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Clinical Research Article

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# Identification and Analysis of a Novel NR0B1 Mutation in Late-Onset Adrenal Hypoplasia Congenita and Hypogonadism

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**Abbreviations:** ACTH, adrenocorticotropic hormone; AHC, adrenal hypoplasia congenita; DHEA-S, dehydroepiandrosterone sulfate; FSH, follicle-stimulating hormone; HHG, hypogonadotropic hypogonadism; LBD, ligand-binding domain; LH, luteinizing hormone; NR0B1/DAX1, nuclear receptor subfamily 0 group B member 1/dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on X chromosome, gene 1; PCR, polymerase chain reaction; SF-1, steroidogenic factor-1; STAR, steroidogenic acute regulatory protein.

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## Abstract

**Objective:** X-linked adrenal hypoplasia congenita (AHC) is a rare disorder characterized by primary adrenal insufficiency and hypogonadotropic hypogonadism (HHG) caused by mutations of the *NR0B1/DAX1* gene. We aimed to search for the presence of any *NR0B1/DAX1* gene mutations in a referred patient and to further characterize the phenotypes of the identified mutation.

**Case Presentation:** Herein, we report a Japanese patient with a novel missense mutation of the *NR0B1/DAX1* gene resulting in adult-onset AHC and HHG. The patient was referred with diffuse skin pigmentation at 28 years of age, presented partial adrenal insufficiency and had undiagnosed incomplete HHG. Urological examination revealed severe oligospermia and testicular microlithiasis.

**Results:** The *NR0B1/DAX1* gene mutation was identified by exome sequencing as a novel missense mutation (c.884A>T, p.Leu295His). We conducted in silico modeling of this mutant NR0B1/DAX1 protein (p.Leu295His) which affected the conserved hydrophobic core of the putative ligand-binding domain (LBD). In addition, functional analysis revealed

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that this mutant showed a decreased ability as a transcriptional repressor to suppress target genes, such as *STAR* and *LHB*. Furthermore, this mutant showed functionally impaired repression of steroidogenesis in human adrenocortical H295R cells.

**Conclusions:** We identified a novel missense mutation of the *NR0B1/DAX1* gene in a patient suffering from late-onset AHC and HHG, who presented with oligospermia and testicular microlithiasis. This mutant NR0B1/DAX1 protein was found to have reduced repressor activity, according to in vitro studies, clinically consistent with the patient's phenotypic features.

**Key Words:** adrenal hypoplasia congenita, hypogonadotropic hypogonadism, NR0B1, testicular microlithiasis, cortisol, steroidogenic acute regulatory protein (STAR)

NR0B1/DAX1 (nuclear receptor subfamily 0 group B member 1/dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on X chromosome, gene 1) is a nuclear receptor encoded by the gene *NR0B1*, located on the short arm of the X chromosome (Xp21). NR0B1/DAX1 plays a pivotal role in the development and function of the adrenal and reproductive axes. Loss of NR0B1/DAX1's inhibitory property due to *NR0B1* mutations was demonstrated to be responsible for the pathology of X-linked adrenal hypoplasia congenita (AHC) and hypogonadotropic hypogonadism (HHG) [1-3]. Recently, numerous *NR0B1/DAX1* gene mutations have been identified in several men and women suffering from insufficient adrenal function and nontypical reproductive phenotypes [4-10]. The degree and onset of adrenal insufficiency and HHG are variable and may be concordant with the identified mutations [10, 11]. Therefore, the identification of *NR0B1/DAX1* gene mutations and characterization of the associated clinical features are important.

Herein, we report the clinical features of a 28-year-old Japanese man diagnosed with adrenal insufficiency who harbored a novel missense mutation of *NR0B1/DAX1* gene, resulting in an adult-onset phenotype. This patient presented with oligospermia and testicular microlithiasis on urological examination.

In addition, we examined the functional properties of the mutant *NR0B1/DAX1* found in this patient. NR0B1/DAX1 predominantly represses steroid biosynthesis by inhibiting the transcription of steroidogenic factor-1 (NR5A1/SF1)-mediated *STAR* (steroidogenic acute regulatory protein), which is a master regulator in the steroid biosynthetic pathway [2]. NR0B1/DAX1 also inhibits luteinizing hormone (LH) β subunit transcription activities, thereby reducing the expression of gonadotropin-releasing hormone [12, 13]. In both the STAR promotor assay and gene expression analysis, this mutant NR0B1/DAX1 (p.Leu295His) showed impaired repression of both steroidogenesis and gonadotropin release.

