FOXYN1 compound heterozygous mutations cause selective thymic hypoplasia in humans

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Graphical abstract

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FOXN1 compound heterozygous mutations cause selective thymic hypoplasia in humans

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We report on 2 patients with compound heterozygous mutations in forkhead box N1 (FOXN1), a transcription factor essential for thymic epithelial cell (TEC) differentiation. TECs are critical for T cell development. Both patients had a presentation consistent with T−B−NK− SCID, with normal hair and nails, distinct from the classic nude/SCID phenotype in individuals with autosomal-recessive FOXN1 mutations. To understand the basis of this phenotype and the effects of the mutations on FOXN1, we generated mice using CRISPR-Cas9 technology to genocopy mutations in 1 of the patients. The mice with the Foxn1 compound heterozygous mutations had thymic hypoplasia, causing a T−B−NK− SCID phenotype, whereas the hair and nails of these mice were normal. Characterization of the functional changes due to the Foxn1 mutations revealed a 5′-amino acid segment at the end of the DNA-binding domain essential for the development of TECs but not keratinocytes. The transcriptional activity of this Foxn1 mutant was partly retained, indicating a region that specifies TEC functions. Analysis of an additional 9 FOXN1 mutations identified in multiple unrelated patients revealed distinct functional consequences contingent on the impact of the mutation on the DNA-binding and transactivation domains of FOXN1.

Introduction

The development of T cells expressing T cell receptors (TCRs) bearing a precise specificity for peptides embedded in self-HLA (MHC) molecules occurs in the thymus. At this site, T cell interactions with thymic epithelial cells (TECs) establish an individualized TCR repertoire. The developmental progression of T cells occurs in several stages and involves 2 distinct epithelial cell (EC) subsets, cortical and medullary TECs (1, 2). Designated by their distribution within the thymus, these subsets are functionally distinct despite developing from a common precursor cell (3). Cortical TECs (cTECs) support early T cell development. They release chemokines such as Ccl19, Ccl21, and Ccl25 to direct early thymocyte precursor entry at a cortical-medullary interface (4). cTECs also secrete IL-7, and in conjunction with the chemokines, combinatorially provide proliferation, differentiation, and directional information to the immature double-negative (DN) CD4 CD8 thymocyte subset (4). This is further determined by the expression on cTECs of delta ligand–like 4 (DDL4), which binds to Notch on immature thymocytes and leads to expression of the Notch-activated transcription factor RBPJκ. The various cTEC-dependent signals promote chromosome rearrangements at the TCRβ locus in the DN thymocytes. Successful expression of a TCRβ protein signal an expansion and differentiation phase of the DN cells into the double-positive (DP) CD4+CD8+ subset. During this transition, the TCRα locus is rearranged, with a productively produced TCRα protein pairing with the TCRβ subunit. A byproduct of this rearrangement event is the TCR excision circle (TREC), which is the newborn screening analyte used to identify patients with SCID (5).

The survival of an αβ TCR–expressing DP thymocyte is enabled after favorable “weak” TCR interactions with self-peptides bound to MHC molecules presented by the cTECs (positive selection). The positively selected thymocytes are directed into the CD4+CD8− and CD4−CD8+ single-positive (SP) subsets that enter the medullary regions. The expansion and function of medullary TECs (mTECs) relies on their crosstalk with SP thymocytes, with CD40L, RANKL, and EGF engaging CD40, RANK, and EGFR, respectively, on the TECs (6, 7). The mTECs present many tissue-restricted antigens on HLA molecules, a process that requires the transcriptional regulator termed AIRE, along with a second transcription factor, FezF2 (8, 9). mTECs purge T cells with high-affinity, self-reactive TCR specificities (negative selection). An added function of mTECs is to support the selection of Tregs (10). The selected Tregs exit into the peripheral lymphoid organs, where they prevent the promiscuous activation of any remaining autoreactive T cells (11). The gauntlet of cTEC- and mTEC-mediated T cell selection processes, aided by DCs, results in pro-

Conflict of interest: MLM developed thymus transplantation intellectual property, which has been licensed to Enzyvant Therapeutics. Both MLM and Duke University may benefit financially if the technology is commercially successful in the future.

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grammed cell death for the vast majority of thymocytes. What emerges from the thymus is a population of mature T cells with a precise TCR specificity unique to a given individual.

The development and differentiation of TECs into the cortical and medullary subsets are established by the transcription factor forkhead box N1 (FOXN1) (1, 12). FOXN1 belongs to a large family of proteins defined by a conserved, 100-amino-acid-long DNA-binding domain called the forkhead or winged helix. Forkhead family proteins are present in species from yeast to humans, but not in plants (13, 14). FOXN1 arose via duplication of FOXN4, an ancestral homolog that first appeared in those early chordates with evidence of a thymus (1, 15, 16). Comprising 3 α helices, 3 β sheets, and 2 loops designated as the wings, the third α helix of FOXN1 contacts the major groove of DNA (17). The second winged segment provides additional binding specificity within the minor groove of DNA. Foxn1 interacts with DNA sequences containing a GAa/cGC consensus and transcriptionally activates nearly 500 genes including keratins, cytokeratins, proteasome components, and cell-surface proteins (2). A transactivation domain near the COOH-terminal half of the protein is also required for Foxn1 functions (18).

The importance of FOXN1 in TEC and keratinocyte development was first uncovered in a spontaneously arising nude phenotype in mice (nu/nu), which was later shown to be caused by autosomal-recessive mutations in this gene (12, 19). The loss of Foxn1 protein expression results in a TEC deficiency in combination with somal-recessive mutations in this gene (12, 19). The loss of Foxn1 type in mice (nu/nu), which was later shown to be caused by autosomal-recessive mutations in this gene (12, 19), was first uncovered in a spontaneously arising nude phenotype (22–26). This phenotype is also reported in approximately 10 patients, all of whom presented with similar nude/SCID phenotypes (22–26). This phenotype is also reported in approximately 10 patients, all of whom presented with similar nude/SCID phenotypes (22–26). This phenotype is also reported in approximately 10 patients, all of whom presented with similar nude/SCID phenotypes (22–26). This phenotype is also reported in approximately 10 patients, all of whom presented with similar nude/SCID phenotypes (22–26). This phenotype is also reported in approximately 10 patients, all of whom presented with similar nude/SCID phenotypes (22–26). This phenotype is also reported in approximately 10 patients, all of whom presented with similar nude/SCID phenotypes (22–26).

