

CI, 1.01-2.41). Children with increased *FOXP3* methylation were also 2 times more likely to have asthma than children with lower *FOXP3* methylation (OR, 2.12; 95% CI, 1.14-3.85).

This study provides the first evidence that *FOXP3* methylation is associated with chronic DEP exposure during childhood and increased risk for persistent wheezing and asthma. Furthermore, we were able to identify epigenetic changes associated with environmental exposure and respiratory disease in saliva. This is an important finding because it represents a less invasive and inexpensive procedure for collecting DNA, which is highly conducive to large epidemiologic studies of children. These findings support future strategies targeting Treg cell expansion through boosting *FOXP3* upregulation for asthma prevention/intervention.

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Combined *DOCK8* and *CLEC7A* mutations causing immunodeficiency in 3 brothers with diarrhea, eczema, and infections

To the Editor:

We report 4 brothers from a consanguineous Italian family with an immunodeficiency syndrome characterized by severe eczema, milk and egg allergies, recurrent infections, intractable diarrhea, failure to thrive, and, in 2 of the 3 surviving brothers, lymphoma. One of the brothers (patient II-2) died at age 12 months of cardiovascular failure caused by intractable diarrhea, and the other 3 were evaluated as part of this study (Table I and Fig 1, A). The proband (patient II-1) presented shortly after birth with severe eczema and intractable diarrhea without gastrointestinal infection. In the first year of life, he had multiple allergies to milk, soy, and egg proteins. He had repeated episodes of pneumonia, leading to bilateral

TABLE I. Clinical features of patients

	Patient II-1	Patient II-2	Patient II-3	Patient II-4	DOCK8 features	CLEC7A features	IPEX syndrome features
Age at genetic diagnosis (y)	27	NA	16	13			
Sex	Male	Male	Male	Male	Male and female	Male and female	Male
Eczema (age of onset)	6 mo	Yes	6 mo	NA	Yes	No	Yes
Food allergies	Multiple	NA	Multiple	Multiple	Yes	No	Rare
Diarrhea (age of onset)	6 mo	Yes	6 mo	6 mo	Rare	No	Yes
Pneumonia	2 episodes	NA	2 episodes	No	Yes	No	Rare
Meningitis/sepsis	Yes	NA	Yes	No	Yes	No	Rare
Upper respiratory tract infections	Yes	NA	Yes	Yes	Yes	No	Rare
Bronchiectasis	Yes	NA	No	No	Yes	No	No
<i>Candida</i> species infection	Yes	NA	No	No	Yes	Yes	No
Lymphoma	Yes, B cell	NA	Yes, T cell	No	Yes	No	No
Cutaneous viral infections	No	NA	No	No	Yes	No	No
Early-onset diabetes	No	NA	No	No	No	No	Yes
Outcome	HSCT	Deceased	Deceased	HSCT			

HSCT, Hematopoietic stem cell transplantation; NA, not applicable.