This is the first study, to our knowledge, focusing on the functional analysis of a novel missense *NR0B1/DAX1* mutation (c.884A>T), identified in a Japanese patient with late-onset AHC and HHG.

## Materials and Methods

### Ethics Statement/Clinical Data

Informed written consent was obtained from the patient. This study was approved by the ethics committee of Iwate Medical University (COA no. HG2018-521). All clinical investigations were conducted according to the principles of the Declaration of Helsinki.

### DNA Extraction and Sequencing of NR0B1/DAX1 Gene

Genomic cDNA was extracted from peripheral blood leukocytes using DNAzol BD Reagent (Thermo Fisher scientific). Exons 1 and 2 of *NR0B1/DAX1* were polymerase chain reaction (PCR)-amplified with specific primers (Supplementary Table 1) [14]. The sequencing results were analyzed by ABI PRISM 3100 Genetic analyzer (Applied Biosystems) and compared with the published DAX1 sequence (accession no. NM_000475).

### Homology Modeling

Models of wild-type and p.Leu259His NR0B1/DAX1 were generated by the SWISS modeling server [15] on the basis of the crystal structure of murine NR0B1/DAX1 [16], with amino acid sequence identity of ~69%. Then, the models were energy-minimized using the minimization routines included in the UCSF Chimera program [17].

### Plasmid Construction and Site-Directed Mutagenesis

Human full-length *NR0B1/DAX1* (Origene), *NR5A1/SF1* (GenScript) and *EGR1* (Addgene) plasmid vectors were commercially obtained. DAX1 cDNA containing the L295H

mutation was created by mutagenesis kit (Toyobo, Osaka, Japan) with the following pairs of primers containing the appropriate nucleotide substitutions (CTC to CAC). 5′-CACATGCTTGAGCTGGCCCAGGACCGCT -3′, 5′-CAGGGACGCCCAGCAGTTGCGCACC -3′. The accuracy of the constructions was confirmed by direct sequencing.

### Cell Culture

Adrenocortical NCI-H295R cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 supplemented with 2.5% Nu-Serum, 1% penicillin-streptomycin, 1% ITES-G and 2.5% fetal bovine serum (FBS) (charcoal stripped). HEK293 cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin. The cells were cultured at 37 °C in 5% $CO_2$. The culture medium was replaced every 2 to 3 days and cells were digested with trypsin for subculturing. For steroidogenesis assays, forskolin (10 μM) was added in the medium. Cortisol concentration in the media was measured with a Cortisol ELISA Kit (Abcam).

### The Dual-Reporter Luciferase Assay and Transient Transfection

STAR promotor sequences (–1501 to –17) from human genomic DNA were cloned into the dual-reporter pEZX-PG04 vector (GeneCopoeia), encoding Gaussia luciferase (GLuc) and secreted alkaline phosphatase (SEAP) genes. H295R and HEK293 cells were transiently co-transfected with 50 ng wild-type or mutant *NR0B1/DAX1* vector or empty vector with 20 ng *NR5A1/SF1* vector and 30 ng pEZX-StAR-GLuc vector using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

### RNA Isolation, cDNA Preparation, and RT-PCR

The total RNA was isolated using the RNeasy Mini Kit (Qiagen) and then 500 ng of total RNA were reverse-transcribed into cDNA employing an iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed using SsoFast EvaGreen Supermix (Bio-Rad). The expression quantity of a particular gene was normalized by *TBP* or *HPRT1*. Primer sequences used for qPCR are provided in Supplementary Table 1 [14]. The RT-PCR of *LHB* was attempted with 3 reverse primers to obtain the whole PCR products [18].

### Western Blotting

Total cell lysates were isolated and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Antibodies for NR0B1/DAX1 (Abcam, RRID:AB_2857966) [19] and α-Tubulin (Abcam, RRID:AB_880625) [20] were used for Western blotting.

### Statistical Analyses

All the data are presented as means ± standard error of the mean (SEM). Statistical significance was defined as $P < 0.05$ and determined by an unpaired Student *t* test or 1-way ANOVA for all data obtained in this study. When the data showed a nonnormal distribution, the Mann-Whitney U test was used to compare differences between groups.