With alopecia universalis and nail plate dystrophy (23, 25, 28). Thymic hypoplasia/aplasia arising from defects with stromal cell populations such as TECs can also occur in individuals with mutations in CHD7, PAX1, TBX1, and TBX2, and in those with chromosome 22q11.2 deletion syndrome (DiGeorge syndrome) (29–35). However, only the autosomal-recessive mutations described for FOXN1 lead to the additional developmental problems of the hair shaft and nail plate.

We report here on the identification of mutations in FOXN1 in 2 patients with a T−B−NK−C SCID without evidence of alopecia or nail dystrophy. We used CRISPR-Cas9 technology to develop Foxn1 compound heterozygous mice that genocopied selected human FOXN1 mutations. A comparison of the various mice harboring homozygous and compound heterozygous Foxn1 mutations revealed differential functions of this transcription factor in TECs versus keratinocytes. Phenotype analyses and functional assays revealed an important 5–amino acid sequence in Foxn1 that differentiates its role in TECs versus keratinocytes. A comparative analysis of 12 distinct FOXN1 mutations, identified in patient 1 (Pt. 1) and patient 2 (Pt. 2) as well as in several unrelated patients with heterozygous mutations, revealed varying transcriptional activities depending on the location and type of mutation. These findings provide structure-function insights into this key transcription factor.

**Results**

**Compound heterozygous mutations in FOXN1 identified in 2 unrelated patients with a SCID phenotype without alopecia.** Pt. 1 was a female born at full term to nonconsanguineous parents and was identified as having SCID following newborn screening that revealed undetectable TRECs. The subsequent clinical work-up was consistent with a typical T B N K − SCID phenotype, using the normal age-matched reference range (Table 1) (36, 37). Exome sequencing revealed 2 mutations in the FOXN1 gene, a duplication

**Table 1. Clinical presentations of 2 patients identified with compound heterozygous FOXN1 mutations**

<table>
<thead>
<tr>
<th>Age</th>
<th>ALC (cells/µL)</th>
<th>CD3+ (cells/µL)</th>
<th>CD4+ (cells/µL)</th>
<th>CD8+ (cells/µL)</th>
<th>CD4+ CD45RA+ (cells/µL)</th>
<th>CD4+ CD45RA–CD62L+ (cells/µL)</th>
<th>NK (cells/µL)</th>
<th>CD19+ (cells/µL)</th>
<th>Mitogen proliferation</th>
</tr>
</thead>
</table>

*The proliferation assays were conducted in 3 different laboratories; the initial mitogen proliferation used anti-CD3, anti-CD28, and anti–IL-2 stimulation, whereas the subsequent 2 assays were performed using phytohemagglutinin stimulation at 2 separate laboratories. ALC, absolute lymphocyte count; PHA, phytohemagglutinin.*
of ACCC at position c.933 (c.933_936dup), and a 15-nt deletion (c.1089_1103del) (Table 2). Analysis of the parental data indicated that the 933 mutation was inherited from the mother, whereas the 1089 mutation was passed down from the father. Both parents were healthy and had no evidence of hair loss or nail changes (Figure 1, A, C, and D; Family 1). The c.933 duplication was identified as a presumed pathogenic variant that was predicted to result in a premature stop codon following a new coding sequence of approximately 169 amino acids extending from the middle of the DNA-binding domain (p.Thr313fsX169). The second allele in Pt. 2 contained a 15-nt deletion near the end of the DNA-binding domain (p.Thr313fs*169). This resulted in a single amino acid conversion (Trp to Cys) followed by a 5–amino acid deletion (p.Trp363_Pro367delin-130). The deletion did not change the reading frame. The clinical course for Pt. 1 was characterized by rhino/enteroviral infection at 2 months of age, norovirus enteritis and submandibular abscess in infancy; S. aureus. The child underwent hematopoietic cell transplantation at 6 months of age (Table 2). Exome sequencing revealed c.C1288T and c.1465delC FOXN1 variants (Figure 1, B and E; family 2). Sanger sequencing confirmed individual FOXN1 allelic mutations in Pt. 2. The nucleotide changes caused a p.Pro430Ser on FOXN1 derived from 1 allele and a p.Gln489Argfs on the second allele that resulted in a frameshift mutation in the protein (Figure 1, B, C, and E). The 1288 variant is a polymorphism found in 4% of the general population (dbSNP: rs61749867), but the impact of this variant on FOXN1 has not been established. Analysis of the parents indicated that the c.C1288T variant was de novo.

The FOXNI mutations described for both Pt. 1 and Pt. 2 are distinct from the classic autosomal-recessive mutations originally identified in FOXNI (Figure 1C) (22–26). To date, previously identified mutations on other genes associated with SCID or cellular immunodeficiency phenotypes were not found in Pt. 1 or Pt. 2 (Table 2).

Mice harboring compound heterozygous mutations in Foxn1 have thymic hypoplasia with normal fur and nail beds. To confirm that the FOXNI mutations in Pt. 1 were causal to the SCID phenotype, we used a CRISPR-Cas9 strategy to create analogous mutations in C57Bl/6 mice. Individual guide RNAs were designed with Cas9 cleavage sites near the equivalent positions in the murine locus

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**Table 2. Clinical presentations of patients with compound heterozygous or monoallelic mutations in FOXN1**

