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## Abnormal SCID Newborn Screening and Spontaneous Recovery Associated with a Novel Haploinsufficiency *IKZF1* Mutation

Hye Sun Kuehn<sup>1</sup>, Nicholas J. Gloude<sup>2,3</sup>, David Dimmock<sup>4</sup>, Mari Tokita<sup>4</sup>, Meredith Wright<sup>4</sup>, Sergio D. Rosenzweig<sup>1</sup>, Cathleen Collins<sup>3,5</sup>

<sup>1</sup> Immunology Service, Department of Laboratory Medicine, NIH Clinical Center, National Institutes of Health, Building 10, Rm 2C306, 10 Center Drive, MSC1508, Bethesda, MD, USA

<sup>2</sup> Division of Hematology Oncology, Department of Pediatrics, University of California San Diego, San Diego, CA, USA

<sup>3</sup> Rady Children's Hospital San Diego, San Diego, CA, USA

<sup>4</sup> Rady Children's Institute for Genomic Medicine, San Diego, CA, USA

<sup>5</sup> Division of Allergy, Immunology, and Rheumatology, Department of Pediatrics, University of California San Diego, San Diego, CA, USA

### Abstract

**Purpose**—IKAROS, encoded by *IKZF1*, is a member of the IKAROS family of zinc-finger transcription factors playing critical roles in lymphocyte development, differentiation, and tumor suppression. Several studies demonstrated that *IKZF1* mutations affecting DNA binding or homo-/hetero-dimerization are mostly associated with common variable immunodeficiency, combined immunodeficiency, or hematologic manifestations. Herein we report a likely de novo, nonsense *IKZF1* mutation (p.C182\*) in a baby with low T cell receptor excision circles (TREC) identified by newborn screening testing for severe combined immunodeficiency. The patient also presented a profound B cell deficiency at birth.

**Methods**—Genetic, functional, immunologic, and clinical outcome data associated with this patient and her mutation were evaluated.

**Results**—Mutant p.C182\* was detected in the cytoplasm of the patient's primary cells, in contrast to wild type (WT) IKAROS protein, only detected in the nucleus. Functional in vitro assessments revealed that p.C182\* was less stable than WT IKAROS protein and failed to bind to

✉ Sergio D. Rosenzweig srosenzweig@cc.nih.gov.

Hye Sun Kuehn and Nicholas J. Gloude contributed equally to this work; Sergio D. Rosenzweig and Cathleen Collins contributed equally to this work.

**Author's Contributions** NJG, DD, and CC diagnosed the patient, collected biologic and clinical data, and wrote the first draft. MT and MW performed WGS and analyzed data. HSK and SDR designed the research project and wrote the manuscript. HSK performed the experiments and analyzed the data with SDR.

**Conflict of Interest** The authors declare no competing interests.

**Ethics Approval** Yes.

**Consent to Participate** All patients or their guardians provided informed consent in accordance with the Declaration of Helsinki under institutional review board-approved protocols of the National Institute of Allergy and Infectious Diseases, NIH, and Western IRB (Pr #:20171726); [ClinicalTrials.gov](https://clinicaltrials.gov) Identifier: NCT03385876.

**Consent for Publication** Yes.

its target DNA binding sequence and dimerize with WT IKAROS protein, resulting in impaired pericentromeric targeting and transcriptional repression by means of haploinsufficiency. During follow-up, while a spontaneous recovery of TREC and T cells was observed, B cells improved but not to sustained normal ranges.

**Conclusions**—Patients with IKAROS-associated diseases can present with SCID-like TREC values through newborn screening testing. *IKZF1* mutations should be added to the low TREC differential, although spontaneous recovery has to be considered.

### Keywords

TREC; T cells; B cells; lymphopenia; CVID; CID; NBS

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## Introduction

Much is understood about the complex biology of the transcription factor IKAROS/IKZF1, but its story continues to unfold. IKAROS is part of a family of zinc-finger proteins (IKZF1–IKZF5) that bind DNA through four N-terminal zinc-finger domains and homo- or heterodimerize through two C-terminal zinc-fingers [1]. IKAROS can act both as an activator and as a repressor of transcription, exerting its function by targeting DNA sequences at pericentromeric heterochromatin (PC-HC) regions of particular genes and regulating the nucleosome remodeling and histone deacetylase complex [2]. In humans, both somatic and germline *IKZF1* mutations have been implicated in distinctive, although related disease presentations: Somatic changes are associated with B cell precursor acute lymphoblastic leukemia (B-ALL) in children and adults [3]; germline heterozygous mutations contribute mostly to common variable immune deficiency (CVID) when acting by haploinsufficiency [4], to a severe form of combined immune deficiency (CID) with susceptibility to pneumocystis pneumonia by a dominant negative effect [5], and mostly to hematologic cytopenia/Evans syndrome and malignancies through dimerization defects [6]. Here, we describe and functionally characterize a novel IKAROS mutant identified in a baby presenting with low T cell receptor excision circles (TREC) through severe combined immunodeficiency (SCID) newborn screening.