## Results

### Case Report

A 28-year-old man was referred with suspected adrenocortical failure. In the past, he had occasionally suffered from general fatigue and sustained fevers of unknown origin. Physical examination revealed diffuse skin pigmentation on the lips and gingiva as well as under the fingernails (Fig. 1A). No additional symptoms, such as episodes of nausea, abdominal pain, orthostatic dizziness, or weight loss, were noted. His height and weight were 190.5 cm and 71.3 kg, respectively, and his blood pressure was 112/62 mmHg with no medication.

His elder brother had been diagnosed with Addison's disease and had died after a norovirus infection, likely associated with adrenal insufficiency, at 30 years of age (Fig. 1B). The patient's mother was not available for genetic testing for personal reasons.

### Endocrinological Investigation and Treatment

As shown in Table 1, clinical laboratory investigations revealed normal levels of cortisol, testosterone, and LH. In contrast, he had a low serum level of dehydroepiandrosterone sulfate (DHEA-S), while plasma adrenocorticotropic hormone (ACTH) was elevated, findings consistent with primary adrenal failure. No remarkable adrenal change was observed on an abdominal computed tomography scan. While basal levels of cortisol and aldosterone remained normal, no response to ACTH was observed on the ACTH stimulation test (Fig. 1C). These clinical laboratory investigation results were compatible with adrenal insufficiency under stressed conditions. Based on these results, the patient was started on a course of hydrocortisone 20 mg, which improved his general condition and reduced the basal level of plasma ACTH.

## Urological Investigation

Puberty had started at the age of 13, with spontaneous virilization, growth spurt, and testicular growth. Urological examination showed normal penile length (7.8 cm). An ultrasound examination of his testes revealed mild-to-moderate reduction in size (right: 3.0 × 1.1 cm; left: 2.9 × 1.3 cm) and volume (right: 7.6 mL; left: 8.4 mL) with bilateral segmental testicular microlithiasis (Fig. 1D), which may be indicative of degeneration of the testicular parenchyma [21]. While he claimed to have a normal libido as well as sexual function, semen analysis revealed oligospermia with a decreased mobility rate (Table 1).

