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–/loB+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+β 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.

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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The importance of \( \text{FOXXN1} \) in TEC and keratinocyte development was first uncovered in a spontaneously arising nude phenotype in mice (\( \text{nu/nu} \)), 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 structural defects of the hair shaft and nail beds (12, 20, 21). This is the basis of the T–B+NK+ nude/SCID phenotype. Due to the role of Foxn1 in TECs, which is independent of 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 heterogeneous 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 TREC levels. The subsequent clinical work-up was consistent with a typical T–B+NK+ SCID phenotype, using the normal age-matched reference range (Table 1) (36, 37). Exome sequencing revealed 2 mutations in the FOXN1 gene, a duplication in patient 1 (Pt. 1) and patient 2 (Pt. 2) as well as in several unrelated patients with heterogeneous mutations, revealed varying transcriptional activities depending on the location and type of mutation. These findings provide structure-function insights into this key transcription factor.
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.Cys) followed by a 5–amino acid deletion (p.Trp363_Pro367delin-frame). This resulted in a single amino acid conversion (Trp to Cys) (Figure 1, A and C). The deletion did not change the reading frame. The clinical course for Pt. 1 was characterized by rhinoenteroviral infection at 2 months of age, norovirus enteritis and parainfluenza virus from parainfluenza virus. The 1089 mutation was inherited from the mother, whereas the c.1465delC variant was de novo.

The FOXN1 mutations described for both Pt. 1 and Pt. 2 are distinct from the classic autosomal-recessive mutations originally identified in FOXN1 (Figure 1C) (22–26). To date, previously identified mutations on other genes associated with SCID or cellular immune deficiency 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 FOXN1 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.

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 1 yr of age from parainfluenza virus.</td>
</tr>
<tr>
<td>Pt. 1</td>
<td>c.1089_1103delT5</td>
<td>p.W363C</td>
<td>31%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Multiple viral infections, failure to thrive. Died at 1 yr of age from parainfluenza virus.</td>
</tr>
<tr>
<td>Pt. 2</td>
<td>c.1288C&gt;T</td>
<td>p.P4305</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. 2</td>
<td>c.1465delC</td>
<td>p.R489fsX61</td>
<td>18%</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>No</td>
<td>Healthy, no recurrent infections.</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>No</td>
<td>Developed S. aureus submandibular abscess in infancy; otherwise healthy.</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>952A&gt;G</td>
<td>p.H21R</td>
<td>82%</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Died in infancy from coronavirus encephalitis. Also had a heterozygous DOCK8 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>No</td>
<td>Healthy, no recurrent infections.</td>
</tr>
<tr>
<td>Pt. 9</td>
<td>1075G&gt;C</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_1206delT6</td>
<td>p.P401AfsX144</td>
<td>3%</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Also had heterozygous DOCK8 and CHD7 mutations with no congenital anomalies.</td>
</tr>
<tr>
<td>Pt. 11</td>
<td>1201_1206delT6</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_1206delT6</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_1206delT6</td>
<td>p.P401AfsX144</td>
<td>3%</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Healthy, no recurrent infections.</td>
</tr>
<tr>
<td>Pt. 14</td>
<td>1201_1206delT6</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>No</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.P473HisX77</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.
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, TCRβ, 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 thymocytes. mouse line (Figure 3A). These findings firmly establish that the clinical phenotypes of Pt. 1 resulted from compound heterozygous mutations in FOXN1.

**Figure 2. Human FOXN1 compound heterozygous mutations genocopied in mice cause thymic aplasia with normal fur and nails.** (A) The human FOXN1 mutations for Pt. 1 were introduced into the murine genome by CRISPR-Cas9 technology. The DNA repair template used for each allele is shown. Silent mutations were introduced into the murine sequence to facilitate genotyping and to prevent premature stop codons. (B) Images of F2-generation mice: WT Foxn1 (Foxn1WT/WT), homozygous mutant (Foxn1933/933 and Foxn11089/1089), and compound heterozygous (Foxn1933/1089) mice, the latter genocopying Pt. 1. The genocopied mice are indicated in red font. The images are representative of 5 independently characterized mice. (C) The overall sizes of the thymi from the various mouse lines are shown for comparative purposes. (D) Thymus weights and overall thymic cellularity were calculated. Data represent the mean ± SEM. n = 22 Foxn1WT/WT, n = 6 Foxn1933/933, n = 10 Foxn11089/1089, and n = 10 Foxn1933/1089 mice. P values of less than 0.05 were considered significant. For the comparisons shown, a Brown–Forsythe and Welch’s 1-way ANOVA was applied.

