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A Novel *FOXE1* Mutation (R73S) in Bamforth–Lazarus Syndrome Causing Increased Thyroidal Gene Expression

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Background: Homozygous loss-of-function mutations in the *FOXE1* gene have been reported in several patients with partial or complete Bamforth–Lazarus syndrome: congenital hypothyroidism (CH) with thyroid dysgenesis (usually athyreosis), cleft palate, spiky hair, with or without choanal atresia, and bifid epiglottis. Here, our objective was to evaluate potential functional consequences of a *FOXE1* mutation in a patient with a similar clinical phenotype.
Methods: *FOXE1* was sequenced in eight patients with thyroid dysgenesis and cleft palate. Transient transfection was performed in HEK293 cells using the thyroglobulin (TG) and thyroid peroxidase (TPO) promoters in luciferase reporter plasmids to assess the functional impact of the *FOXE1* mutations. Primary human thyrocytes transfected with wild type and mutant *FOXE1* served to assess the impact of the mutation on endogenous TG and TPO expression.
Results: We identified and characterized the function of a new homozygous *FOXE1* missense mutation (p.R73S) in a boy with a typical phenotype (athyreosis, cleft palate, and partial choanal atresia). This new mutation located within the forkhead domain was inherited from the heterozygous healthy consanguineous parents. *In vitro* functional studies in HEK293 cells showed that this mutant gene enhanced the activity of the *TG* and *TPO* gene promoters (1.5-fold and 1.7-fold respectively vs. wild type *FOXE1*; $p < 0.05$), unlike the five mutations previously reported in Bamforth–Lazarus syndrome. The gain-of-function effect of the *FOXE1*-p.R73S mutant gene was confirmed by an increase in endogenous TG production in primary human thyrocytes.
Conclusion: We identified a new homozygous *FOXE1* mutation responsible for enhanced expression of the *TG* and *TPO* genes in a boy whose phenotype is similar to that reported previously in patients with loss-of-function *FOXE1* mutations. This finding further delineates the role for *FOXE1* in both thyroid and palate development, and shows that enhanced gene activity should be considered among the mechanisms underlying Bamforth–Lazarus syndrome.

Introduction

Forkhead box E1/thyroid transcription factor 2 (FOXE1, also known as TTF2 or TITF2) is a forkhead/winged helix-domain containing protein (1). FOXE1 was initially characterized as a thyroid-specific transcription factor that binds to both the thyroglobulin (*TG*) and thyroid peroxidase (*TPO*) gene promoters, two thyroid differentiation markers (2,3). During development, *FOXE1* is expressed in the thyroid and oropharyngeal epithelium of humans and mice, and in the thymus of humans only (4,5). *FOXE1* is also expressed in the adult human thyroid, as well as in the hair follicles and prepubertal testis (6).

Homozygous *Foxe1*-null mice exhibit thyroid agenesis or ectopia and cleft palate (7). In humans, Bamforth–Lazarus syndrome is a rare inherited condition characterized chiefly by congenital hypothyroidism (CH) due to thyroid dysgenesis (usually agenesis), cleft palate, spiky hair, with or without choanal atresia, and bifid epiglottis (OMIM access number 241850). To date, five missense *FOXE1* mutations have been

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reported in patients with Bamforth–Lazarus syndrome (8–12). These mutations were typically inherited from heterozygous carrier parents who were usually consanguineous, although a uniparental isodisomy mechanism was reported recently in a nonconsanguineous family (9). In all reported cases of Bamforth–Lazarus syndrome caused by *FOXE1* mutations, the mutations affected the forkhead domain of the protein, whose activity was decreased or abolished.

Here, we describe the identification and characterization of a new homozygous missense *FOXE1* mutation in a patient born from consanguineous parents and exhibiting features similar to those previously reported in *FOXE1*-mutated patients. In contrast to previously reported mutations, this new mutation results in increased transcriptional activity of the mutant protein on the *TG* and *TPO* promoters. Thus, two different molecular mechanisms, gain and loss of function, are associated with the same phenotype (Bamforth–Lazarus syndrome).

Patients and Methods

The study complied with French law on medical research and was approved by our local ethics committee. The parents gave their written informed consent for the inclusion of their children in the study and for the genetic tests required for the study.