<table>
<thead>
<tr>
<th>Patient</th>
<th>DNA mutation</th>
<th>Protein-coding mutation</th>
<th>Protein function (% of WT) (luciferase assay)</th>
<th>Abnormal hair</th>
<th>Abnormal nails</th>
<th>Transplant</th>
<th>Other clinical notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>None</td>
<td>None</td>
<td>100%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pt. 1</td>
<td>c.933_936dupACCC</td>
<td>p.T313fsX169</td>
<td>1.5%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Multiple viral infections, failure to thrive. Died at 1yr of age from parainfluenza virus.</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>c.1288C&gt;T</td>
<td>p.P430S</td>
<td>100%</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Received BM transplant. Healthy, no recurrent infections, on s.c. gamma globulin therapy.</td>
</tr>
<tr>
<td>Pt. 3</td>
<td>c.1465delC</td>
<td>p.R489fsX61</td>
<td>18%</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Developed S. aureus submandibular abscess in infancy; otherwise healthy.</td>
</tr>
<tr>
<td>Pt. 4</td>
<td>c.1465delC</td>
<td>p.R489fsX61</td>
<td>18%</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Healthy, no recurrent infections.</td>
</tr>
<tr>
<td>Pt. 5</td>
<td>724C&gt;T</td>
<td>p.P242S</td>
<td>95%</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Died in infancy from coronavirus encephalitis. Also had a heterozygous D0C8 mutation.</td>
</tr>
<tr>
<td>Pt. 6</td>
<td>958C&gt;T</td>
<td>p.R320W</td>
<td>2%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Healthy, no recurrent infections.</td>
</tr>
<tr>
<td>Pt. 7</td>
<td>962A&gt;G</td>
<td>p.H321R</td>
<td>82%</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Died in infancy from coronavirus encephalitis. Also had a heterozygous D0C8 mutation.</td>
</tr>
<tr>
<td>Pt. 8</td>
<td>982T&gt;C</td>
<td>p.C328R</td>
<td>16%</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Healthy, no recurrent infections.</td>
</tr>
<tr>
<td>Pt. 9</td>
<td>1075G&gt;A</td>
<td>p.E359K</td>
<td>63%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Healthy, no recurrent infections.</td>
</tr>
<tr>
<td>Pt. 10</td>
<td>1201_1206del16</td>
<td>p.P401AfsX144</td>
<td>3%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Also had heterogeneous D0C8 and CHD7 mutations with no congenital anomalies.</td>
</tr>
<tr>
<td>Pt. 11</td>
<td>1201_1206del16</td>
<td>p.P401AfsX144</td>
<td>3%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Healthy, no recurrent infections.</td>
</tr>
<tr>
<td>Pt. 12</td>
<td>1201_1206del16</td>
<td>p.P401AfsX144</td>
<td>3%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Healthy, no recurrent infections. Received live virus vaccines.</td>
</tr>
<tr>
<td>Pt. 13</td>
<td>1201_1206del16</td>
<td>p.P401AfsX144</td>
<td>3%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Healthy, no recurrent infections.</td>
</tr>
<tr>
<td>Pt. 14</td>
<td>1201_1206del16</td>
<td>p.P401AfsX144</td>
<td>3%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Born prematurely due to maternal infection. Currently healthy with no recurrent infections.</td>
</tr>
<tr>
<td>Pt. 15</td>
<td>1293delC</td>
<td>p.P432fsX118</td>
<td>2%</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Low numbers through ~3 mo when alternate therapy was given.</td>
</tr>
<tr>
<td>Pt. 16</td>
<td>1418delC</td>
<td>p.P473HfsX77</td>
<td>12%</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Low numbers through ~4 mo when alternative therapy was given.</td>
</tr>
</tbody>
</table>

S. aureus, Staphylococcus aureus.
mental material available online with this article; https://doi.org/10.1172/JCI127565DS1). DNA sequencing of these runts revealed deletions within the DNA-binding domain on both Foxn1 alleles. In contrast, the 1089 founders appeared visually indistinguishable from normal mice (Figure 2B and Supplemental Figure 1A). Founders with the correct nucleotide duplication (933 nt) or deletion (1089 nt) in Foxn1 were bred with WT mice, followed by intercrossing of the mice to establish the appropriate homozygous and compound heterozygous lines.

F2-generation mice homozygous for the 933 mutation (Foxn1933/933) were small and hairless and had diminished nail lengths and shortened whiskers compared with littermate controls (Figure 2B and Supplemental Figure 1B). These phenotypes matched that of the classic nu/nu mouse (12). In contrast, the physical appearance of the Foxn11089/1089 and Foxn1933/1089 mice matched that of their littermate controls (Figure 2B). The Foxn1933/933 mice had thymic aplasia, with a 35- to 50-fold reduction in thymus weights compared with littermate control thymus weights (Figure 2, C and D). The Foxn11089/1089 and Foxn1933/1089 compound hetero-

Figure 1. Compound heterozygous mutations in FOXN1 identified in 2 patients. (A and B) Pt. 1 and Pt. 2 were identified from 2 independent and unrelated families. (C) Domain structure of FOXN1, with the 2 domains characterized to date: a DNA-binding region in the middle of the protein and a transactivation domain near the COOH terminus. Three Foxn1 autosomal-recessive mutations previously reported in patients with nude/SCID phenotypes are listed in red. Two patients, Pt. 1 and Pt. 2, presented with compound heterozygous mutations in FOXN1 at distinct sites, which are indicated above the exon assembly. (D and E) The DNA sequence mutations in FOXN1 and the corresponding effects on the amino acid sequence are shown for Pt. 1 (D) and Pt. 2 (E). The amino acid changes resulting from the various FOXN1 mutations are illustrated.

for Pt. 1, at positions 933 and 1089 (Figure 2A). Silent mutations were included to create new DNA restriction sites for genotyping purposes. The more difficult design was for position 933 (Pt. 1). At this location, the 4-nt duplication (ACCC) in human FOXN1 creates a frameshift that begins mid-way through the DNA-binding domain. The corresponding TCCT insertion within the same location in the murine Foxn1 locus creates an immediate stop codon. Consequently, a TCCT insertion was engineered into the murine locus along with several additional nucleotide substitutions within the repair template to better mimic the read-through seen with the patient’s allele. The resulting coding sequences for both the human and murine genes following the insertion site were novel, with a new stop codon introduced within exon 6. The corresponding mutations in the human genome led to a longer transcript, again with codons unrelated to WT FOXN1.