zygous mice had pronounced thymic hypoplasia, with a 10- and 17-fold reduction in thymic 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 (Foxn1WT/1089 and Foxn1WT/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, TCRβ, CD25, CD44, CD45, and CD69 by flow cytometry. As shown in the littermate controls (Foxn1WT/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 Foxn1933/933 mouse line (Figure 3A). The DN and CD8lo 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 Foxn1 locus lead to some differentiation of thymocytes to the CD4+CD8+ stage, at which point there was a developmental block. Thymopoiesis in the Foxn1933/933 mice, genocopying nu/nu mice, which was devoid of thymocytes (38). The Foxn1933/933 homozygous knockin mice had an increased percentage of DP thymocytes (90%–95%) with severely reduced SP subsets compared with controls. This result suggests that homozygous mutations at the Foxn1 locus lead to some differentiation of thymocytes to the CD4+CD8+ stage, at which point there was a developmental block. Thymopoiesis in the Foxn1933/1089 mice, genocopying Pt. 1, was more severely affected. A significant reduction in the percentage of both DP and SP subsets was obvious, albeit not as extreme as that seen in the Foxn1933/933 mouse line, a finding consistent with the presence of a small thymic structure (Figure 2C). These data confirm that the distinct Foxn1 mutations have differential consequences for early T cell development and positive selection.

Figure 3. Thymic hypoplasia results from compound heterozygous mutations in Foxn1. (A–D) WT Foxn1 (Foxn1WT/WT), homozygous mutant (Foxn1933/933, Foxn11089/1089), and compound heterozygous (Foxn1933/1089) mice, the latter genocopying Pt. 1, were obtained by intercrossing the Foxn1933/933 and Foxn11089/1089 lines. The thymus was isolated and single-cell suspensions prepared for flow cytometry. (A) Fluorochrome-labeled antibodies against CD4 and CD8 were used to detect DN, DP, and SP thymocyte subsets. (B) The progression of thymocytes from the DN1–DN4 stages of thymocytes was assessed by cell-surface staining for CD44 and CD25 following exclusion of cells expressing CD3, CD4, CD8, B220, NK1.1, TCRβ, Ter119, CD11b, and CD11c. (C) Positive selection of thymocytes was assessed by staining thymocytes for TCRβ and CD69. (D) Graphs show the percentage of various cell subsets, determined from pooled experiments using a minimum of 5 mice per line. Data indicate the mean ± SEM. n = 22 Foxn1WT/WT mice, n = 6 Foxn1933/933 mice, n = 6 Foxn11089/1089 mice, and n = 5 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. ****P < 0.0001 for most comparisons, with the following exceptions: P = 0.002 for DN comparisons of Foxn1933/1089 versus Foxn1933/933; P = NS for DP comparisons of Foxn1933/1089 versus Foxn1933/1089 and Foxn1933/1089 versus Foxn11089/1089; P = 0.002 for CD4+ SP Foxn1933/1089 versus Foxn1933/1089 and Foxn1933/1089 versus Foxn1933/1089, P = NS for Foxn1933/1089 versus Foxn11089/1089, P = 0.05 for CD8+ SP Foxn1933/1089 versus Foxn1933/1089, comparisons for DN1–DN4 were as follows: P = NS for DN1 differences between Foxn1933/933 and Foxn11089/1089, P = 0.008 for DN2 comparisons of Foxn1933/933 versus Foxn1933/1089 versus Foxn11089/1089, P = 0.002 for DN3 comparisons of Foxn1933/933 versus Foxn1933/1089 versus Foxn11089/1089, P = 0.002 for DN3 comparisons of Foxn1933/933 versus Foxn1933/1089 versus Foxn11089/1089, P = NS for DN2 comparisons of Foxn1933/933 versus Foxn1933/1089 versus Foxn11089/1089, P = NS for DN3 comparisons of Foxn1933/933 versus Foxn1933/1089 versus Foxn11089/1089, P = 0.002 for DN4 comparisons of Foxn1933/933 versus Foxn1933/1089 and P = NS for DN4 comparisons of Foxn1933/933 versus Foxn1933/1089. ND, not detected; PE, phycoerythrin.
heterozygous mouse line (Foxn1<sup>933/933</sup>) and in one of the homozygous mutant lines (Foxn1<sup>1089/1089</sup>) (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 (Foxn1<sup>933/933</sup> mice) and hypoplasia (Foxn1<sup>1089/1089</sup> and Foxn1<sup>933/1089</sup> lines) is an almost complete loss of mature CD4+ and CD8+ SP 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 (Foxn1<sup>WT/933</sup> and Foxn1<sup>WT/1089</sup>) 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 Foxn1<sup>WT/WT</sup>, n = 5 Foxn1<sup>933/933</sup>, n = 11 Foxn1<sup>1089/1089</sup>, and n = 21 Foxn1<sup>933/1089</sup> mice. The percentages of NK1.1+ TCRβ– cells were calculated; n = 26 Foxn1<sup>WT/WT</sup>, n = 3 Foxn1<sup>933/933</sup>, n = 11 Foxn1<sup>1089/1089</sup>, and n = 11 Foxn1<sup>933/1089</sup> 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<sup>933/933</sup> and Foxn1<sup>1089/1089</sup> 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<sup>933/933</sup> and Foxn1<sup>1089/1089</sup> mice relative to what we observed in the littermate controls (Figure 5B). Thymi from the Foxn1<sup>1089/1089</sup> 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<sup>WT/WT</sup> mice had
The insertion of the 4-nt sequence identified in Pt. 1 (Foxn1933) and performed protein or deletions into the cDNA of murine expression and function of the protein, we introduced substitutions distinct approximately 13% and 81% cTECs and mTECs (EpCam+CD45–), DNA-binding and transactivation domains.