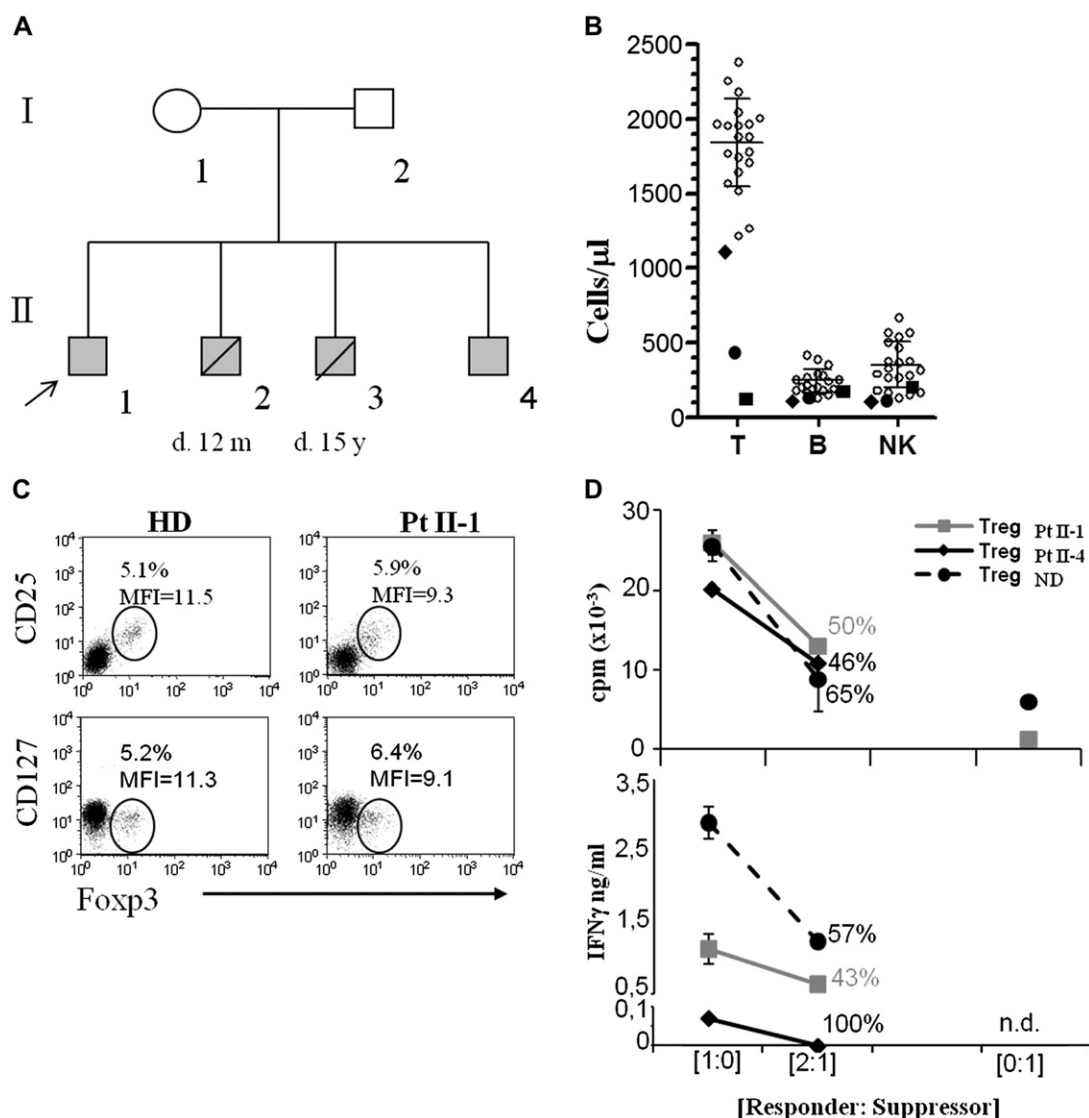


FIG 1. **A**, Family tree. The proband is indicated by the arrow. **B**, Scatter plot of absolute counts of T, B, and natural killer (NK) cells (solid diamond, patient II-1; solid circles, patient II-3; solid square, patient II-4) compared with 20 age-matched healthy donors (open circles; means $\pm$ SDs). **C**, *FOXP3* expression in CD4⁺ of patient II-1 versus a healthy donor. *MFI*, Mean fluorescence intensity. **D**, Capacity of CD4⁺CD25⁺ Treg cells from patients II-1 and II-4 to suppress the autologous CD4⁺CD25⁺ T-cell response as inhibition percentage of tritiated thymidine incorporation (upper panel) and IFN- γ production (lower panel).

bronchiectasis at 16 years of age. At age 25 years, he had lymphoma involving the tonsils, lungs, and spleen. This was defined as a high-grade, large-cell, B-cell lymphoma (EBV negative) and was successfully treated with immune chemotherapy. At age 27 years, he had *Pseudomonas aeruginosa*-induced sepsis. At age 28 years, he underwent successful transplantation with hematopoietic stem cells from a matched unrelated donor.