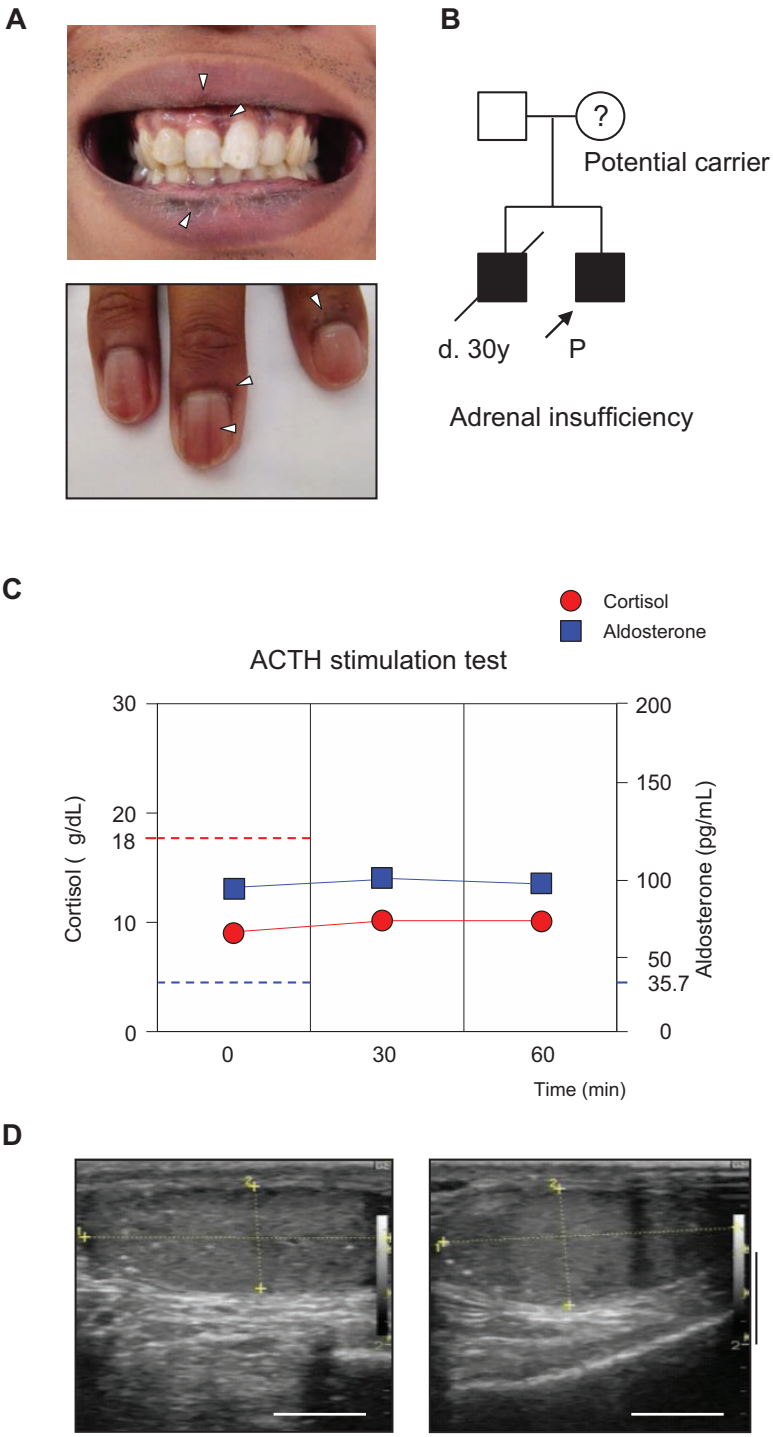


**Figure 1. Clinical features of a patient with adrenal insufficiency and hypogonadotropic hypogonadism.** (A) Skin pigmentation on the lips and gingiva (upper image) and under the fingernails (lower image). (B) Pedigree of a Japanese family with X-linked adrenal hypoplasia congenita with a mutation in the *NR0B1/DAX1* gene. (C) ACTH stimulation tests. Plasma cortisol and aldosterone levels before and after intravenous injection of ACTH (250 μg). (D) Ultrasonographic image of testicular microlithiasis (Left: left testis, Right: right testis). Scale bars, 1 cm.

**Table 1.** Summary of Endocrinological and Urological Results

| | Component | Result | Reference range |
|---|---|---|---|
| Plasma | ACTH 8am (pg/mL) | 539 | 7.2–63.3 |
| | Cortisol 8am (μg/dL) | 8.38 | 0.24-18 |
| | Testosterone (ng/mL) | 5.57 | 1.31-8.71 |
| | DHEA-S (μg/dL) | 16 | 85-690 (age 21-30) |
| | LH (mIU/ml) | 5.3 | 0.79-5.72 |
| | FSH (mIU/ml) | 13.1 | 2.0-8.3 |
| | Estradiol E2 | 11.5 | 14.6-48.8 |
| | Plasma aldosterone concentration (pg/mL) | 104 | 35.7-240 |
| | Plasma renin activity | 5.1 | 0.3-5.4 |
| | TSH (μIU/mL) | 1.19 | 0.5-5 |
| | Free T4 (ng/dL) | 1.5 | 0.9-1.7 |
| | Free T3 (pg/mL) | 3.51 | 2.3-4 |
| | Adrenal cortex autoantibody | <10 | <10 |
| | T-SPOT | Negative | Negative |
| Urine | Cortisol (μg/day) | 28.6 | 11.2-80.3 |
| Semen | Volume (mL) | 2.3 | >1.5 |
| | pH | 8.6 | >7.2 |
| | Concentration ($10^6$/mL) | <2.0 | >15.0 |
| | Count | <2.0 | >40 million |
| | Motility (%) | 0 | >40 |

Abbreviations: ACTH, adrenocorticotropic hormone; DHEA-S, dehydroepiandrosterone sulfate; LH, luteinizing hormone; FSH, follicle-stimulating hormone; TSH, thyroid stimulating hormone; T-SPOT, T-SPOT tuberculosis test.

Furthermore, elevated serum level of follicle-stimulating hormone (FSH) reflected the impairment of spermatogenesis [22].