Patients

We sequenced the *FOXE1* gene in eight patients with thyroid dysgenesis (four with athyreosis, one with an ectopic gland, and three with an orthotopic gland) and cleft palate. Two patients had congenital heart defects, and two were born from consanguineous parents.

DNA sequencing and multiplex ligation-dependent probe amplification

Genomic DNA was isolated from whole blood. The entire coding exon of *FOXE1* (NM_004473.3) was amplified by PCR, and DNA sequencing was performed (ABI Prism 377 automatic sequencer; Life Technologies Corporation, Carlsbad, CA; primer sequences and PCR conditions available on request). No mutations were found in the *PAX8* or *NKX2-1* exonic regions. We screened 100 normal individuals for the identified *FOXE1* alterations. Multiplex ligation-dependent probe amplification (MLPA) was performed using the SALSA MLPA KIT P319A1 thyroid kit (MRC-Holland, Amsterdam, The Netherlands) as previously described (13).

Functional characterization of the new FOXE1 *mutation*

For the functional analysis, the p.R73S mutant identified in one of the eight patients was generated from a wild type *FOXE1* cDNA template as described previously, using the Stratagene Quikchange® kit (Agilent Technologies, Santa Clara, CA) (14). Transient co-transfection assays with wild type and p.R73S mutant *FOXE1* were performed as described previously (14), using the two natural human *TPO* and *TG* gene promoters upstream of the luciferase gene (kindly provided by S. Refetoff and G. Vassart) in nonthyroid cells (HEK293, ATCC, CRL-1573). Cells were plated at 2×10^5/well in a 24-well plate 24 h before transfection. We used lipofectamine to co-transfect cells transiently with 520 ng of reporter plasmid containing *TG* or *TPO*, 260 ng of pFlag, or wild type or mutated *FOXE1*. We chose to co-transfect *FOXE1* with *PAX8* and *NKX2-1* because interactions between these transcription factors have been demonstrated (15), and their simultaneous expression is unique to thyroid follicular cells (5). Normalization of transfection efficiency was done using 150 ng of plasmid Bosβ-galactosidase [kindly provided by K.C. Chatterjee (11)]. After 48 h, cells were harvested, and luciferase and β-galactosidase assays were performed. The Mann–Whitney *U* test was used to evaluate differences in transcriptional activities. Statistical analyses were performed using Statview v5.0 statistical package (SAS Institute, Cary, NC). Bar graphs represent mean ± standard error of the mean (SEM) of four independent experiments in triplicate, as indicated in the figure legends. Western blot was performed as previously described (14).

Normal human thyroid tissue specimens were collected at the Institut Gustave Roussy, Villejuif, France, in accordance with local and national ethical requirements. Informed consent was obtained from all donors. Primary human thyrocytes cultured as previously described (16) were transfected using XtremeGENE HP DNA, as recommended by the manufacturer (Roche Diagnostics, Meylan, France).

EMSA was performed as previously described (17) using biotinylated labeled TG (5′-GAG GGA GCT CCT TTT GAC CAG CAG AGA AAA CAG GAT GGG GCA C-3′) and TPO 5′-TGT TCC CAA CAG TAA CTC ATC ATT GAG CCA TGG AAT CC-3′) probes derived from the corresponding human promoters, as recommended by the manufacturer. For competition incubations, a 100-fold excess of cold probe was used. An unrelated protein (EBNA; Pierce Antibodies, Rockford, IL) was used to establish the specificity of the *TPO* and *TG* DNA sequences (data not shown).

Results

Among our eight patients with thyroid dysgenesis and cleft palate, one had a previously unreported *FOXE1* mutation, c.217C > A, p.R73S. Importantly, MLPA revealed no instances of *FOXE1* deletion in our cohort.

Patient having the new mutation

The patient with the new mutation was a boy born at full term, after an uneventful pregnancy, to first cousins of Egyptian origin. At birth, he was noted to have partial choanal atresia on the right side and cleft palate, which was repaired at the age of two months. At one month of age, excessive sleepiness and poor feeding were noted. Serum total thyroxine (T4) was undetectable (< 0.02 ng/dL), and thyrotropin (TSH) was markedly elevated (> 100 μU/mL). Thyroid ultrasound findings were consistent with athyreosis, and thyroid scintigraphy showed no tracer uptake along the normal thyroid migration trajectory. Thyroxine therapy was started. Both parents and the patient's brother were euthyroid with normal thyroid gland morphology and no birth defects detected by otorhinolaryngological investigations.