The phenotypes of the founder pups derived from the CRISPR-Cas9 genome editing of the Foxn1 933-nt position were distinct from those targeted at position 1089. Many of the 933 pups had a nude, runted phenotype (Supplemental Figure 1A; supplement.
how the diverse FOXN1 mutations affect thymopoiesis, we compared the developmental progression of thymocytes in the different mouse lines (Figure 3A). We monitored the various stages by comparing the cell-surface expression of the CD4, CD8, TCR β, CD25, CD44, CD45, and CD69 by flow cytometry. As shown in the littermate controls (Foxn1 WT/WT), thymopoiesis was characterized by a subset of immature CD4–CD8– cells (DN, 4%) that developed into a CD4 +CD8+ cell population (DP, 80%–87%), of which a small number were positively selected into the CD4+CD8– (CD4 SP, 3%–8%) and CD4–CD8+ lineages (CD8 SP, 2%–5%) (Figure 3A). With the exception of some DN and CD8lo cells, the DP and SP cell populations were virtually nonexistent in the Foxn1 933/933 mouse line (Figure 3A). The DN and CD8lo cell populations lacked CD45+ cell-surface expression, indicating that they were not thy

zygous mice had pronounced thymic hypoplasia, with a 10- and 17-fold reduction in thymus weights, respectively (Figure 2, C and D). This was confirmed when we compared the total thymic cellularity of 6 to 22 mice per group (Figure 2D). Sex was not a determinant for the hypoplasia, as males and females were equally affected (Supplemental Figure 2A). A comparison of the various heterozygous mice derived from the intercrosses (Foxn1 WT/1089 and Foxn1 WT/933) revealed no impact of a single-allele mutation on thymic cellularity, T cell development, and/or peripheral lymphocytes compared with littermate controls (Supplemental Figure 2, B–E). These findings firmly establish that the clinical phenotypes of Pt. 1 resulted from compound heterozygous mutations in FOXN1.

Thymopoiesis is severely attenuated in mouse lines with compound heterozygous mutations in Foxn1 that genocopy Pt. 1. To understand how the diverse FOXN1 mutations affect thymopoiesis, we compared the developmental progression of thymocytes in the different mouse lines (Figure 3A). We monitored the various stages by comparing the cell-surface expression of the CD4, CD8, TCR β, CD25, CD44, CD45, and CD69 by flow cytometry. As shown in the littermate controls (Foxn1 WT/WT), thymopoiesis was characterized by a subset of immature CD4–CD8– cells (DN, 4%) that developed into a CD4+CD8+ cell population (DP, 80%–87%), of which a small number were positively selected into the CD4+CD8– (CD4 SP, 3%–8%) and CD4–CD8+ lineages (CD8 SP, 2%–5%) (Figure 3A). With the exception of some DN and CD8 SP cells, the DP and SP cell populations were virtually nonexistent in the Foxn1 WT/933 mouse line (Figure 3A). The DN and CD8 SP cell populations lacked CD45+ cell-surface expression, indicating that they were not thy-
These data confirm that the distinct mutations have differential consequences for early T cell development and positive selection. We further explored this by characterizing the 4 stages of DN thymocyte progression as DN1–DN4 (DN1: CD44+CD25–, DN2: CD44+CD25+, DN3: CD44–CD25+, DN4: CD44–CD25–). We noted similar percentages of each of the DN1–DN4 subsets when we compared Foxn1933/1089 thymocytes with those of normal controls. Such results suggest that homozygous mutations at the Foxn1933/1089 position of the Foxn1 WT/WT mice, genocopying Pt. 1, had a selective block at the DN1 stage (Figure 3B). However, the mouse line that genocopied Pt. 1 (Foxn1933/933) had a selective block at the DN1 stage (Figure 3B). The percentage of CD69+ thymocytes expressing intermediate levels of TCRβ on cells expressing intermediate levels of TCRβ was reduced in the Foxn1933/1089 mice, genocopying nu/nu mice, which showed a severe lack of immature DN thymocytes. We assessed the effect on positive selection by screening for the upregulation of CD69 on cells expressing intermediate levels of TCRβ (Figure 3C). The percentage of CD69+ thymocytes expressing intermediate or high levels of the TCRβ subunit was reduced in the Foxn1933/1089 mice.
heterozygous mouse line (Foxn1933/1089) and in one of the homozygous mutant lines (Foxn11089/1089) (Figure 3C). These developmental abnormalities were of statistical significance, as revealed by comparisons of the different thymocyte subpopulations with multiple mice per group (Figure 3D).

The consequence of the thymic aplasia (Foxn1933/933 mice) and hypoplasia (Foxn11089/1089 and Foxn1933/1089 lines) is an almost complete loss of mature CD4+ and CD8+ T cells in the peripheral lymph nodes of these mice (Figure 4A). B cells and NK cells were present, confirming the T-B-NK+ SCID phenotype of Pt. 1 (Figure 4, B–D). Comparison of the cell populations using 3–26 mice per group confirmed the statistical significance of the findings (Figure 4D). The various cell populations in 6-week-old heterozygous mice (Foxn1WT/933 and Foxn1WT/1089) were similar to normal control cell populations, revealing that 1 functional allele of Foxn1 was sufficient to support normal T cell development (Supplemental Figure 2, B–E). Taken together, our data demonstrate that T cell development is severely compromised in mice with compound heterozygous mutations in Foxn1 matching Pt. 1 and results in severe T cell lymphopenia.