## Identification of a Novel Missense NR0B1/DAX1 Mutation by Exome Sequencing

After obtaining written informed consent from the patient, we performed direct sequencing. Whole exome sequencing of the *NR0B1* gene revealed 1 missense mutation, ie, c.884T>A (p.Leu295His) (Fig. 2A). This missense mutation was located in the C-terminus amino acids (253-470) of NR0B1, which contains the ligand-binding domain (LBD) of the nuclear receptor super family [23] (Fig. 2B) (UniProt database https://www.uniprot.org/). This variant has not previously been reported and is thus not in the Human Genome Mutation Database (http://www.hgmd.cf.ac.uk) or genome variant databases such as ClinVar (NCBI), the International Genome Sample Resource (IGSR), and the Genome Aggregation Database (https://gnomad.broadinstitute.org/). Therefore, this variant represents a novel mutation, to the best of our knowledge.

Furthermore, in silico analysis by Mutation Taster (http://www.mutationtaster.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) predicted p.L295H to be a disease-causing mutation and probably damaging with a score of 1.0, respectively (Fig. 2C and Fig. S1) [14]. Alignment analysis of this residue revealed Leu295 to be highly conserved among human, monkey, rat, mouse, chicken, and frog genomes (Fig. 2D). Taken together, these results indicate Leu295 to be a highly conserved residue and suggest that the mutant p.Leu295His NR0B1 could have exerted pathogenic effects in this patient.

## Structural Analysis of p.Leu295His Mutant in NR0B1/DAX1

Most of the reported missense mutations in NR0B1 gene were clustered in the putative LBD, which is predicted to bind to other nuclear hormone receptors [5, 16]. Based on our model structure of NR0B1/DAX1, the Leu295 is positioned at the center of the LBD and is far (≥20 Å) from the co-repressor interaction surface. This residue is almost completely buried inside the protein product and participates in forming the hydrophobic core (Fig. 3A). The main-chain structure of p.Leu295His is essentially identical to that of wild-type NR0B1/DAX1 (Fig. 3B). However, the mutated histidine at residue 295 affects the side chain orientations of some surrounding residues in the hydrophobic core due to the change in its size and less hydrophobic character.

## p.Leu295His NR0B1/DAX1 Mutant Showed Impaired Suppression of NR5A1/SF1-Mediated Steroidogenesis

It is well known that NR0B1/DAX1 is a negative regulator of NR5A1/SF1-mediated transactivation of steroid biosynthetic genes [24, 25]. Therefore, to investigate whether the p.Leu295His NR0B1 mutant impaired this repressor function, a STAR-luciferase assay was performed in H295R cells and HEK293 cells (Fig. 3C and Fig. S2) [14]. Transfection of wild-type and mutant *NR0B1* revealed NR0B1/DAX1 protein expression was equivalent, confirmed by N-terminal NR0B1/DAX1 antibody (Fig. 3D and Fig. S3) [14]. Of note, HEK293 cells presumably lack endogenous NR0B1/DAX1 expression. As shown in Fig. 3E, transfection of NR5A1/SF1 significantly activated STAR promotor activity. While wild-type NR0B1/DAX1 significantly suppressed NR5A1/SF1-mediated STAR promotor activity, ie, by 63.2%, the p.Leu295His mutant showed significantly impaired repressor function, suppressing NR5A1/SF1-mediated STAR promotor activities by 48.4% in H295R cells (Fig. 3E). Since *STAR* is a key gene in steroid hormone synthesis [2, 26], we