## Methods

### Patients and Samples

All patients or their guardians provided informed consent in accordance with the Declaration of Helsinki under institutional review board–approved protocols of the National Institute of Allergy and Infectious Diseases, NIH, and Western IRB (Pr #:20171726); [ClinicalTrials.gov Identifier: NCT03385876](https://clinicaltrials.gov/ct2/show/study/NCT03385876). Blood from healthy donors and patients were obtained under approved protocols. All procedures were based on standard of care and established clinical guidelines were followed.

### Whole Genome Sequencing (WGS) and Sanger Validation

Consent for rapid WGS was obtained for the proband and her parents. Following DNA extraction from whole blood, samples were processed and analyzed according to the

ACMG/AMP guidelines for the interpretation of sequence variants as described elsewhere [7, 8]. *IKZF1* mutations were Sanger validated from gDNA using specific primers and standard techniques (primers sequence available upon request).

### cDNA Sequencing

Total RNA was isolated from total PBMCs using the Qiagen RNeasy Plus mini kit and reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). cDNA was PCR-amplified and sequenced using *IKZF1* primers, forward: 5'-CAAATCCACATAACCTGAGGAC-3'; forward: 5'-TTCCAGTGCAATCAGTGCGGGGCCT-3'; and reverse: 5'-CTGGGTACAGTGTGC-3'. To quantify the ratio of *IKZF1* WT and mutant transcripts, the cDNA was first PCR-amplified for 30 cycles using primers (forward: 5'-CAAA TCCACATAACCTGAGGAC-3', reverse: 5'-CTGG GTACAGTGTGC-3'), and an aliquot of this reaction was further amplified for 30 cycles using primers (forward: 5'-TTCCAGTGCAATCAGTGCGGGGCCT-3'; reverse: 5'-CTGGGTACAGTGTGC-3'). PCR products were cloned into *pGEM-TA Easy Vector* by TA cloning (Promega, A1360) according to the manufacturer's instruction. Following bacterial transformation, 27 colonies were sequenced for *IKZF1* using primers spanning the mutated area.

### Western Blotting and Immunoprecipitation

To generate T cell blasts, total PBMCs were stimulated with anti-CD3 and anti-CD28 (1 µg/ml) in the presence of IL-2 (10 ng/ml) for 7 days. IL-2 was added every 2–3 days. Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). For the immunoprecipitation, 293 T cells were transfected with HA tagged IKAROS WT or mutant together with flag-tagged IKAROS WT. Twenty-four hours after transfection, cell lysates were prepared in IP buffer (50-mM Tris pH 7.4, 150-mM NaCl, 2-mM EDTA, 0.5% Triton X-100 protease, and phosphatase inhibitor cocktail [Sigma]), and protein lysates were incubated with a rabbit anti-FLAG antibody and 40 µl protein A/G agarose beads (Pierce) for 4 h at 4 °C. Beads were washed three times with IP buffer; samples were prepared and separated on NuPAGE® Novex® 4–12% Bis-Tris Protein Gels (Life Technology). The following antibodies were used in the study: anti-IKAROS (Abcam; 229275); anti-HA antibody (Biolegend; 901501); anti-flag antibody (cell signaling; 14793); Lamin A/C (cell signaling; 4777); and GAPDH (cell signaling; 2118).

### Fluorescence Microscopy

Immunofluorescence was performed essentially as described previously [4–6]. Briefly, NIH3T3 cells were transfected with pcDNA3-HA WT or mutant IKAROS with or without pCMV6-AC- MYC-DDK-WT IKAROS using Effectene (Qiagen). The cells were fixed, permeabilized, and stained with anti-HA antibody (Biolegend; 901501) and/or anti-flag antibody (cell signaling; 14793) for 2 h. The cells were then washed and stained with Alexa Fluor 488 or Alexa Fluor 568 - conjugated secondary antibodies for an hour. Images were obtained with an EVOS M5000 fluorescence microscope (× 40 objective, Thermo Fisher scientific).

### LightShift Chemiluminescent EMSA

HEK293T cells were transfected with pCMV6-AC-MYC-DDK-WT or mutant C182\* IKAROS. Forty-eight hours after transfection, nuclear extracts were prepared, and gel mobility shift assays were performed using LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) as described previously [5, 6]. The mutant protein was detected in the nuclear extracts, and the same amount of nuclear extracts were allowed to bind to IKAROS probes. Sequences for the probes were as follows:  $\gamma$  satellite from human chromosome 8 ( $\gamma$ -Sat8) (5'-BIOTIN-GCGAGACCGCAGGG AATGCTGGGAGCCTCCC) and IKAROS consensus-binding sequence 1 (IKBS1) (5'-BIOTIN- TCAGCTTT TGGGAATACCCTGTCA). The EMSA images were acquired with C-Digit Scanner using Image Studio Software (Li-Cor).