Figure 4. Mice with compound heterozygous mutations in Foxn1 have severe peripheral T cell lymphopenia. (A–C) Lymph nodes were collected from the indicated mice and stained with antibodies detecting cell-surface CD4, CD8, B220, TCRβ, and NK1.1 expression. The cells were analyzed by flow cytometry comparing the cell-surface expression of (A) CD4+ and CD8+ SP cells; (B) B220 (marker of B cells) and CD3ε cell-surface expression; and (C) NK1.1 cell-surface expression and TCRβ. n = 3 to 26 mice per genotype for A–C. APC, allophycocyanin. (D) The percentages of CD4+ SP cells, CD8+ SP cells, B220+ B cells, and CD3ε+ T cells were calculated; n = 24 Foxn1WT/WT, n = 5 Foxn1933/933, n = 11 Foxn11089/1089, and n = 21 Foxn1933/1089 mice. The percentages of NK1.1+ TCRβ+ cells were calculated; n = 26 Foxn1WT/WT, n = 3 Foxn1933/933, n = 11 Foxn11089/1089, and n = 11 Foxn1933/1089 mice. For the comparisons shown, a Brown-Forsythe and Welch’s 1-way ANOVA was applied. P values of less than 0.05 were considered significant.
Cortical and medullary TECs are affected by mutations in Foxn1. In the thymus, Foxn1 expression is restricted to ECs. To determine how the diverse Foxn1 mutations impact TECs, we compared cTEC and mTEC subsets. H&E staining of the tissue in the Foxn1\textsuperscript{933/933} and Foxn1\textsuperscript{1089/1089} lines revealed hypoplastic/aplastic tissue with poorly defined cortical and medullary segments, cystic regions, and increased adipose tissue (Figure 5A). Immunofluorescent staining with antibodies specific for cTECs (cytokeratin 8) and mTECs (cytokeratin 5) revealed an almost complete absence of these cells in Foxn1\textsuperscript{933/933} and Foxn1\textsuperscript{1089/1089} mice relative to what we observed in the littermate controls (Figure 5B). Thymi from the Foxn1\textsuperscript{1089/1089} line were not as severely affected, as small clusters of cTECs and mTECs were evident in the hypoplastic tissue. The number and percentage of cTECs and mTECs in these mice relative to littermate controls were determined by flow cytometry (Figure 5C). With regard to cell percentages, the Foxn1\textsuperscript{WT/WT} mice had
approximately 13% and 81% cTECs and mTECs (EpCam+CD45+), respectively (Figure 5, C and F). In contrast, the Foxn11089/1089 thymi had 4% and 31% cTEC and mTEC representation, respectively (Figure 5F). The lower percentages in the Foxn11089/1089 mice were matched with an increased percentage of EpCam+CD45+ TECs from the Foxn11089/1089 mouse line (62%), which lacked the cytokeratin markers that distinguish cTECs and mTECs (Figure 5C). In normal mice, the mTECs were divided into the less mature MHC class IIlo (MHC CIIlo) and more mature MHC class IIhi (MHC CIIhi) cells (Figure 5, D and E). We detected a significant reduction in the percentage of MHC CIIlo cells in the Foxn11089/1089 lines, and the MHC CII+ cells present had a reduced cell-surface expression of this molecule relative to littermate controls (Figure 5, D and E). These TEC comparisons indicate that the mutations at the 1089 position of FOXN1 are not as damaging as those that disrupt the DNA-binding domain (position 933), providing some functionality for TEC development.

The transcriptional activity of Foxn1 is regulated by both the DNA-binding and transactivation domains. To determine how the distinct FOXN1 mutations identified in Pt. 1 and Pt. 2 affect the expression and function of the protein, we introduced substitutions or deletions into the cDNA of murine Foxn1 and performed protein expression and promoter-based reporter assay comparisons (2, 39). The insertion of the 4-nt sequence identified in Pt. 1 (Foxn1933) resulted in the expression of a truncated 50-kDa protein (Figure 6A). The 15-nt deletion, corresponding to the one identified in the second allele of Pt. 1 (Foxn11465), was almost identical in molecular mass to that of the WT control (Figure 6A). Foxn11465 was also similar in size to Foxn1WT, whereas a single nucleotide deletion at position 1465 created a truncated protein with a mass of 65 kDa as a result of the frameshift (Figure 6B). As our studies were progressing, several additional FOXN1 mutations were identified in unrelated patients presenting with low T cell counts for whom exome sequencing was undertaken (Table 2 and Supplemental Table 1). Unlike the compound heterozygous genotypes for Pt. 1 and Pt. 2, these patients had FOXN1 mutations evident only on a single allele (Table 2 and Supplemental Table 2). One subject had a previously reported R320W mutation in FOXN1, whereas 2 patients had the 1465delC that was noted in Pt. 2. We characterized FOXN1 with SNPs (FOXN1c24, FOXN1b8, FOXN1d2, FOXN1d2, FOXN1c05) and deletions (FOXN1d120,126, FOXN1d2a2, FOXN1d2a6c), with the human mutations introduced into the murine cDNA (Supplemental Table 2). Consistent with the location and type of mutation, either full-length or truncated Foxn1 proteins were expressed (Supplemental Figure 3, A and B).

The functional activity of Foxn1 mutations corresponding to those in Pt. 1 and Pt. 2, along with the additional mutations, were compared by transcriptional reporter assays using the promoter sequence for the Pumbl1 gene, which encodes β1t (2). We found that Foxn1933 had virtually no transcriptional activity (Figure 6C). The Foxn1 constructs with mutations at positions 1089 and 1465 had 31% and 18% WT activity, respectively (Figure 6D). Interestingly, coexpression of the corresponding heterozygous mutation (Foxn11089 with Foxn1933) or the polymorphism (Foxn1d1288 with Foxn1d1465) did not attenuate the transcriptional activity of the functional allele, suggesting that Foxn1 functions as a monomer (Figure 6, C and D). Foxn1d1288 was inactive, whereas Foxn1d1288 and Foxn1d1465 had activity comparable to that of Foxn1WT, at 95% and 82% normal values, respectively (Table 2 and Supplemental Figure 3C). Foxn1d1465 exhibited 16% and 63% control activity, respectively (Table 2 and Supplemental Figure 3C). The deletions at positions 1201, 1293, and 1418 had a more severe effect,
identical to that of the classic nu/nu mouse, consistent with an essential role for the FOXN1 DNA–binding domain in both TEC and keratinocyte differentiation and gene expression. Contrasting with this, the Foxn1 933/1089-knockin mouse line, genocopying Pt. 1, had a normal hair coat and whiskers despite the dramatic thymic hypoplasia. This suggests that the Foxn1933/1089 mutations transcriptionally affect TECs differently than they do keratinocytes. We considered 2 explanations for the divergent consequences of harboring Foxn1933/933 versus Foxn1933/1089 genotypes for hair and nail reducing the functional activities of Foxn1 to 3%, 2%, and 12% of normal values, respectively (Table 2 and Supplemental Figure 3D). Taken together, these results confirm crucial roles for both the DNA-binding and transactivation domains in the transcriptional activity of Foxn1, with the extent of functional incapacitation being dependent on the location and type of Foxn1 mutation (Figure 1C and Table 2).