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 functional-ity for TEC development. These functional impairments were dependent on the location and type of mutation. (A–C) Transient transfection assays in HEK293T cells were performed with expression vectors for WT Foxn1 or Foxn1 constructs harboring the indicated mutations that matched the FOXN1 mutations identified in patients. The mutations were divided into those identified in Pt. 1 (A) (Foxn11089, Foxn11288) and Pt. 2 (B) (Foxn11288, Foxn11465) as well as the indicated controls. Forty-eight hours after transfection, the cells were lysed, and the proteins were extracted and resolved by SDS-PAGE. Western blotting was performed with antibodies against Foxn1, followed by antibodies detecting GAPDH, which was used as a loading control.

The transcriptional activity of Foxn1 is regulated by both the transcriptional reporter assays using the promoter sequence for the gene, which encodes β2m (2). We fragmented cTECs and mTECs (EpCam+CD45–) 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 IIα (MHC CIIα) and more mature MHC class IIβ (MHC CIIβ) cells (Figure 5, D and E). We detected a significant reduction in the percentage of MHC CIIα cells in the Foxn11089/1089 mice, with expression vectors for WT Foxn1, followed by antibodies detecting GAPDH, which was used as a loading control.

We characterized FOXN1 with SNPs (FOXN1224, FOXN1982, FOXN11293, FOXN11418, FOXN11465) and deletions (FOXN11201,1216, FOXN11296delC, FOXN11436delC), 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 Psmb11 gene, which encodes β2m (2). We found that Foxn1982 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 (Foxn11465) and deletions (FOXN11201,1216, FOXN11296delC, FOXN11436delC), 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 Psmb11 gene, which encodes β2m (2). We found that Foxn1982 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 (Foxn11465) and deletions (FOXN11201,1216, FOXN11296delC, FOXN11436delC), 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).
identical to that of the classic \textit{nu/nu} mouse, consistent with an essential role for the \textit{FOXN1} DNA–binding domain in both TEC and keratinocyte differentiation and gene expression. Contrasting with this, the \textit{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 \textit{Foxn1}^{933/1089} mutations transcriptionally affect TECs differently than they do keratinocytes. We considered 2 explanations for the divergent consequences of harboring \textit{Foxn1}^{933/933} versus \textit{Foxn1}^{933/1089} genotypes for hair and nail

reducing the functional activities of \textit{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 \textit{Foxn1} mutation (Figure 1C and Table 2).

\textit{FOXN1} differentially regulates genes in the thymus versus the skin. The \textit{Foxn1}^{933/933} homozygous knockin mice have a phenotype identical to that of the classic \textit{nu/nu} mouse, consistent with an essential role for the \textit{FOXN1} DNA–binding domain in both TEC and keratinocyte differentiation and gene expression. Contrasting with this, the \textit{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 \textit{Foxn1}^{933/1089} mutations transcriptionally affect TECs differently than they do keratinocytes. We considered 2 explanations for the divergent consequences of harboring \textit{Foxn1}^{933/933} versus \textit{Foxn1}^{933/1089} genotypes for hair and nail