Likewise, a younger sibling (patient II-3) presented at 6 months of age with allergy to milk proteins and intractable diarrhea, despite a milk protein-free diet. He had 2 episodes of pneumonia and several upper respiratory tract infections. At age 15 years, he had an anaplastic peripheral T-cell lymphoma involving the skin, lymph nodes, liver, and spleen, which proved refractory to chemotherapy. The patient died of infection while receiving high-dose methotrexate and cytarabine.

Patient II-4 was fed with an extensively hydrolyzed formula but presented with noninfectious diarrhea as an infant. He had respiratory tract infections and otitis. At age 13 years, he underwent successful transplantations with hematopoietic stem cells from a matched unrelated donor.

Immunologic studies in the 3 siblings surviving infancy showed severe T-cell lymphopenia (160–1140 cells/ μ L), with normal relative percentages of B and natural killer cells (Fig 1, B). Immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (OMIM no. 304790) was suspected,^{1,2} but forkhead box protein 3 (*FOXP3*) sequencing did not reveal any mutation in the coding region except a previously reported but silent single nucleotide polymorphism (SNP) at serine 181 (543C>T, rs2232367 in the National Center for Biotechnology Information dbSNP database) in all of the affected siblings.

A previous study had suggested this variant to be associated with IPEX syndrome.³ However, analysis of 100 chromosomes from healthy subjects showed the allele frequency of rs2232367 to be 5% in the Italian population. Sequencing of reverse transcribed mRNA did not reveal any splicing variant of *FOXP3* (data not shown), and expression of *FOXP3* protein was normal in CD25⁺/CD4⁺ T cells in the proband (Fig 1, C). Next, we examined regulatory T (Treg) functional properties of CD4⁺CD25^{hi} Treg cells and CD4⁺CD25^{lo} effector cells from 2 of the siblings surviving infancy (the proband II-1 and sibling II-4). Treg cells isolated by means of magnetic bead cell sorting in parallel with those of a healthy donors and stimulated with anti-CD3 mAb in the presence of allogeneic antigen-presenting cells strongly suppressed both the proliferation and IFN- γ secretion of cocultured autologous effector T cells at a ratio (responder/suppressor) of 2:1 (Fig 1, D). The proliferative capacity of Treg cells from the proband was also examined, revealing them to be anergic. Altogether, these genetic and functional data ruled out the diagnosis of IPEX syndrome caused by *FOXP3* mutation.

Genome-wide genotyping of SNPs in the 3 siblings and their mother with the Affymetrix 6.0 microarray (Affymetrix, Santa Clara, Calif) and analysis with GenCol⁴ revealed regions with an identical genotype, including chromosomes 9p24 and 12p13 (see Figs E1, A, and E2 in this article's Online Repository at www.jacionline.org), suggesting an autosomal recessive disorder. Exome enrichment (37.7 million nucleotides; Agilent SureSelect version 1; Agilent, Santa Clara, Calif), and next-generation sequencing were performed on the proband, as previously described.⁵ Mean enrichment of the exome was 44-fold. Next-generation sequencing yielded 171 million sequences, each of which was 130 nucleotides long. Approximately 95% of sequences aligned to the reference human genome uniquely, covering each exonic nucleotide approximately 135 times. Approximately 1.45% of target nucleotides were not sequenced, whereas approximately 88.3% had at least 20-fold sequence coverage. A bioinformatic decision tree was used to identify nucleotide variants in the aligned sequences.⁶