FOXN1 differentially regulates genes in the thymus versus the skin. The Foxn1933/933 homozygous knockin mice have a phenotype identical to that of the classic nu/nu mouse, consistent with an essential role for the FOXN1 DNA–binding domain in both TEC and keratinocyte differentiation and gene expression. Contrastingly, with this, the Foxn1933/1089-knockin mouse line, genocopying Pt. 1, had a normal hair coat and whiskers despite the dramatic thymic hypoplasia. This suggests that the Foxn1933/1089 mutations transcriptionally affect TECs differently than they do keratinocytes. We considered 2 explanations for the divergent consequences of harboring Foxn1933/933 versus Foxn1933/1089 genotypes for hair and nail
phenotypes. First, the FOXN1933/933 mutations could selectively affect the TEC transcriptome as opposed to the keratinocyte transcriptome. Alternatively, the loss-of-function mutations in FOXN1 could modulate RNA transcripts in both TECs and keratinocytes in a quantitative manner, with the latter cell population maintaining reduced yet sufficient levels of transcripts to enable extrusion of the hair shaft and nail beds. To address these possibilities and characterize the transcript changes that resulted from the compound heterozygous mutations in FOXN1, we performed comparative gene expression analyses. RNA was extracted from embryonic thymic lobes at E13.5 and used for gene analyses. The E13.5 time point coincides with an expansion/differentiation phase of TECs prior to significant thymocyte expansion. We made gene expression comparisons with 3 control thymi (Foxn11089/1089) and 3 hypoplastic thymi (Foxn1933/933) (Figure 7A). Gene array comparisons revealed 417 upregulated and 500 downregulated genes in the Foxn1933/933 line relative to the littermate controls (Figure 7A). These differences were not surprising, given the severe hypoplasia evident in the Foxn1933/933 mice (Figure 3B). Our focus on a select subset of transcripts with roles in the development of the thymus and parathyroids, which are linked during the patterning of the third pharyngeal apparatus, revealed some surprising differences (Figure 7B). Tbx1 and Eya1 levels were higher in mutant mice, suggesting impaired differentiation of the thymus within the third pharyngeal pouch region (Figure 7B and Supplemental Table 3). In contrast to this, we found that Pax1 and Dcs2 levels were substantially lower in mutant mice (Supplemental Table 3). Although Pax1 participates in the formation of the thymic lobes, Dsc2 has only been described as a Foxn1 target in the skin (3, 40). Taken together, our data are consistent with previous reports comparing normal and Foxn1-mutant mice and provide further insights into the transcript changes in embryonic hypoplastic thymi (2).

To determine how the same mutations in Foxn1 affect hair follicle development, we isolated skin from 4-day-old pups. This is a stage when hair follicle extrusion and whisker formation is first evident in normal mice (Figure 7C). As previously reported, the Foxn1933/933 pups showed no hair follicle extrusion (Figure 7C). In contrast, the hair in the Foxn1933/933 mice was visually similar to that of littermate controls (Figure 7C). We performed quantitative reverse transcription PCR (qRT-PCR) to compare a number of key genes expressed along the developing hair shaft needed for hair follicle extrusion. The nude mice (Foxn11089/1089) had reduced expression of mouse Krt84 (mKrt84), mKrt33, mKrt33b, and mDsc2, consistent with the central role of the DNA-binding activity of Foxn1 in enabling keratinocyte functions (Figure 7D). Foxn1933/933 mice have no nude phenotype. The transcripts coupled to hair follicle extrusion in these mice were expressed at levels comparable to those seen in littermate controls (Figure 7E). This indicates a functional dichotomy in the requirements for Foxn1 in controlling extrusion of the hair shaft in comparison with expression of thymus-selective genes.

Discussion
FOXN1 is a master transcriptional regulator of TECs that controls the expression of genes critical for both cTEC and mTEC differentiation and expansion (1, 28, 41). Although autosomal-recessive mutations in FOXN1 can result in a nude/SCID phenotype, even hypomorphic alleles can reduce the effectiveness of thymopoiesis (2, 42). This is best revealed in mouse models, in which enforced expression of Foxn1 in aged mice prevents the characteristic age-related thymic involution and improves T cell output (43–45). We identified what to our knowledge are several previously unreported mutations in human FOXN1 and, for 2 patients, distinct compound heterozygous mutations. Genocopying the compound heterozygous mutations identified in Pt. 1 using a mouse model was consistent with a novel clinical presentation of T’B’NK’ SCID without evidence of alopecia or nail dystrophy. We identified the FOXN1 variants in Pt. 2 using targeted exome sequencing. However, this approach did not establish whether both variants were restricted to 1 allele, with the possibility that a normal FOXN1 sequence was present on the second allele. To answer this, we cloned the region comprising exon 8 and used more than 16 independently isolated bacterial clones to sequence the FOXN1 region comprising positions 1288 and 1465. Consistent with a compound heterozygous genotype, we confirmed individual allelic variants for positions 1288 and 1465. We also had an unexpected finding of some clones with a WT FOXN1 sequence. Although molecular analysis for maternal engraftment when the child was 2 months of age was negative, we suspect that the WT FOXN1 sequences were maternally derived, since DNA was obtained from the child’s blood at 5 months of age (subsequent studies for maternal engraftment were not obtained at this time point). Moreover, the mother did not have any clinical indications of a T-/- phenotype. The allelic variant at position 1288 did not affect the expression or function of full-length FOXN1, as established through Western blot analysis and a β-gal luciferase reporter assay. The 1288 polymorphism is in fact reported in approximately 3% of the general population (dbSNP: rs61749867), suggesting that it is benign. However, the Pro-to-Ser amino acid change due to the 1288 polymorphism would result in a conformational modification in the protein. This change could affect FOXN1 interactions with other proteins involved in thymopoiesis. Consequently, we cannot rule out the possibility that the 1288 variant has a functional impact in vivo in the context of a second allelic FOXN1 pathogenic variant. We are currently testing this in additional mouse models.