FOXE1 *gene sequencing*

Sequencing of *FOXE1* amplified from the patient's DNA showed a biallelic, single-nucleotide substitution (c.217C > A) responsible for an arginine-to-serine change at codon 73 (p.R73S, numbering relative to NP_004464) within the forkhead

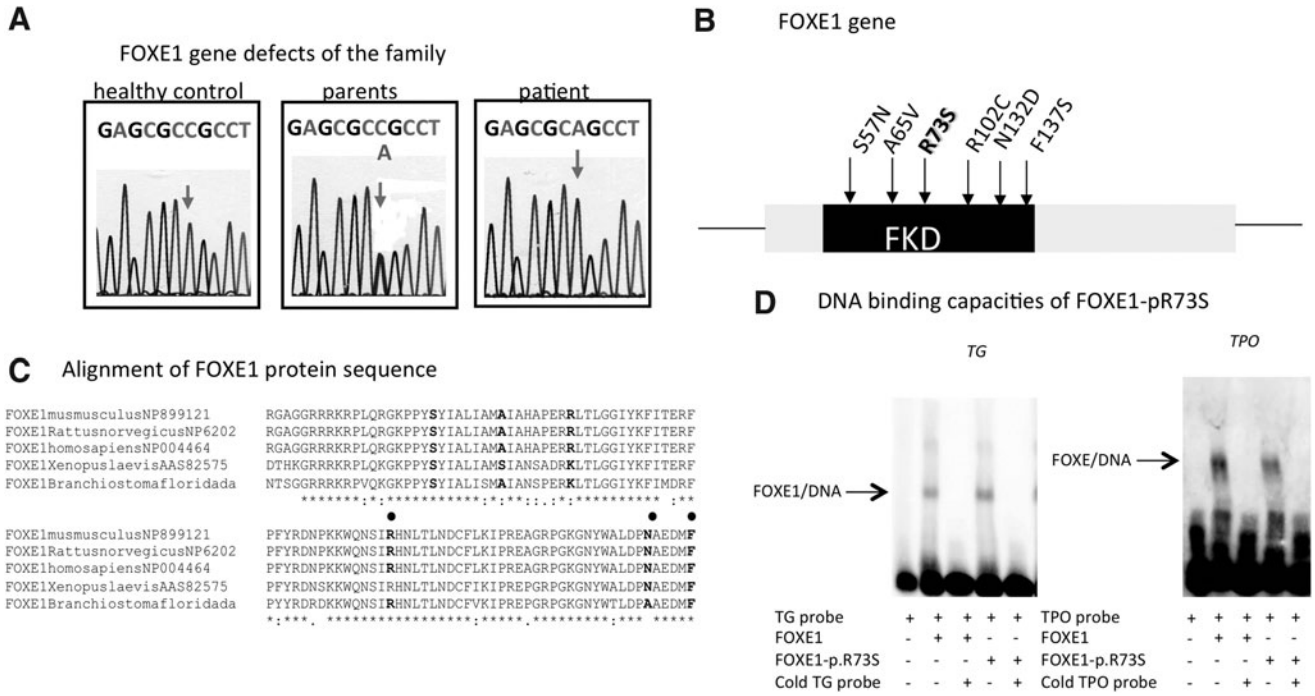

FIG. 1. Identification and analysis of *FOXE1* mutations. **(A)** *FOXE1* gene defects: chromatograms showing the heterozygous or homozygous *FOXE1* mutations. **(B)** *FOXE1* gene: position of the mutations identified in previous studies and in our cohort. **(C)** Alignment of the forkhead domain (FKD) sequence of human *FOXE1* with sequences from other species, indicating marked conservation (arrow) of the different mutations (spot). **(D)** DNA-binding capacities of *FOXE1*-p.R73S to the human *TG* and *TPO* promoters; 2 μg of nuclear extract from HEK293 cells transfected with wild type or mutated *FOXE1* were incubated with biotinylated *TG* and *TPO* probes with (+) or without (−) a competitor (cold probe).