### Dual Luciferase Reporter Assay

The IKBS1 reporter plasmid (pGL4.11-Luc2P, Promega) containing four repeats of the high affinity IKAROS binding site (IKBS1, TCAGCTTTTGGGAATACCCTGTCA) was described previously [6]. HEK293T cells were transiently transfected with pcDNA3-HA-IKAROS WT and/or mutant C182\*, pGL4.11-IKBS1, and pRL-TK (Renilla luciferase) using Effectine (Qiagen). Cells were harvested after 20–24 h and analyzed for luciferase activity using a Dual-Luciferase reporter assay system (Promega) on a GloMax microplate reader (Promega) according to manufacturer's instructions. The luciferase activity was calculated by dividing the luciferase activity of IKBS1 by the internal Renilla luciferase activity.

### Protein Stability Test

HEK293T cells were transfected with pcDNA3-HA-WT or C182\* IKAROS. After 20–24 h, cells were treated/untreated with cycloheximide (CHX, 20  $\mu$ g/ml) for additional 24 h. Whole cell lysates were prepared and analyzed by immunoblot analysis using an anti-HA antibody to test for IKAROS expression. Anti-vinculin (Santa Cruz, sc-73614) was used as a loading control. The relative IKAROS protein stability was calculated by dividing CHX treated sample by untreated sample ( $\times 100$ ) after normalization with vinculin to show the remaining protein amount after CHX treatment.

### T and B Cell Proliferation

Total PBMCs were incubated with CellTrace Violet Cell Proliferation Kit (Invitrogen, following manufacturer's instructions). Total of  $1 \times 10^5$  cells were seeded into 96-well plates, stimulated with anti-CD3 and anti-CD28 antibodies (1  $\mu$ g/ml each, clones OKT3 and CD28.2, respectively, eBioscience), CD40L (Enzo 100 ng/ml), IL-21 (Peprotech, 50 ng/ml), CpG (Enzo 1  $\mu$ M), and anti-IgM (Jackson Lab, 10  $\mu$ g/ml) as indicated in the figure. After 3 days (for T cells) or 4 days (for B cells), cells were stained with fluorochrome-conjugated CD4, CD8, or CD19 antibodies (BD Biosciences). Cells were washed two times with PBS and acquired and analyzed by flow cytometry (Becton Dickinson FACSCanto II) and FlowJo software (TreeStar).

## Results

A full-term Hispanic girl born at 40 weeks gestational age was found to have a confirmed abnormal newborn screen for SCID, with low TREC at 17 copies/ $\mu$ L (reference range > 18). Call back for liquid blood testing with a complete blood count and peripheral blood flow cytometry as requested by the State of California [9] was normal for age at 10 days of life (Table 1). Flow cytometry demonstrated normal CD3+, CD4+, CD8+ T, and NK cells numbers but low B cells. Most CD4+ and CD8+ T cells expressed CD45RA indicating normal thymic generation of naïve T cells (Table 1). Mitogen testing showed normal proliferation to PHA and PWM for CD3+ and CD45+ cells but was unable to be done on B cells due to low numbers. Repeat analysis at 8 weeks of life showed persistently low but improving B cell counts from 30/ $\mu$ L (day 10) to 368/ $\mu$ L (8 weeks) (Table 1). Recent thymic emigrants (CD3 + CD4 + CD45RA + CD31+; 79% in CD4+ T cells) were normal at age 21 weeks.

A commercially available 207 primary immunodeficiency gene panel showed heterozygous variants of unknown significance in *CD8A*, *LYST*, *SEMA3E*, *STAT5B*, and *TTC7A* in the patient that did not correlate or fully explained the patient's phenotype (Table 2). Due to low TREC and persistently low B cells at 8 weeks of life, parents were consented to an institutional review board-approved study for rapid WGS for the trio. A pathogenic heterozygous mutation was identified in *IKZF1* c.546C > A (p.C182\*) in the patient, but not in the parents (likely de novo; parental gonadal mosaicism was not ruled out); the mutation was confirmed by Sanger sequencing. This nonsense variant, mapping to exon 5 (of 8) of the gene, has not been previously reported. Mutation p.C182\* is predicted to encode for an ~ 20 kDa truncated protein lacking part of the DNA binding domain (partial ZF3 and complete ZF4-deletion) and the whole C-terminus dimerization domain (ZF5 and ZF6), likely severely impacting protein function. Of note, *IKZF1* was not included in the 207 gene panel tested in the patient or the updated 452 gene panel from this vendor.