Homozygous variants were detected in 2 genes in the proband (see Fig E1, B and C). The first was a known nonsense mutation in the pattern recognition receptor gene (C-type lectin domain family 7, member A [*CLEC7A*], p.Tyr238X, rs16910526) that signals through caspase recruitment domain-containing protein 9 (see Fig E3, C, in this article's Online Repository at www.jacionline.org). This mutation has been reported to cause familial chronic mucocutaneous candidiasis (CANDF4; OMIM no. 613108).⁷ The second was a novel homozygous frameshift variant in exon 27 of the dedicator of cytokinesis 8 (*DOCK8*) gene (c.3193delA). *DOCK8* mutations cause autosomal recessive hyper-IgE syndrome (OMIM no. 243700), which is characterized by T lymphopenia, eosinophilia, increased IgE levels, eczema, recurrent infections, and severe food allergies.^{8,9} Sanger sequencing confirmed homozygosity for both the *DOCK8* and *CLEC7A* variants in the 3 siblings and heterozygosity in their parents. The *DOCK8* mutation is predicted to result in a premature stop codon 17 residues downstream of the deletion (Ser1065AlafsX17). However, Sanger sequencing of *DOCK8* mRNA revealed a small number of transcripts that read through the premature stop codon (see Fig E3, A, and data not shown). Indeed, immunoblots of T cells from the 3 patients showed low-level *DOCK8* protein

expression (see Fig E3, B), suggesting residual protein synthesis by means of alternative splicing.

Exome sequencing, when combined with confirmatory testing, has previously shown efficacy for molecular elucidation of Mendelian diseases. Here we describe another application, namely the identification of potentially causative mutations in 2 or more loci in inherited diseases. Patients with *DOCK8* mutations display skin infections, pneumonia, and increased serum IgE levels but do not usually have diarrhea. The latter is typically associated with IPEX syndrome. The phenotypic severity and enteropathy despite residual *DOCK8* protein expression might reflect the combined defects in 2 genes acting in parallel immunologic pathways. Interestingly, this unusual phenotype partially resembles IPEX syndrome. The prevalence of mutations in 2 genes in apparently Mendelian disorders is not known. Such interactions would not be discovered by using conventional, serial, univariate molecular testing because the *DOCK8* result would be interpreted as a definitive diagnosis, and further molecular evidence would not be sought. We suggest that exome sequencing be considered in patients with atypical presentations of Mendelian diseases for examination of possible underpinning mutations in more than 1 locus.

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The study was approved by the Institutional Review Board of Spedali Civili, Brescia, and informed consent was obtained from the patients and/or the parents.

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ζ Chain-associated protein of 70 kDa (ZAP70) deficiency in human subjects is associated with abnormalities of thymic stromal cells: Implications for T-cell tolerance

To the Editor:

Thymic stromal cells play a critical role in permitting the development, maturation, and selection of T lymphocytes.¹ In particular, cortical thymic epithelial cells (TECs) promote differentiation and positive selection of thymocytes, whereas medullary thymic epithelial cells (mTECs) and dendritic cells (DCs) participate in the negative selection of self-reactive T cells and the generation of regulatory T (Treg) cells (see Fig E1 in this article's Online Repository at www.jacionline.org).^{1,2} Maturation of mTECs is marked by expression of claudin 4 (CLD4), upregulation of HLA-DR, and binding to *Ulex europaeus* agglutinin 1 (UEA-1). A subset of mature mTECs express autoimmune regulator (AIRE), a transcription factor that promotes expression of tissue-restricted antigens, thereby permitting negative selection of T cells recognizing tissue-restricted antigens presented by AIRE⁺ mTECs and thymic DCs.¹ Furthermore, terminally differentiated mTECs in Hassall corpuscles from normal human thymus express involucrin (IVL) and secrete thymic stromal lymphopoietin.^{2,3} This cytokine acts on myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs), which instruct differentiation of natural regulatory T (nTreg) cells expressing the transcription factor forkhead box protein 3 (FOXP3).² Selection of nTreg cells is also contributed by AIRE⁺ mTECs. Therefore cross-talk between thymocytes and thymic stromal cells is essential to establish immune tolerance.

