression in mammalian cells. *Mol Cell Biol* 7, 725-737.

26  
27 27 Di Palma, T., D'Andrea, B., Liguori, G.L., Liguoro, A., de Cristofaro, T., Del Prete, D.,  
28  
29 Pappalardo, A., Mascia, A. & Zannini, M. (2009) TAZ is a coactivator for Pax8 and TTF-1, two  
30  
31 transcription factors involved in thyroid differentiation. *Exp Cell Res* 315, 162-175.

32  
33 28 Lefevre, F., Remy, M.H. & Masson, J.M. (1997) Alanine-stretch scanning mutagenesis: a  
34  
35 simple and efficient method to probe protein structure and function. *Nucleic Acids Res* 25, 447-  
36  
37 448.

38  
39  
40 29 Xu, H.E., Rould, M.A., Xu, W., Epstein, J.A., Maas, R.L. & Pabo, C.O. (1999) Crystal  
41  
42 structure of the human Pax6 paired domain-DNA complex reveals specific roles for the linker  
43  
44 region and carboxy-terminal subdomain in DNA binding. *Genes Dev* 13, 1263-1275.

45  
46  
47 30 Xu, W., Rould, M.A., Jun, S., Desplan, C. & Pabo, C.O. (1995) Crystal structure of a  
48  
49 paired domain-DNA complex at 2.5 Å resolution reveals structural basis for Pax developmental  
50  
51 mutations. *Cell* 80, 639-650.

52  
53 31 Codutti, L., van Ingen, H., Vascotto, C., Fogolari, F., Corazza, A., Tell, G., Quadrifoglio,  
54  
55 F., Viglino, P., Boelens, R. & Esposito, G. (2008) The solution structure of DNA-free Pax-8  
56  
57  
58  
59  
60

1  
2  
3 paired box domain accounts for redox regulation of transcriptional activity in the pax protein  
4 family. *J Biol Chem* 283, 33321-33328.  
5  
6

7  
8 32 Di Palma, T., de Cristofaro, T., D'Ambrosio, C., Del Prete, D., Scaloni, A. & Zannini, M.  
9 (2008) Poly(ADP-ribose) polymerase 1 binds to Pax8 and inhibits its transcriptional activity. *J*  
10 *Mol Endocrinol* 41, 379-388.  
11  
12

13  
14 33 Amendola, E., De Luca, P., Macchia, P.E., Terracciano, D., Rosica, A., Chiappetta, G.,  
15 Kimura, S., Mansouri, A., Affuso, A., Arra, C., Macchia, V., Di Lauro, R. & De Felice, M.  
16 (2005) A mouse model demonstrates a multigenic origin of congenital hypothyroidism.  
17 *Endocrinology* 146, 5038-5047.  
18  
19

20  
21 34 Vilain, C., Rydlewski, C., Duprez, L., Heinrichs, C., Abramowicz, M., Malvaux, P.,  
22 Renneboog, B., Parma, J., Costagliola, S. & Vassart, G. (2001) Autosomal dominant transmission  
23 of congenital thyroid hypoplasia due to loss-of-function mutation of PAX8. *J Clin Endocrinol*  
24 *Metab* 86, 234-238.  
25  
26  
27  
28  
29

30  
31 35 Nutt, S.L., Vambrie, S., Steinlein, P., Kozmik, Z., Rolink, A., Weith, A. & Busslinger, M.  
32 (1999) Independent regulation of the two Pax5 alleles during B-cell development. *Nat Genet* 21,  
33 390-395.  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

**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  $\alpha$ -helix 3 ( $\alpha$ 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  $\mu$ 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.

1  
2  
3 B, HeLa cells were transiently transfected with the reporter plasmid TgCAT (2  $\mu$ 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.  
9  
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).  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