At 14 weeks of life, B lymphocytes increased to 913 cells/ $\mu$ L. B cell subsets were essentially normal except for increased transitional B cells (CD19+/CD38+/IgM+) and decreased class-switched memory B cells (CD19+/CD27+/IgM-/IgD-). TREC analysis was also tested and was normal at 27,040 copies per  $10^6$  CD3+ T cells (Table 1). Repeated T and B cell proliferation upon TCR and BCR stimulation (respectively) showed a normal-to-slightly-diminished response (Fig. 1 a and b). A revertant mutation in T- and monocyte-enriched cells from a 21-week sample was ruled out (Fig. 1c; limited sample volume did not allow for B cell enrichment and testing).

When *IKZF1* mRNA/cDNA was evaluated, both WT and mutant transcripts were detected at similar levels in the patient's PBMCs after subcloning and sequencing (Fig. 2a). In terms of IKAROS protein, the patient showed reduced levels of WT IKAROS expression in the nucleus compared to the normal controls, while the mutant protein was readily found in the cytosol (Fig. 2b). To further assess the function of the variant protein, a mutant construct was tested for IKAROS PC-HC targeting, DNA binding, transcription activity, and protein stability. While WT IKAROS exhibited the characteristic punctate staining pattern of PC-HC localization, the mutant lost its ability to target this region and was abundantly

detected in the cytoplasm (Fig. 2c). When co-expressed with the WT vector, IKAROS p.C182\* did not exert any dominant effect on pericentromeric targeting, and its mechanism of action was determined as haploinsufficiency for this function (Fig. 2d). The mutant also failed to dimerize with WT IKAROS due to the lack of ZF5 and ZF6 (Fig. 2e, and as suggested in Fig. 2d), and to bind to specific DNA targets (Fig. 2f). While WT IKAROS repressed basal IKBS1 transcription activity by ~ 60%, mutant C182\* completely failed to do so. In WT/C182\* co-transfection experiments at three different ratios (WT/C182\* 25/75; 50/50 and 75/25), WT expression repressed the transcription activity in a progressive and dose-dependent manner (WT/C182\*: 25/75, ~ 37%, 50/50, ~ 40%, 75/25, ~ 47%), arguing against a dominant negative effect but in favor of a haploinsufficiency mechanism by C182\* for this function too (Fig. 2g). Mutant C182\* protein stability was also reduced when compared to WT IKAROS (Fig. 2h), being this feature common in dimerization defective, but not in classical haploinsufficient variants [6]. This reduced protein stability may as well contribute to explain the apparent discrepancy between C182\* nuclear localization in primary cells (absent; Fig. 2b) vs. transfected and overexpressed in HEK293T cells (present, although failing to PC-HC localization, targeted DNA binding and transcription activity; Fig. 2 c, d, f, and g). These results suggest that mutant p.C182\* is retained in the cytosol, is less stable than WT IKAROS, fails to efficiently localize to the nucleus or to dimerize with the WT protein, and is also unable to bind target DNA sequences, without directly affecting the WT protein. As shown, this new *IKZF1* mutation displays distinctive mechanistic and clinical features when compared with previously reported allelic variants [4–6, 10].

## Discussion

Clinically, our patient presented with two main immune characteristics: very low B cells and abnormally low TREC at newborn screen testing. Low B cells, either since very early in life or progressively declining, have been previously described to be associated with all *IKZF1* allelic variants [4–6, 10]. During the first months of follow-up in our patient, B cells progressively increased although not completely normalized for age. In association with the low TREC, low T cell numbers, maturation, or functional T cell defects were not detected when evaluated at 10 days (or later timepoints) after the SCID newborn screen test was collected. While the chance of a “false positive” SCID newborn screen test remains possible, the sequential rather than simultaneous testing does not allow us to definitively confirm or rule out this hypothesis based exclusively in those tests. On the other hand, the experience accumulated from SCID newborn screening demonstrated that primary immunodeficiencies as DiGeorge syndrome, Ataxia-Telangiectasia, or Wiskott-Aldrich syndrome (as well as secondary forms of lymphocyte loss) can present with low TREC and lymphopenia at birth with subsequent values normalization [9, 11, 12]. More closely to our case, the fact that two previously reported *IKZF1*-mutated infants presented with SCID-like manifestations followed by spontaneous immunologic recovery raises the possibility of “transient SCID” being part of the IKAROS-associated diseases spectrum (Fig. 2a). The patient herein described is the third *IKZF1*-mutated infant to present with such features in a disease with less than 100 cases described in the literature [13]. In 2016, Hoshino et al. reported a newborn male patient diagnosed with pancytopenia and virtual alymphocytosis at birth (absolute lymphocyte count (ALC), 150/μL) in whom bone