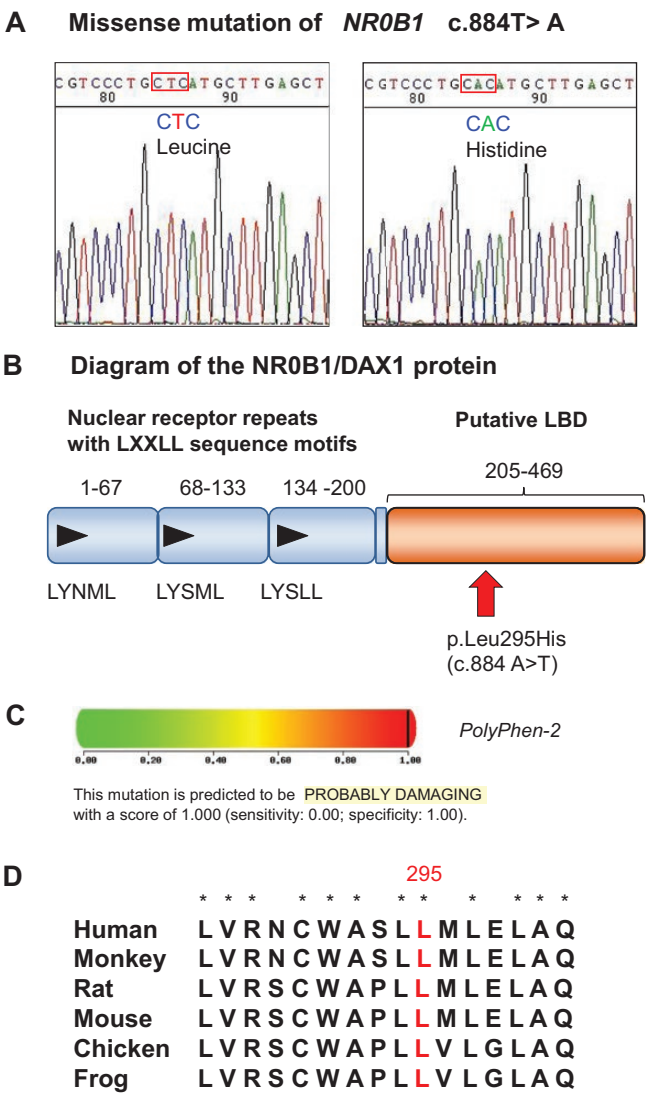


**Figure 2. Identification of the novel *NR0B1/DAX1* missense mutation.** (A) Direct sequencing revealed a homozygous p.Leu295His mutation in the *NR0B1/DAX1* gene from a control male (left) and a patient with AHC and HHG (right). Missense mutation involving 3 nucleotides (CAC) at position 883-885 leads to a shift of the amino acid from leucine to histidine at position 295. (B) Diagram of the NR0B1/DAX1 mutant protein, composed of nuclear receptor repeats with LXXLL sequence motifs and the putative LBD. Red arrow indicates the position of the p.Leu295His mutation. LBD: ligand-binding domain. (C) The p.Leu295His mutation in the NR0B1/DAX1 gene was scored "probably damaging" based on Ployphen-2. (D) Evolutionary conservation of amino acids at the novel missense mutant position p.Leu295His. The identification numbers of the NR0B1 nucleotides were as follows: Human (NP_000475.4), Monkey (NM_00195285.1), Rat (NM_053317.1), Mouse (NM_007430.5), Chicken (NM_204593.1), Xenopus (XM_002933615.4). The leucine at the mutant position are shown in red. The star (*) indicates the conserved residue.

next investigate the steroidogenic changes produced by the p.Leu295His mutant in H295R cells. As shown in Fig. 3F, expression profiles revealed that several steroidogenic genes in forskolin-stimulated H295R cells as being upregulated when mutant is transfected. These data indicate that repressor activity of NR0B1/DAX1 in synergetic steroid production was impaired by mutant *NR0B1/DAX1*. Consistent with steroidogenic gene expression, we also found that the repressor activity of NR0B1/DAX1 in cortisol secretion from H295R cells was impaired by mutant *NR0B1/DAX1* (Fig. 3G).

## The p.Leu295His NR0B1/DAX1 Mutant Suppressed Spermatogenesis, Resulting in Oligospermia

Recent characterization of NR0B1/DAX1 has elucidated that NR0B1/DAX1 plays a pivotal role in the initiation and maintenance of spermatogenesis and is required for testis cord organization [27, 28]. We next examined NR0B1/DAX1-mediated repression of the NR5A1/EGR1 synergistic activation of *LHB* gene expression (Fig. S4) [14]. Wild-type NR0B1/DAX1 repressed this synergistic

