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1 **A boy with a novel homozygous ZAP70 mutation presenting with a**
2 **dermatological phenotype and a postnatal decrease in CD8⁺ T cells**

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6 Roya Babayeva, MD^{1,2,3*}, Cedric Mongellaz, MSc^{4*}, Ibrahim Serhat Karakus, MD^{5*},
7 Murat Cansever, MD⁶, Sevgi Bilgic Eltan, MD^{1,2,3}, Mehmet Cihangir Catak, MSc^{1,2,3},
8 Alper Bulutoglu, MSc^{1,2,3}, Yasemin Kendir Demirkol, MD⁷, Metin Eser, MD⁷, Elif
9 Karakoc-Aydiner, MD^{1,2,3}, Ahmet Ozen, MD^{1,2,3}, Valérie S Zimmermann, PhD⁴, Naomi
10 Taylor, MD, PhD^{4,8}, Safa Baris, MD^{1,2,3}

11 *Pediatr. Allergy Immunol.*, 2022, 33:e13756; doi:10.1111/pai.13756

12
13 ¹Marmara University, School of Medicine, Division of Pediatric Allergy and Immunology,
14 Istanbul, Turkey; ²Istanbul Jeffrey Modell Diagnostic and Research Center
15 for Primary Immunodeficiencies, Istanbul, Turkey; ³The Isil Berat Barlan Center for
16 Translational Medicine, Istanbul, Turkey; ⁴Institut de Genetique Moleculaire de Montpellier,
17 University of Montpellier, CNRS, Montpellier, France; ⁵Marmara University, School of
18 Medicine, Istanbul, Turkey; ⁶Ministry of Health, Kayseri City Hospital, Pediatric Allergy and
19 Immunology; ⁷University of Health Sciences, Umraniye Education and Research Hospital,
20 Division of Pediatric Genetics, Istanbul, Turkey; ⁸Pediatric Oncology Branch, Center for Cancer
21 Research (CCR), National Cancer Institute (NCI), NIH, Bethesda, MD
22

23 * Equally contributed to this study
24
25
26

27 **Correspondence:**

28 Safa Baris, MD, Marmara University, Division of Pediatric Allergy/Immunology, Fevzi Çakmak
29 Mah. No: 41, Pendik/Istanbul/Turkey. Phone: +90 216 625 78 11, Email:
30 safabaris@hotmail.com
31
32

33 **Key words:** Inborn errors of immunity, ZAP70, dermatitis, transplantation
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37 **Letter to The Editor**

38

39 Patients with deficiency of zeta-chain-associated protein kinase 70 (ZAP-70) protein
40 generally present as combined immunodeficiency (CID) with severe recurrent
41 infections and dermatological findings during the first years of life. They also suffer
42 from diarrhea, mainly resulting from viral agents, lymphoproliferation, and
43 autoimmunity (autoimmune cytopenia, bullous pemphigoid, nephrotic syndrome, and
44 adrenal insufficiency). The most striking immunological findings are severely
45 decreased CD3⁺CD8⁺ T-cell counts with decreased proliferation. The current definitive
46 treatment of ZAP-70 deficiency is hematopoietic stem cell transplantation (HSCT) ¹.

47

48 To date, 52 patients with biallelic mutations in the *ZAP70* gene have been described
49 in the literature ^{1,2}. Herein, we report a patient with a novel missense mutation in the
50 *ZAP70* who presented with atypical skin lesions and a rapid decrease in CD8⁺ T-cell
51 counts on immunological evaluations between 6 and 9 months of age. Despite
52 undetectable ZAP-70 protein, the patient did not present severe infections in the first
53 year of life. This description expands the spectrum of disease caused by mutations in
54 the *ZAP70*, thus providing vigilance for early diagnosis and treatment.