DNA binding domain of the predicted protein sequence (Fig. 1A and B). Both consanguineous parents were heterozygous for this *FOXE1* mutation, suggesting autosomal recessive inheritance. DNA from the unaffected sibling was not available for analysis. Alignment of amino acid sequences within forkhead domains of a number of proteins indicated marked cross-species conservation of the residue corresponding to arginine 73 in FOXE1 (Fig. 1C), suggesting functional significance of this amino acid.

Functional characterization of the FOXE1*-p.R73S mutant*

EMSA showed no difference in binding to the *TG* and *TPO* promoters between wild type *FOXE1* and *FOXE1*-pR73S (Fig. 1D).

FOXE1-p.R73S mutant co-transfection in HEK293 cells resulted in enhanced transcriptional activity of both the *TG* and the *TPO* promoters compared to the wild type expression vector (1.7-fold with the *TG* promoter and 1.5-fold with the *TPO* promoter, $p < 0.05$; Fig. 2A and B). As previously described, the transcriptional activity of wild type *FOXE1* was significantly higher in the thyroidal context (i.e., using human *PAX8*- and *NKX2-1*-expressing vectors) than when co-transfected alone. This transcriptional activity was markedly increased with mutant *FOXE1* (2.2-fold compared to the wild type gene, for both the *TG* and the *TPO* promoters, $p < 0.05$). Western blot confirmed that the amounts of wild type FOXE1 and mutated FOXE1 proteins were identical when the genes were transfected alone or with *PAX8* and *NKX2-1* in HEK293 cells (Fig. 2C). Moreover, *FOXE*-p.R73S transfection in primary human thyrocytes confirmed the increase in endogenous *TG* transcriptional activity in the thyroid cells (Fig. 3), whereas *TPO* transcriptional activity was not increased (data not shown). In primary human thyrocytes, the mutant FOXE1 protein was expressed at a higher level than the wild type FOXE1 protein after transfection with the same amounts of plasmid. These results were found in five independent experiments.

Discussion

We have identified the first *FOXE1* mutation leading to increased activity associated with CH and cleft palate. Interestingly, this homozygous missense gain-of-function mutation resulted in the same phenotype as previously described loss-of-function *FOXE1* mutations, suggesting that both mechanisms can induce CH.

All the *FOXE1* mutations identified so far produce proteins with decreased activity. In contrast, the new mutation identified here in a patient with Bamforth–Lazarus syndrome enhanced transcriptional activity as studied in a similar cellular context to that used previously (8–11). Thus, functional studies showed a marked increase in mutant FOXE1 protein activity in a nonthyroidal context, a simulated thyroidal context (with *NKX2-1* and *PAX8*), and a true thyroidal context (primary human thyrocytes). In HEK293 cells, mutant FOXE1 resulted in overactivation of the *TG* and *TPO* promoters. Moreover, in primary human thyrocytes,