marrow transplantation was recommended [10]. However, after 1 month, the patient showed spontaneous recovery of all hematologic parameters including lymphocyte counts (ALC, 6030/ $\mu$ L). This patient carried a germline heterozygous *IKZF1* p.Y210C mutation believed to be acting by DNA binding haploinsufficiency. In 2017, Yoshida et al. reported a 9-month-old female patient presenting with a severe pulmonary infection/acute respiratory distress and a T-/B-SCID immunophenotype (260/ $\mu$ L lymphocytes, 192/ $\mu$ L T cells; 15/ $\mu$ L B cells; agammaglobulinemia; and markedly reduced T cell proliferation) [14]. While the patient underwent a purified CD34-positive bone marrow transplant from her haploidentical mother without preconditioning at 10 months of age, the graft was rejected and followed with complete autologous recovery. At the age of 4 years, the patient presented an ALC of 2054/ $\mu$ L; 1970/ $\mu$ L T cells; 26/ $\mu$ L B cells; and normal-low proliferative responses. This patient carried a germline heterozygous *IKZF1* mutation p.N159S, later reported to act in a dominant negative manner [5].

Interestingly, in our patient, the number of B and T cells (assuming a direct correlation between low TREC and low thymic output/T cell lymphopenia) increased over her first weeks/months of life. These results may suggest that having one WT and one mutant IKAROS copy (i.e., pY210C, p.N159S, or p.C182\*) during the prenatal period and under certain circumstances might not be sufficient to achieve normal B- or T- cell production at birth but might be enough to partially or completely restore lymphocyte development postnatally. On the other hand, as IKAROS-associated disease manifestations could be progressive and appear later in life, long-term follow-up for B- and T cell-related defects (e.g., B cell lymphopenia, hypogammaglobulinemia, B or T cell malignancy) is warranted in this patient, as evidenced by decreasing B cells and IgG levels at 17 months evaluation [4, 6, 13]. Although the patient remains asymptomatic, her clinical and immune status is regularly monitored in case any medical intervention is needed (e.g., IgG replacement therapy). Besides, *IKZF1* mutations should be added to the list of genes associated with abnormal SCID newborn screens although spontaneous recovery should be considered.

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## Abbreviations

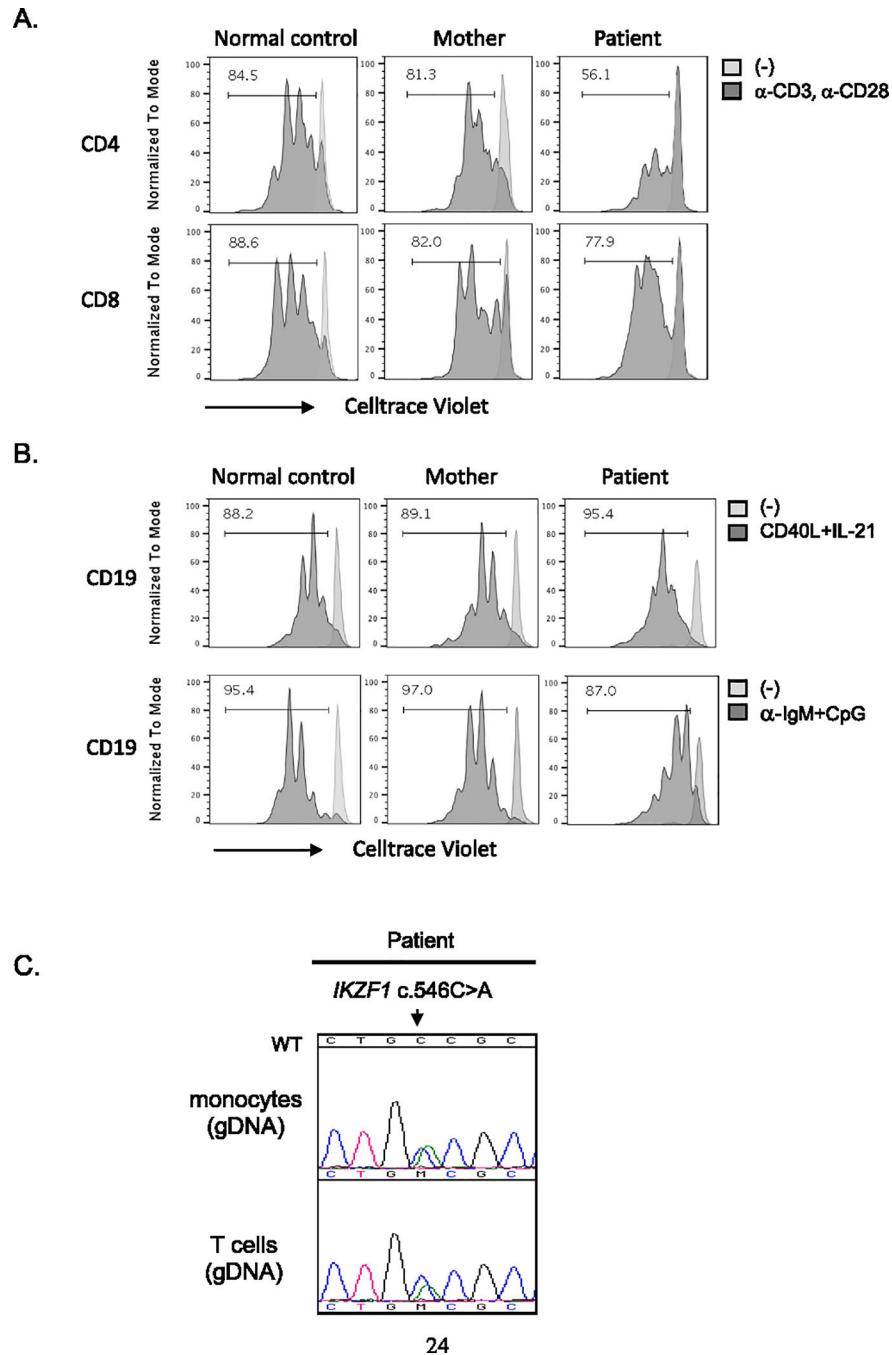
<b>CVID</b>	Common variable immunodeficiency
<b>CID</b>	Combined immunodeficiency
<b>TREC</b>	T cell receptor excision circles