Our characterization of the human FOXN1 mutations in mouse models has uncovered what we believe to be a novel region at the COOH-terminal end of the DNA-binding domain that uncouples TEC development from keratinocyte differentiation. Comparison of the thymic hypoplasia in the Foxn11089/1089 and Foxn1933/933 mouse lines revealed a critical 5–amino acid stretch in Foxn1 that was deleted as a result of the 1089 mutation in Pt. 1. This 5–amino acid segment is highly conserved with Foxn4, an ancestral gene present in all species that evolved with a thymus or thymus-like structure (thymoid) (Supplemental Figure 4) (16). The thymus/thymoid-like structures are present in cartilaginous fish (sharks) and agnathans (lampreys), but not in cephalochordates (segmented marine animals such as lancelets) (19). Interestingly, the 5–amino acid sequence in the lancelet Foxn4 diverges from that in lamprey, potentially explaining the emergence of the thymus-like structure (16). Possible interactions between this 5–amino acid stretch and distinct transcription factors and/or other DNA-binding proteins could account for the differential function in TECs versus keratinocytes. Although one nuclear localization site (NLS) prediction program suggested that this site was an NLS, we noted that the
Foxn11069 protein could translocate into the nucleus. Further supporting our suggestion that the nuclear localization remains intact with the 15-nt deletion in Foxn11069 is the observation that the compound heterozygous mice (Foxn1935/1069) genocopying Pt. 1 had normal transcript levels of genes necessary for hair follicle extrusion. In most studies to date, Foxn1 appears to have overlapping requirements for both TEC and keratinocyte development (46). To our knowledge, only 1 report has revealed a differential contribution of Foxn1 in TECs versus keratinocytes (18, 47). In the published study, the removal of exon 3 selectively caused thymic aplasia, with no effects on hair shaft or nail bed development (47). It remains unknown how the exon 3 deletion results in the differential effects of Foxn1 on TECs versus keratinocytes.

A second important region in Foxn1 is the transactivation domain, encoded within exons 8–9 (18). The compound heterozygous mutations in Pt. 1 are proximal to this transactivation domain. In addition, Pt. 2 presented with a mutation at 1465, which affects the transactivation domain. The introduction of this 1465 deletion mutation in murine Foxn1 resulted in the expression of a truncated protein that had only 18% normal transcriptional activity, even though the DNA-binding domain remained intact. This confirms a critical role for the transactivation domain in TEC development, possibly via interactions with a yet-to-be identified coactivator. Alternatively, the mutations near the transactivation domain may provide additional DNA-binding specificity for induction of TEC-selective genes, and keratinocytes may not require this binding specificity.

Several additional patients were identified through SCID screening of newborns with low TREC numbers and were found to have mutations on just 1 allele of FOXN1. These mutations affected multiple regions in FOXN1, including the DNA-binding and/or transactivation domains (Table 2 and Supplemental Table 2). Although not predicted to be causal to a SCID phenotype because of the presence of 1 WT allele, such mutations could lead to reduced T cell output, either during stress at birth or through aging. In mouse models, hypomorphic mutations in murine Foxn1 result in thymic involution as the mice age, contributing to reduced T cell output (42, 45, 48, 49). It remains to be seen whether patients with single-allele mutations in FOXN1 have an earlier onset of thymic involution at birth due to stress and later in life. Although our comparisons of 24-week-old Foxn1 heterozygous mice (Foxn1935/Wt) failed to reveal evidence of premature thymic involution, the impact of stress on thymopoiesis still needs to be assessed in these mice (50). Among the patients with single-allele mutations were 2 individuals with the same mutation as that in Pt. 2, at position 1465. Pt. 7 has a mutation in the DNA-binding region of FOXN1 and was clinically reported to have very low T cell numbers and normal hair and nail beds. Such findings suggest that additional polymorphisms and/or mutations in noncoding regions of FOXN1 (intron, promoter, enhancer elements) could contribute to the low T cell counts noted in those with only 1 affected allele. Alternatively, mutations in other genes coupled to FOXN1 function may further compound the effects of the identified single-allele mutations, as exemplified with the reported DOCK8 and/or CHD7 mutations in 2 of the patients (Table 2).

In summary, we describe what we believe to be a new clinical presentation of a primary immunodeficiency related to compound heterozygous mutations in FOXN1, causing TδBδNK− SCID without the typical nude phenotype. Therefore, a careful structure-function analysis is warranted before assuming that low TREC and T cell counts in patients are caused by FOXN1 mutations, particularly in individuals with monoallelic mutations. Consideration of maternal T cell engraftment is also important if most of the cells are memory T cells and a low TREC output is maintained. A challenge is to know whether a given FOXN1 mutation may allow for the development of sufficient numbers of T cells to prevent infections. Thymus transplantation should not be undertaken if the T cell numbers are likely to increase with time (51, 52). In view of the likelihood that the 2 alleles of FOXN1 produce proteins that function independently, patients with single-allele mutations should be followed at least for several months to a year prior to considering thymus transplantation.

Methods

Antibodies, oligonucleotides, and plasmids. The antibodies used in the experiments as well as their sources and the plasmids used for transfections are listed in Supplemental Table 4. Oligonucleotide sequences used for genotyping, PCR reactions, qRT-PCR reactions, and sequencing are listed in Supplemental Table 5. The murine Foxn1 cDNA was cloned from genomic DNA using E14.5 fetal thymi isolated from C57BL/6 timed pregnant mice. The sequence was confirmed by Sanger sequencing after the RT-PCR product was subcloned into the pCR2.1-TOPO TA cloning vector (Thermo Fisher Scientific). The murine Foxn1 cDNA was subcloned into the pcMV-FLAG-vector (MilliporeSigma) using the Bgl II/Kpn I restriction enzyme cloning sites. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The human FOXN1 mutations were introduced into the murine equivalent location with complementary oligonucleotide primers, which are listed in Supplemental Table 5. All mutated sequences were confirmed with DNA sequencing. Exon 8 from genomic DNA obtained from Pt. 2 was prepared and subcloned by PCR reactions using LA-Taq (Takara). The PCR reactions were resolved on agarose gels, and a 479-bp fragment was isolated and subcloned into pCR2.1 using the TOPO-TA Cloning System (Thermo Fisher Scientific). Eighteen independent clones were expanded and used for sequencing with T7 primers.