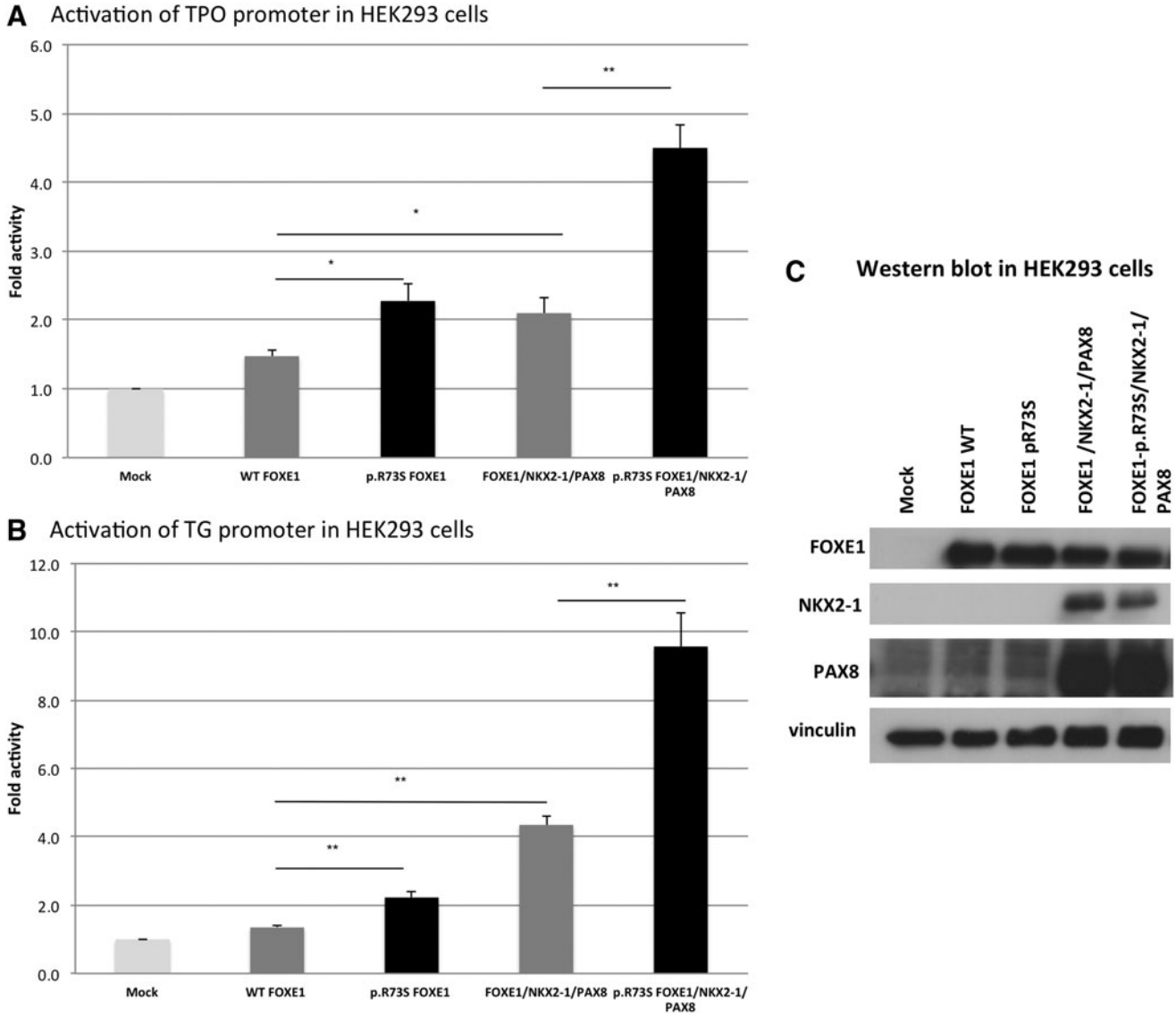

FIG. 2. Functional activity of wild type (WT) versus mutant *FOXE1* on *TPO* **(A)** and *TG* **(B)** luciferase reporter constructs. Promoter constructs were co-transfected in HEK293 cells with the empty pFlag vector or the expression vector for WT or mutated *FOXE1*. Effects on transcriptional activity of mutant *FOXE1* co-expressed with *PAX8* and *NKX2-1*. Luciferase activities are expressed as the fold increase relative to the activity obtained with the empty vector and assigned a value of 1. Results are the mean ± standard error of the mean of four independent experiments performed in triplicate. $*p < 0.05$, $**p < 0.01$ (Mann–Whitney *U* test). **(C)** Expression of FOXE1 WT or mutant proteins when transfected alone or with PAX8 and NKX2-1 proteins in HEK293 cells, as assessed by Western blot. pFlag was the empty vector.

mutant *FOXE1* was overexpressed and resulted in *TG* promoter overactivation. However, we were unable to identify the exact mechanism responsible for overactivation. Increased binding is one possibility, but EMSA may not be well suited to demonstrate it (data not shown). We can only speculate that FOXE1 DNA interaction become more efficient in TG transactivation.

In the primary thyrocytes, a FOXE1-pR73S induced activity increase was shown but not in the HEK293 cells. We can hypothesized different interactions with FOXE1 could be modifying the FOXE1 expression by stabilization in primary thyrocytes and thus make it resistant to proteasomal degradation.

Moreover, the FOXE1 gain of function due to the mutation could be leading to the thyroid bud degeneration by apoptosis. In fact, the link between Pax8 and apoptosis was clearly shown. However, this link between FOXE1 and apoptosis remains speculative (18–20).

Cleft palate has been reported in both *Foxe1* null mice and mice with *Foxe1* overexpression (7,21). Unfortunately, the thyroid phenotype of these mice was either not studied or not reported (personal communication from the authors) (21). Nevertheless, the findings in this mouse model support our observation of impaired palate and thyroid development in a patient with a *FOXE1* gain-of-function mutation.