<b>PC-HC</b>	Pericentromeric heterochromatin
<b>SCID</b>	Severe combined immunodeficiency

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**Fig. 1.**

T and B cell proliferation. **a** Total PBMCs (21-week sample from the patient) stained with CellTrace Violet reagent and stimulated with anti-CD3 and anti-CD28 antibodies for 3 days. **b** For B cell proliferation, cells were stimulated with CD40L and IL-21, or CpG and  $\alpha$ -IgM as indicated in the figure. After 3 (for T cells) or 4 days (for B cells), cells were acquired and analyzed by flow cytometry and FlowJo software, respectively. Numbers in the box indicate the percentage of cells having undergone at least one cellular division assessed by CellTrace Violet dye dilution. **c** CD3 T cells and monocytes were enriched from the patient's PBMCs

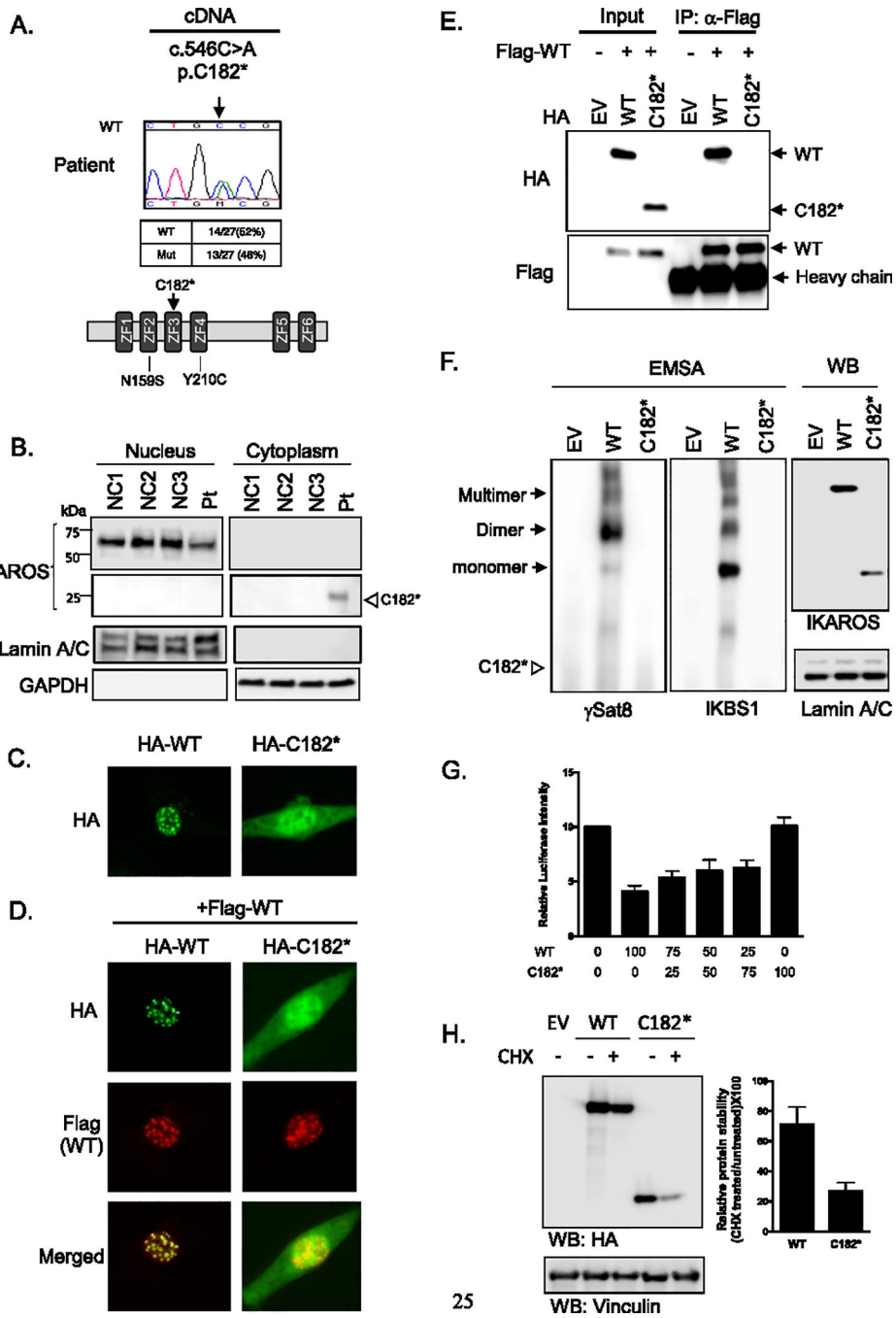
(21-week sample). Genomic DNA was prepared from the cells, and Sanger sequencing was performed. WT indicates reference sequence for wild type IKZF1