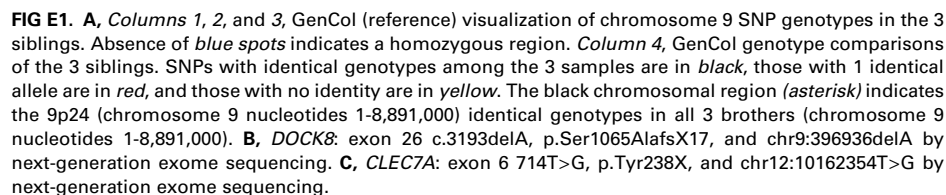
We have previously shown that various forms of severe combined immunodeficiency in human subjects are associated with impaired differentiation of TECs, reduced expression of AIRE, and low numbers of thymic mDCs.⁴ In addition, similar abnormalities are observed in patients with hypomorphic

mutations in severe combined immunodeficiency-causing genes and might contribute to the immune dysregulation of Omenn syndrome.⁴ By contrast, little is known on the thymic stromal compartment in patients with late defects in human T-cell development. We have used morphometric analysis and immunohistochemistry to study the thymic microenvironment of 2 infants with ZAP70 deficiency. Both presented with recurrent infections, had palpable lymph nodes, and had a small-sized thymic shadow detectable by imaging. Characteristic of ZAP70 deficiency, both infants had a selective deficiency of CD8⁺ lymphocytes and preserved generation (full Vβ repertoire) but impaired function of CD4⁺ lymphocytes.⁵ Both patients were previously reported and carried a homozygous intronic mutation that resulted in the creation of a cryptic acceptor splice site and a 9-nt insertion in the ZAP70 mRNA. ZAP70 protein was not detected in patients' lymphocytes, indicating that this truly is a null mutation. Thymic biopsy specimens were obtained without complications by using a minimally invasive technique after obtaining informed consent. The study was approved by the Institutional Review Board of the Hospital for Sick Children, Toronto, Ontario, Canada.

Evaluation of thymic architecture in both patients showed preserved corticomedullary demarcation, as well as the presence of Hassall corpuscles (Fig 1, A), with normal compartmentalization of cytokeratin (CK) 8⁺ CK5[−] cortical TECs and CK8[−] CK5⁺ mTECs (data not shown). However, careful morphometric analysis showed that the thymic medullary area was smaller in ZAP70-deficient (ZAP70^{−/−}) patients than in control subjects (Fig 1, A; ZAP70^{−/−} thymus: 5.52 vs control thymus: 1.05). mTECs from ZAP70^{−/−} patients expressed both CLD4 and the ligand for UEA-1, 2 markers of mature mTECs. Although AIRE⁺ cells were present, their numbers were significantly reduced in ZAP70^{−/−} thymuses (Fig 1, B; ZAP70^{−/−} thymus: 3.3 ± 1.49 vs control thymus: 7.6 ± 1.95; *P* < .001). Hassall corpuscles were present in normal numbers, although some appeared small. Importantly, Hassall corpuscle mTECs from ZAP70^{−/−} thymuses expressed minimal amounts of thymic stromal lymphopoietin and IVL (Fig 1, B), indicating failure to sustain terminal differentiation of mTECs. Of note, staining for AIRE and IVL in control thymuses identified a nonoverlapping set of cells in the thymic medulla, confirming previous observations.³

In the normal thymus FOXP3⁺ thymocytes are abundantly present in the medulla, especially in close proximity to Hassall corpuscles (Fig 1, C, left panels), as previously reported.² A similar pattern was detected also in ZAP70^{−/−} patients, although the number of FOXP3⁺ cells was reduced compared with the number seen in control thymuses (Fig 1, C; ZAP70^{−/−} thymus: 39.3 ± 6.75 vs control thymus: 108.85 ± 10.57; *P* < .001).