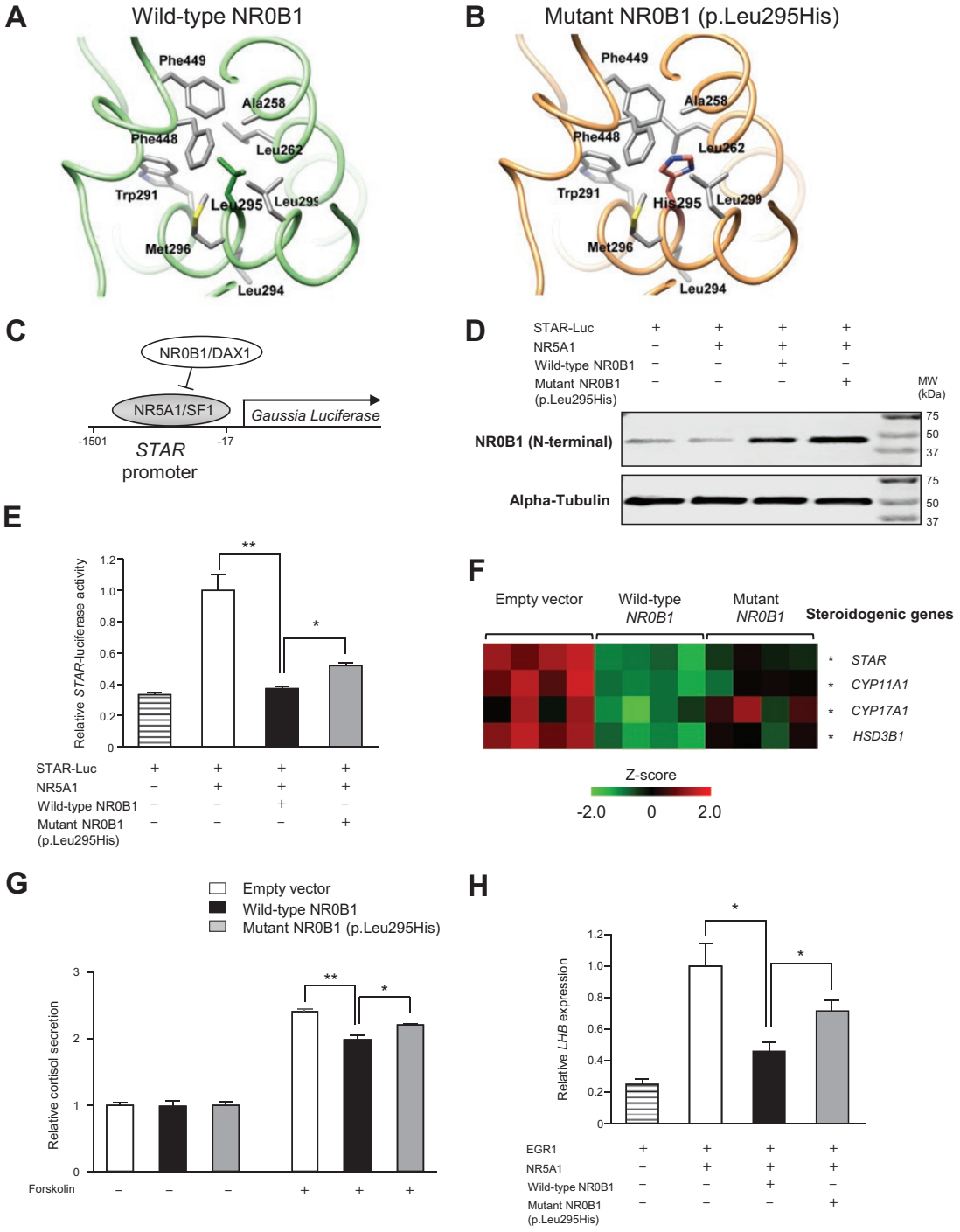


**Figure 3. The structural model and functional analysis of mutant p.Leu295His NR0B1/DAX1.** Model of wild-type NR0B1 (A) and p.Leu295His mutant NR0B1 (B). (C) Luciferase assay for NR5A1/SF1-mediated STAR transcriptional activity. STAR-luc, NR5A1/SF1 and NR0B1/DAX1 (wild-type and mutant p.Leu295His) expression vectors were co-transfected into adrenocortical H295R cells. (D) Immunoblotting of H295R cells with indicated transfections. α-Tubulin was used as loading control. (E) Repression capacities of the wild-type and mutant NR0B1/DAX1 proteins on NR5A1-mediated STAR promotor activity in H295R cells. Results are expressed as the average percentage of STAR promotor activity normalized to SEAP encoded by the pEZX-PG04 vector, as compared to cells transfected with NR5A1/SF1 and empty pEZX-PG04 vectors. (F) Expression profiles of steroidogenic genes (as indicated) in forskolin-stimulated H295R cells transfected with empty control, wild-type and mutant NR0B1/DAX1 (n = 4). The color scale shows the z-score representing the mRNA level of each gene employing a green (low expression)-black-red (high expression) scheme. * $P < 0.05$ between wild-type and mutant NR0B1/DAX1. (G) NR5A1/SF1-mediated relative cortisol secretion from forskolin-stimulated H295R cells transfected with indicated vectors. (H) Relative gene expressions of *LHB* in H295R cells co-transfected with EGR1, NR5A1 and wild-type/mutant NR0B1/DAX1. Values are expressed as mean ± SEM (n = 4). * $P < 0.05$, ** $P < 0.01$.

activation by 52.2%, whereas the p.Leu295His mutant repressed it by only 27.9% (Fig. 3H). It is suggested that this partial loss of repression impacted the exposure to LH, which alters Leydig cell proliferation and maturation, resulting in the pathogenic feature of oligospermia.