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**Fig. 2.** Mutant IKAROS p.C182\* expression and its functional tests. **a** Sequence chromatograms from cDNA from PBMCs in the patient. To quantify the wild type and mutant ratio, PCR products were cloned into *pGEM-TA Easy Vectors*, and 27 colonies were sequenced. Schematic representation of the IKAROS protein. ZF indicates zinc finger. Previously reported *IKZF1* mutations associated with transient SCID presentation are indicated below the ZF2 and ZF4. **b** IKAROS expression in T cell blasts from the patient and normal controls (NC). Immunoblotting of Lamin A/C and GAPDH was used as a marker for the

nuclear and cytoplasmic fraction, respectively. **c,d** Immunofluorescence staining of WT and mutant proteins transfected into NIH3T3 cells. Cells were visualized by using an EVOS fluorescence microscope ( $\times 40$  objective). **e** HEK 293 T cells were co-transfected with flag-tagged wild-type IKAROS WT and HA-IKAROS WT or mutant. EV indicates empty vector control. Immunoprecipitations and immunoblotting were performed using indicated antibodies. **f** HEK 293 T cells were transfected with WT and mutant IKAROS DNA, and nuclear extracts were used for the EMSA assay. The IKAROS WT and C182\* expression was tested from the nuclear extracts. Lamin A/C was used as a marker for the nuclear fraction. Triangle indicates the expected mutant protein binding site. **g** HEK293T cells were co-transfected with expression plasmids for IKAROS WT and/or mutant and pGL4.11-IKBS1 and pRL-TK (Renilla). Numbers indicate the percentage of WT and mutant IKAROS DNA used for the co-transfection. The firefly luciferase activity was normalized to the *Renilla* luciferase activity, and then the results were normalized to the empty vector control. Data are means  $\pm$  SEM from four independent experiments. **h** HEK293T cells transfected with WT or mutant IKAROS were treated with cycloheximide (CHX) for additional 24 h. IKAROS expression after CHX treatment was analyzed by immunoblotting. Vinculin was used as a loading control. The relative IKAROS protein stability was shown in the graph. Data are mean value + SD of three independent experiments. Data shown are representative of 2 (Fig. 1e) or 3 (Fig. 1 c, d, f, and h) independent experiments