Thymic DCs are predominantly located in the medulla and at the corticomedullary junction⁶ and play an important role in negative selection and the generation of nTreg cells.^{1,2} Staining for markers that identify the 3 major thymic DC types (mDCs, pDCs, and Langerhans cells) showed that the number of medullary DCs belonging to each one of these subsets was significantly reduced in ZAP70^{−/−} patients compared with the number seen in healthy control subjects (Fig 2 and see Fig E2 in this article's Online Repository at www.jacionline.org). Depletion of thymic DCs was more prominent in the deep intramedullary area (data not shown) and was especially pronounced for pDCs. Interaction between XCL1 (a chemokine secreted by mature mTECs) and DCs expressing the chemokine receptor XCR1 has been suggested to facilitate trafficking of DCs to the deeper medulla, generation



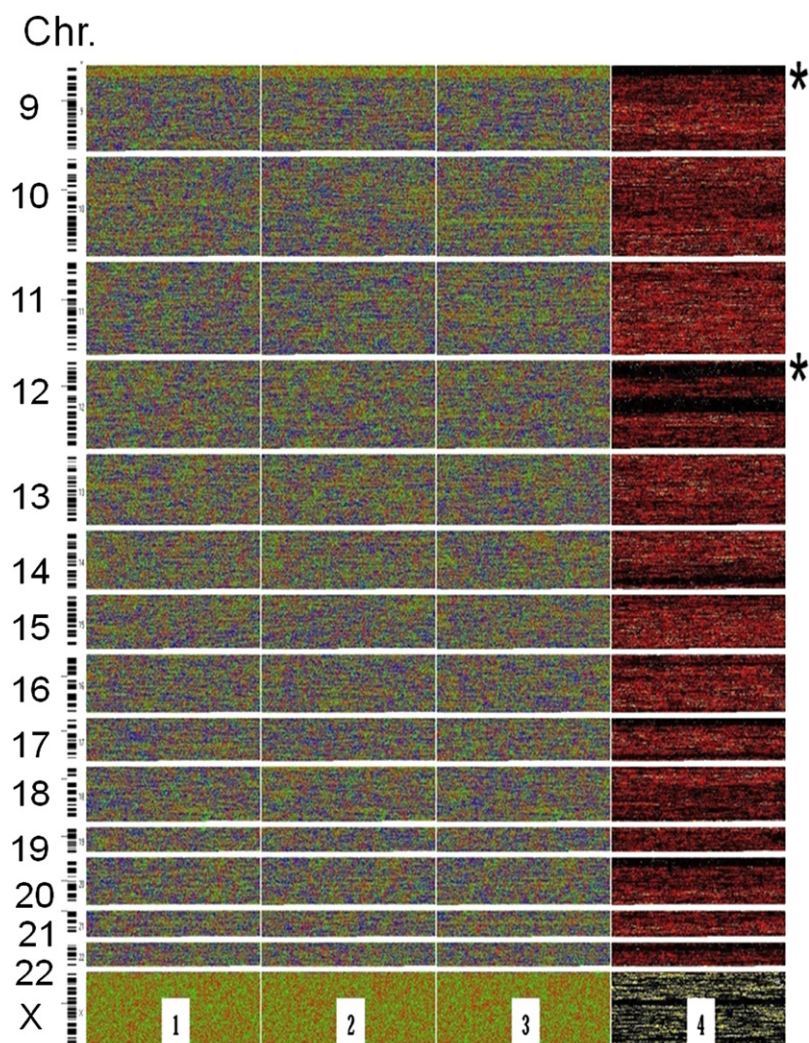


FIG E2. GenCol whole-genome visualization. *Columns 1 to 3* correspond to the genotype of the 3 siblings, whereas *column 4* corresponds to the genotype comparison. In the first 3 columns *red*, *green*, and *blue spots* have been assigned to the AA, BB, and AB genotypes, respectively. Absence of blue pixels for large segments corresponds to regions of homozygosity. In the genotype comparison (*column 4*) the SNPs with identical genotypes between the siblings are in *black*, those with 1 shared allele are in *red*, and those with both alleles different are in *yellow*. The large black areas correspond to chromosomal regions, with identical genotypes in the 3 brothers. The *DOCK8* and *CLEC7A* genes are included in the black regions (*asterisks*) of chromosomes 9 and 12, respectively.