Thymocyte and EC preparations. Thymocyte and peripheral T cell populations were processed and stained for flow cytometric analyses as previously described (53, 54). TECs were isolated from individual thymic lobes by digestion in Liberase (Roche) in the presence of DNase I (Roche) as described previously (55). The cells were stained with antibodies against CD45 (Tombo Scientific), MHC CII (I-A/I-E) (Tombo Scientific), EpCAM (eBioscience), BP-1 (eBioscience), and UEA-1 (Vector Laboratories). Samples were analyzed on a FACSCanto II Flow Cytometer (BD Bioscience). FlowJo software (Tree Star) was used to analyze the data. TEC subsets were analyzed by selection of CD45 EpCAM+ MHCII+ cells with either UEA1+BPI for medullary TECs or BPI+UEA1+ for cortical TECs. MHC II+ and MHC II− cells were used to discriminate between the 2 cortical and 2 medullary TEC subsets. Thymocyte subsets and peripheral T cells were analyzed for cell-surface expression of various proteins including CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, CD44, CD45, CD69, B220, NK1.1, αβ TCR, γδ TCR, and Ter119 (see Supplemental Table 4 for antibody sources).

RNA isolation and analysis. RNA was isolated from adult thymic tissues and P4 skin with miRNAeasy kits (QIAGEN). The tissues were
initially homogenized with a Dounce homogenizer in the presence of QIAzol (QIAGEN). For embryonic thymi, a miRNA isolation kit (Ambion, Thermo Fisher Scientific) was used to isolate the RNA. Contaminating DNA from all the RNA preparations was eliminated by DNase I digestion (Ambion, Thermo Fisher Scientific) using on-column digestion and/or DNase treatment followed by purification with an RNA clean-up step (RNA-5 Quick and Clean, Zymo Research Inc.). cDNA was generated with 100 ng to 1 μg RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Random priming was used for reverse transcription. qRT-PCR was performed with 100 ng cDNA using SYBR Green (Thermo Fisher Scientific). The various PCR reactions and conditions were performed as described previously (56). For gene expression comparisons, the Affymetrix Murine Clarion S mouse array was used, which focuses on 20,000 well annotated genes (Thermo Fisher Scientific). The data on comparisons of the differentially expressed transcripts are available in the Gene Expression Ontology (GES) database (GES 134458; https://bioportal.bioontology.org/ontologies/GEXO).

**IHC and Western blot analysis.** Thymic tissue was fixed and embedded in paraffin. H&E staining was performed as standard protocol, and tissue was imaged on an Axiovert 200M inverted fluorescence microscope. For immunofluorescence staining, sections were incubated with rabbit anti-cytokeratin 5 (1:100, Abcam) and mouse anti-cytokeratin 8 (1:100, BioLegend) at 4°C overnight. The next day, sections were washed with PBS and stained with goat anti–mouse AF488 and donkey anti–rabbit AF555 secondary antibody (1:200, Invitrogen, Thermo Fisher Scientific). Tissues were then stained with DAPI and mounted with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were taken on a Leica confocal microscope. Western blotting was performed as described elsewhere using RIPA lysis procedures with antibodies against Foxn1, GAPDH, and the FLAG epitope listed in Supplemental Table 4 (87).

**Transfections and luciferase reporter assays.** A luciferase reporter containing the promoter of the Psmb11 gene was provided by S. Zuklys and G. Holländer (University of Oxford, Oxford, United Kingdom) (2). The Psmb11 luciferase reporter construct (0.5 μg) was cotransfected into HEK 293T cells (2.5 × 10⁵ cells/well) together with pCMV-FLAG (0.5 μg) expression vectors containing Foxn1 WT or mutants, using Fugene 6 Reagent (Promega). A separate construct containing β-gal (0.1 μg) was included in the transfections to normalize each well for transfection efficiency. Forty-eight hours after transfection, the cells were harvested and the luciferase activity measured using a luciferase assay kit (Promega). Luciferase activity was normalized to β-gal activity, which was used as an internal control.

**Statistics.** Statistical analyses were performed using GraphPad Prism software, versions 7.0d and 8.2 (GraphPad Software). A P value of less than 0.05 was considered statistically significant. For Figure 2D, Figure 3D, and Figure 4D, a Brown-Forsythe and Welch’s 1-way ANOVA was applied instead of an ordinary 1-way ANOVA, since the variances were quite extreme, because the various knockin mouse groups had extreme differences. Applied instead of an ordinary 1-way ANOVA, since the variances were rather unusual, we used a Brown-Forsythe and Welch’s 1-way ANOVA. A P value of less than 0.05 was considered statistically significant. For Figure 2D, Figure 3D, and Figure 4D, a Brown-Forsythe and Welch’s 1-way ANOVA was applied instead of an ordinary 1-way ANOVA, since the variances were quite extreme, because the various knockin mouse groups had extreme differences. For the various Foxn1-targeted mice were generated with the help of Robert Hammer of the Transgenic and Knockout Core at UT Southwestern Medical Center. Salius Züklys and Georg Holländer (University Children’s Hospital and University of Basel, Basel, Switzerland) provided the βS1 transcriptional reporter construct. We further acknowledge the following physicians who provided genetic and clinical data on deidentified individuals with diverse FOXN1 mutations. Patient information was provided to M. Louise Markert at Duke University (Durham, North Carolina, USA); The physicians included Jennifer Heimall (Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania, USA); Elena Perez (Allergy Associates of the Palm Beaches, North Palm Beach Florida, USA); John Bohnsack and Karin Chen (University of Utah Health, Salt Lake City, Utah, USA).

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**Author contributions**

MTDLM and CMS provided care for Pt. 1 and Pt. 2, respectively. MLM, GTP, and TRY acquired deidentified genetic data under an IRB-exempt protocol for Pt. 3–Pt. 9, who were found to have mutations in FOXN1. QD, LKH, MLM, MDLM, and NSCVO conceived the experiments. QD, LKH, FC, ID, PR, EM, MAK, and NSCVO performed the experiments. ID, PR, and SK analyzed the RNA-Seq and array data. QD, LKH, FC, PR, ID, GTP, TRY, MLM, MTDLM, CAW, and NSCVO analyzed the data. QD, MDLM, and NSCVO wrote the manuscript. MLM and CMS reviewed the manuscript.
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