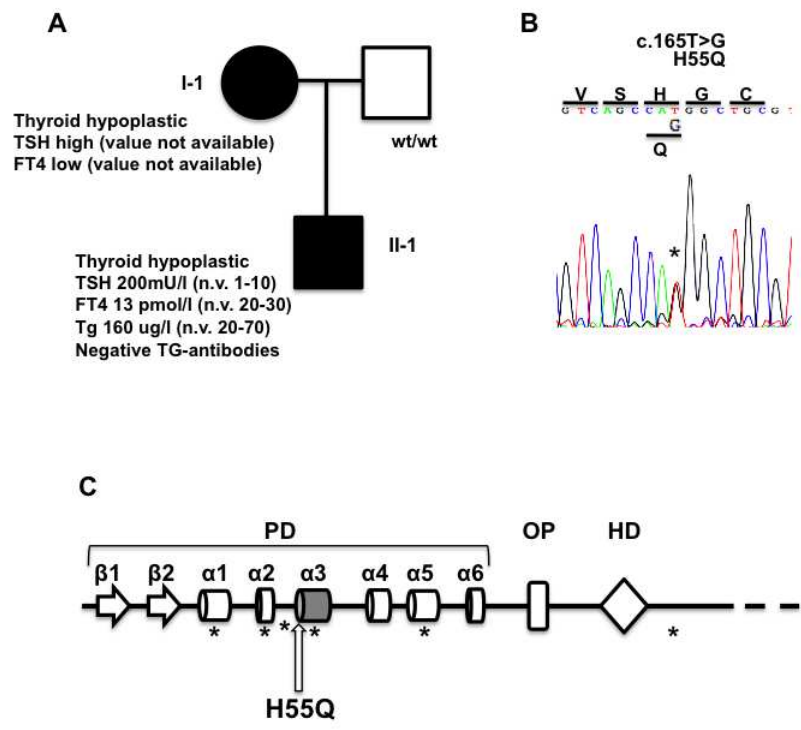


Fig. 1

264x352mm (72 x 72 DPI)

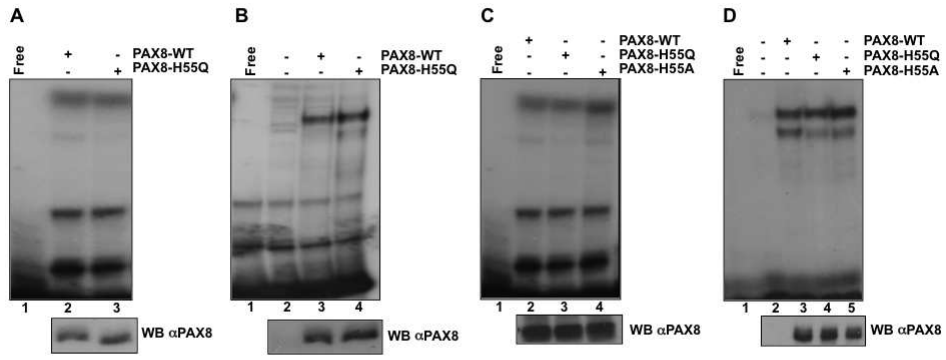


Figure 2

352x264mm (72 x 72 DPI)

Review

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

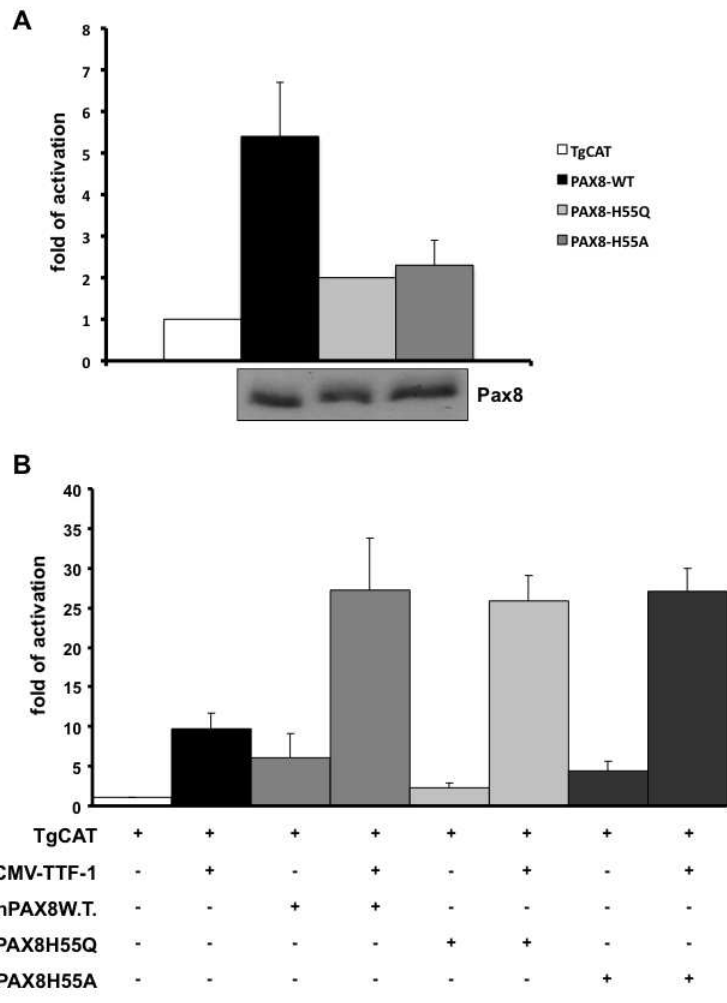


Fig. 3

264x352mm (72 x 72 DPI)