Figure 7. Differential functions of \textit{Foxn1} in TECs versus keratinocytes. (A) Gene expression comparisons were made with 3 independently isolated fetal thymi, obtained from E13.5 embryos of the indicated genotypes. The presence of a hypoplastic thymi was confirmed by subsequent genotyping to select for \textit{Foxn1}^{933} and \textit{Foxn1}^{1089} alleles. A heatmap revealed a subset of up- and downregulated genes, with a selection criterion of a 1.5-fold difference as a cut-off. (B) Data on differentially expressed transcripts, particularly those with reported functions in embryonic thymic development. (C) Images of postembryonic day–4 mice of the indicated genotypes were processed with bright-field imaging on a dark background, revealing hair and fur extending out from the skin. Scale bars: 1 mm. (D and E) qRT-PCR was performed to compare the levels of genes previously reported to be involved in hair shaft extension. This was performed to compare (D) 3 littermate controls and 3 \textit{Foxn1}^{933/933} mice and, in a separate experiment, (E) 3 controls and 3 \textit{Foxn1}^{933/1089} mice. The data shown reflect results from 1 of 2 independent experiments with 2 to 3 mice per group. *\(P = 0.02\), **\(P = 0.001\), and ***\(P = 0.00006\), by Student’s t test. There were no significant differences in any of the transcripts compared between the 2 mouse lines in E.
phenotypes. First, the FOXN1<sup>933/1089</sup> 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 (Foxn1<sup>WT/WT</sup>) and 3 hypoplastic thymi (Foxn1<sup>933/1089</sup>) (Figure 7A). Gene array comparisons revealed 417 upregulated and 500 downregulated genes in the Foxn1<sup>933/1089</sup> line relative to the littermate controls (Figure 7A). These differences were not surprising, given the severe hypoplasia evident in the Foxn1<sup>933/1089</sup> 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 Foxn1<sup>933/933</sup> pups showed no hair follicle extrusion (Figure 7C). In contrast, the hair in the Foxn1<sup>933/1089</sup> 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 in the transcript changes in embryonic hypoplastic thymi (2).

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 Foxn1<sup>933/1089</sup> and Foxn1<sup>991/991</sup> 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
Foxn1\textsuperscript{1089} protein could translocate into the nucleus. Further supporting our suggestion that the nuclear localization remains intact with the 15-nt deletion in Foxn1\textsuperscript{1089} is the observation that the compound heterozygous mice (Foxn1\textsuperscript{1089/1089}) genocopying Pt. 1 had normal transcript levels of genes necessary for hair follicle apoptosis. 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 (Foxn1\textsuperscript{1089/W}) 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 functions 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 T-\(\gamma\)B’NK- 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 thymus isolated from C57BL/6 timed pregnant mice. The sequence was confirmed by Sanger sequencing after the RT-PCR product was subcloned into the pcGR2.1-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 QuickChange 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 17 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 (Tonbo Scientific), MHC CII (I-A/I-E) (Tonbo 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 flow data. TEC subsets were analyzed by selection of CD45\textsuperscript{+} MHC CII\textsuperscript{lo} cells with either UEA1\textsuperscript{+} or B220\textsuperscript{+} for cortical TECs. MHC CII\textsuperscript{hi} and MHC CII\textsuperscript{lo} 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, \(\gamma\)TcR, \(\delta\)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 miRVANA RNA 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 in-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 (GEO) database (GEO 134458; https://biportal.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 (57).

**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 extremely low cell numbers and percentages relative to the control Foxn1WT/WT line (ordinary ANOVA assumes equal variances). For Figure 6, C and D, a normal 1-way ANOVA analysis was applied. Standard Student’s t tests were used for data analysis in Figure 5F and Figure 7, D and E.

**Study approval.** The IRB of UT Southwestern Medical Center approved this study (approval nos. 072010-009 and 112010-015). MLM, GTP, and TRY acquired deidentified genetic data under an IRB-exempt protocol for Pt. 3–Pt. 9, who were taken after initialization was approved by the Duke University Health System. The data were forwarded by various clinicians throughout the United States. Animal experiments described in this work were approved by and conducted with oversight of the IACUC of UT Southwestern (APN nos. 2015-101247 and 2015-101163). Mice were housed in a specific pathogen-free facility at UT Southwestern Medical Center. The Foxn1-targeted mice were developed entirely on a C57BL/6 background. Although more than 40 founders were screened for each construct, 2 independent mouse lines were expanded for each of the mutations generated by CRISPR-Cas: the Foxn1WT mutations (Foxn1-933 no. 17 and Foxn1-933 no. 30) and the Foxn1mutations (Foxn1-1089 no. B4 and Foxn1-1089 no. B15). The mice are described further in Supplemental Table 4. The Foxn1 mouse lines were crossed for 1 to 2 generations with C57BL/6 mice prior to intercrossing.
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42. Chen L, Xiao S, Manley NR. Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner. Blood. 2009;113(3):567–574.