**Table 1**

Laboratory findings

	Day of life 1	Day of life 10	Day of life 18	8 Weeks	14 Weeks	17 Months
TREC (reference range)	17 (L) * (> 18)				27,040# (> 6794)	
Lymphocyte subsets (absolute cells/ $\mu$ L and %)						
Lymphocytes absolute (reference range)		4300 (2000–17,000)	4070 (1930–7460)	4908 (3300–15,000)	7948 (4000–13,500)	4815 (4000–10,500)
CD3 absolute (reference range)		4048 (2500–5500)	3768 (1484–5327)	4092 (2160–5540)	5777 (H) (2160–5540)	3804 (2030–5150)
CD3% (reference range)		94.1% (H) (53–84)	93% (H) (54–82)	83% (H) (54–76)	73% (54–76)	79% (H) / (49–75)
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup> (reference range)		203 (170–1100)	245 (43–526)	363 (160–930)	1037 (H) (160–930)	266 (95–620)
CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup> (%) (reference range)		4.7% (4–18)	6% (2–10)	7% (3–14)	14% (3–14)	5% (2–16)
CD4 absolute (reference range)		3357 (1600–4000)	3037 (733–3181)	3229 (1390–4080)	4610 (H) (1390–4080)	2576 (1220–3550)
CD4% (reference range)		78.1% (H) (35–64)	75% (H) (34–62)	66% (H) (36–55)	58% (H) (36–55)	56% (30–60)
CD4:CD8 ratio (reference range)		5.0	4.3 ( 1.0)	3.7 (1.7–3.9)	4.1 (H) (1.7–3.9)	2.8 (1.4–3.9)
CD8 absolute (reference range)		673 (560–1700)	710 (370–2555)	868 (600–1490)	1159 (600–1490)	912 (530–1590)
CD8% (reference range)		15.7% (12–28)	17% (15–36)	18% (12–24)	14% (12–24)	20% (13–28)
CD3 <sup>+</sup> CD4 <sup>+</sup> CD45RA <sup>+</sup> absolute (reference range)		2746 (1200–3700)				
CD45RA <sup>+</sup> (%) of CD4		81.8%				
CD3 <sup>+</sup> CD8 <sup>+</sup> CD45RA <sup>+</sup> absolute (reference range)		572 (450–1500)				
CD45RA <sup>+</sup> (%) of CD8		85.0%				
CD19 absolute (reference range)		30 (L) (300–2000)	36 (L) (370–2306)	368 (L) (740–2560)	913 (740–2560)	651 (L) (830–1880)
CD19% (reference range)		0.7% (L) (6–32)	1% (L) (13–39)	7% (L) (17–36)	12% (L) (17–36)	13% (L) (20–38)
CD27 <sup>+</sup> IgM <sup>+</sup> IgD <sup>+</sup> (%) of CD19 (reference range)					0.9% (L) (1.9–30.4)	
CD38 <sup>+</sup> IgM <sup>+</sup> (%) of CD19 (reference range)					69.5% (H) (7.6–48.6)	
Immunoglobulins						
Total IgG (reference range)			691 (162–872)	943 (311–664)		382 (L) (451–1202)
IgM (reference range)			31 (1–57)	25 (0–127)		51 (35–184)
IgA (reference range)			8 (0–10)	85 (H) (0–42)		18(15–111)
IgE (reference range)			< 2.00 (0.00–8.00)			

	Day of life 1	Day of life 10	Day of life 18	8 Weeks	14 Weeks	17 Months
Vaccine titers (IU/ml)						Tetanus: $0.24^2$ Diphtheria: $1.64^3$ HBs: reactive
Mitogens						
Viab. of lymphs at day 0			89.7%			
Max prolif of PWM as %CD45			56.1%			
Max prolif of PWM as %CD3			63%			
Max prolif of PHA as %CD45			92.5%			
Max prolif of PHA as %CD3			95.2%			

H and L indicate values above/below the age matched normal range (respectively) for inhouse or CLIA-certified commercially available tests. *HBs* hepatitis B surface.

\* EnLite (PerkinElmer) kit was used to measure TREC copies per  $\mu\text{L}$  of peripheral blood as previously described [9].

# Copies per  $10^6$  CD3<sup>+</sup> T cells.

<sup>1</sup> A CD3<sup>+</sup>/CD4<sup>-</sup>/CD8<sup>-</sup> double negative T cell population at 316 cells/ $\mu\text{L}$ ; 6.6% of lymphocytes was detected; no  $\alpha\beta/\gamma\delta$  markers were included in this test.

<sup>2</sup> Protective titers, > 0.15 IU/ml.

<sup>3</sup> Protective titers, > 0.1 IU/ml

**Table 2**

Variants of uncertain significance

Gene	Transcript	Variant	Zygosity	GnomAD allele frequency	dbSNP ID
CD8A	NM_001768.6	c.63_64delinsTG (p.Ser22Gly)	Heterozygous	0.000039	rs776230766, rs766042221
LYST	NM_000081.3	c.8806G>A (p.Val2936Ile)	Heterozygous	0.000580	rs2753327
SEMA3E	NM_012431.2	c.475G>C (p.Glu159Gln)	Heterozygous	0.000012	rs1241693530
STAT5B	NM_012448.3	c.650G>A (p.Arg217His)	Heterozygous	0.000021	rs200900246
TTC7A	NM_020458.3	c.154C>A (p.Pro52Thr)	Heterozygous	0.000014	rs906523542