## Discussion

NR0B1/DAX1 plays a crucial role in the development and function of the adrenal gland and hypothalamic-pituitary gonadal axis (20). The NR0B1/DAX1 expression pattern is restricted to tissues directly involved in steroid hormone production and reproductive function, ie, adrenal cortex, testicular Leydig and Sertoli cells, ovarian theca and granulosa cells, pituitary gonadotropes, and ventromedial hypothalamic nucleus [29, 30]. Mutations in the NR0B1/DAX1 gene cause X-linked AHC and HHG. However, the onset and severity of phenotypic features are highly variable [31].We identified a novel NR0B1/DAX1 missense mutation (c.884A>T, p.Leu295His) in a Japanese patient who was referred to us for detailed evaluation of adrenal insufficiency. This mutation has not previously been reported and thus has not been included in the currently available databases. This patient showed adult-onset adrenal insufficiency with features such as ACTH elevation with a low DHEA-S level, as well as exhibiting neither cortisol nor aldosterone secretion after ACTH stimulation, while the basal levels of cortisol and aldosterone remained within normal limits. Of note, his elder brother, who had died suddenly with a viral infection, had also shown adrenal insufficiency. These data strongly suggest that his mother will have been a carrier of this mutation.

It is worth noting that the patient was referred for detailed evaluation of suspected adrenal failure at the age of 28, which is a much later onset than usually observed. To date, several missense mutations as well as frameshift and nonsense mutations have been reported in late-onset AHC and HHG [5, 32-34]. This variability in time of onset indicated the site and shift of genetical mutation to both be important factors for determining patient age at clinical onset as well as the phenotypic features of X-linked AHC and HHG.

The frameshift, nonsense, and missense mutations were distributed throughout the NR0B1/DAX1 coding region. To date, however, most missense mutations have been identified within the C-terminal of NR0B1/DAX1, putative LBD [3, 5, 16]. The mutation (p.Leu295His) found in our study was located within the hydrophobic core of this putative LBD [3, 16]. Since histidine is less hydrophobic than leucine, this mutation would likely affect the rigidity of the core, leading to decreased protein stability and/or protein misfolding. Such putative structural perturbations could affect the function of NR0B1/DAX1. Mechanistically, this NR0B1/DAX1 mutant showed impaired repression of SF1-mediated steroidogenesis in both H295R and HEK293 cells. The partial loss of repression capacity in mutant NR0B1/DAX1, demonstrated in functional studies, is clearly consistent with the phenotype in this patient.

In addition, subjects carrying NR0B1/DAX1 mutations with adult-onset AHC have presented a phenotypical varieties of HHG [35]. Infertility due to maturational arrest of spermatogenesis was documented in previous AHC case reports. Interestingly, not only oligospermia but also concomitant testicular microlithiasis, identified by ultrasonographic examination, was present in our patient. This is the first description, so far as we know, of an apparently rare case of late-onset X-linked AHC with testicular microlithiasis. Since testicular microlithiasis with male infertility is known to be associated with testicular malignancy [36, 37], regular medical follow-up is essential for this patient.

In conclusion, we have reported the first case with a novel NR0B1/DAX1 missense mutation (c.884A>T), resulting in adult-onset AHC and HHG. Mutant p.Leu295His NR0B1/DAX1 protein from this patient showed an altered hydrophobic core and this alteration impaired the repressor capacity of NR5A1/SF1-mediated transcription. These findings provide new insights into the structural/functional analysis of NR0B1/DAX1 mutation and the prediction of related clinical manifestations in AHC and HHG. Thus, identification and a functional analysis of NR0B1/DAX1 gene mutations are important not only for understanding the etiologies of AHC and HHG but also for informing patients of their risks of acute adrenal insufficiency and fertility problems.

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## Additional Information

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***Disclosure Summary:*** The authors declare no conflict of interest.

***Data Availability:*** The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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