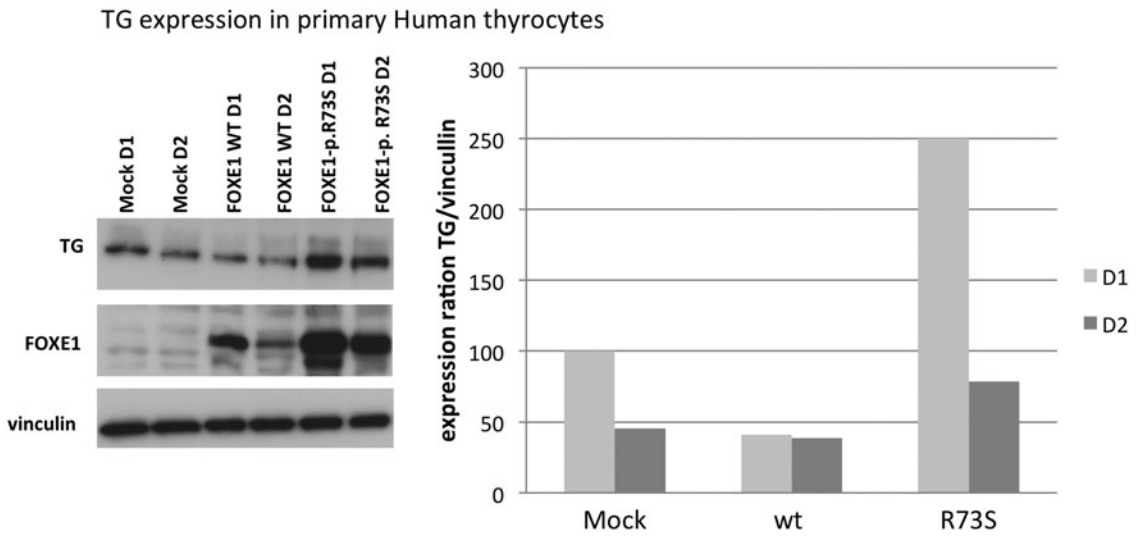

FIG. 3. Expression of FOXE1 WT or mutant proteins, TG, and vinculin in primary human thyrocytes after one (D1) or two days (D2) of transfection, as assessed by Western blot. Expression ratios are shown in graphical form. These results were representative of five experiments in primary human thyrocytes.

In several other diseases, transcription factor mutations resulting in loss or gain of function have been shown to produce similar phenotypes. Missense mutations in the human T Box transcription factor gene (*TBX1*) gene have been identified in patients exhibiting the same phenotype as those with 22q11.2 deletion (22). Zweier *et al.* hypothesized that TBX1 missense mutations may alter the transcriptional activity of the TBX1 protein, possibly through stabilization of the protein–protein or protein–DNA interaction than that of increased 22q11.2 dosage (22). Moreover, in mice, both over- and underexpression of *Tbx1* resulted in a phenotype similar to that in humans (23). *HESX1* mutations were usually identified as leading to mutant proteins with decreased activity, but mutations that enhance DNA binding resulting in increasing interaction with *PROP1* have been also found in patients harboring congenital pituitary disorder (24). In other species, Armstrong *et al.*, working on amyotrophic lateral sclerosis, submitted results which implicate both a gain of toxic function following expression of mutant (but not wild type) human fused in sarcoma (FUS) and a loss of function following antisense morpholino knockdown of zebrafish Fus generate locomotor impairment by reducing presynaptic function at the neuromuscular junctions (25). Moreover, another example of such a gain- or loss-of-function was the Triplo-lethal locus (Tpl) of *Drosophila melanogaster*, which causes lethality when it is present in either three copies or one copy (26,27).

In conclusion, we report the sixth missense *FOXE1* mutation and provide first evidence that a *FOXE1* gain-of-function mutation can result in the same phenotype as that caused in humans by *FOXE1* loss-of-function mutations. Enhanced transcriptional activity should be considered among the mechanisms capable of producing thyroid and other developmental abnormalities.

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Author Disclosure Statement

The authors declare that there is no conflict of interest that could be perceived as jeopardizing the impartiality of the reported research.

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