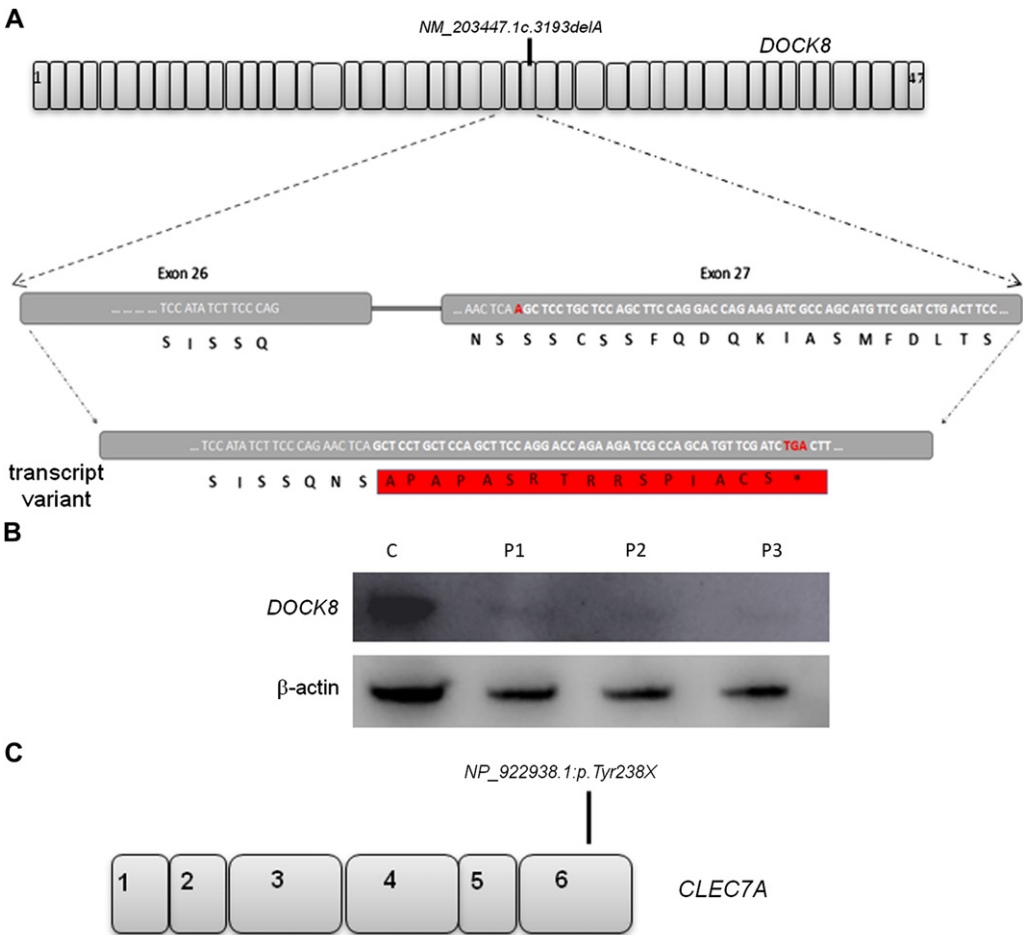


FIG E3. Sanger sequence analysis and protein expression of DOCK8 in patients with primary immunodeficiency. **A**, Structure of DOCK8 mRNA, identification of indel mutation in exon 26, and analysis of DOCK8 transcripts. mRNA isolated from lymphocytes was amplified by using RT-PCR, cloned, and sequenced. **B**, Immunoblots of DOCK8 and β-actin expression in lymphocytes from a control subject (lane C) and the 3 siblings (lanes P1, P2, and P3). **C**, Structure of CLEC7A mRNA identification of nonsense mutation.