55

56 A male infant born in February, 2020 to consanguineous parents of Turkish descent,
57 was admitted to the hospital due to an extensive rash covering his body, starting on
58 the third day of life. He also had continual diarrhea, with a frequency of two times per
59 day since birth. Local treatments and antihistamines did not improve his skin
60 symptoms. Physical examination revealed no specific features other than
61 erythematous papular lesions on the dorsum of arms, malar rash like lesions, and
62 livedo reticularis formation on his legs (**Fig.1A**). Complete blood count showed a
63 lymphocyte count of 4700/mm³, eosinophil of 2100/mm³, hemoglobin of 10.7 g/dl, and
64 platelet of 988,000/mm³. Immunoglobulin levels were normal except for an IgE level of
65 499 mg/dl. Lymphocyte immunophenotyping revealed moderate low CD3⁺ and CD8⁺ T
66 cells, accompanied by decreased naive and increased memory CD4⁺ and CD8⁺ T cells.
67 Levels of recent thymic emigrants were also diminished compared to healthy controls.
68 Detailed blood test results and immunophenotyping are shown in **Table 1**. Skin prick

69 test and specific IgE levels were all negative and biopsy from the skin lesions showed
70 minimal vacuolar degenerations with focal parakeratosis. Immunohistochemical
71 examination of the biopsy revealed infiltrating T lymphocytes, predominantly CD4⁺ T
72 cells. Maternal engraftment was excluded by using short tandem repeat analysis in the
73 patient. Viral serology for *Cytomegalovirus* (CMV), *Epstein-Barr virus* (EBV) were
74 negative. Interestingly, a follow-up lymphocyte subset analysis at 9 months of age
75 showed a severe decrease in CD8⁺ T cells (1.2%). The patient's family history revealed
76 an older sister with EBV-related B-cell lymphoproliferative disease; she died at 22
77 months of age, due to systemic aspergillosis leading to multiorgan failure.

78

79 Next-generation sequencing revealed a novel homozygous missense mutation in the
80 *ZAP70* gene (c.37G>A), confirmed by Sanger sequencing. Both parents were
81 heterozygous for the mutation. The other healthy brother had 2 WT alleles, as
82 assessed by high-resolution melting analysis (**Fig.1B** and **Fig.S1A**). The mutation led
83 to a Gly13Ser alteration in the N-SH2 domain of ZAP-70 (**Fig.1C**) and was associated
84 with a loss of protein expression analyzed by both western blot and flow cytometry
85 (**Fig.1D**). Furthermore, phytohemagglutinin (PHA)- as well as anti-CD3/CD28-induced
86 CD4⁺ T cell proliferation was defective in the patient, compared to healthy family
87 members (**Fig.S1B** and **Fig.S1C**). Methodological details are provided in the
88 Supplementary data.

89

90 Although the patient presented with mild clinical features, he was considered for HSCT
91 due to the absence of ZAP-70 protein and suspected ZAP70 deficiency in the older
92 sibling who died from severe fungal infection. At 9 months of age, he was transplanted
93 using a bone marrow stem cell source without conditioning from a fully-matched male
94 sibling. However, he failed to engraft. One month later, he received a second bone
95 marrow transplantation from the same donor following a reduced toxicity conditioning
96 regimen (treosulfan-10 mg/m² from days -5 to -3 and fludarabine-30 mg/m² from days
97 -5 to -2) with cyclosporine A prophylaxis for graft-versus-host disease (GVHD). Full
98 donor engraftment was achieved and the patient did not experience GVHD. Allele-
99 specific DNA polymerase chain reaction analysis of the *ZAP70* gene showed a normal
100 high-resolution melting analysis and restriction profile after successful HSCT (**Fig.1E**).

101 Currently, the patient is doing well, 10 months after transplantation, without any
102 complications.

103

104 In the literature, there are three types of described mutations in ZAP70 deficiency¹.
105 The most common is a complete loss-of-function mutation, often targeting the kinase
106 domain. Hypomorphic mutations also exist, having been described in five patients.
107 These patients present with less severe symptoms compared to patients with complete
108 loss but also exhibit low levels of poorly functioning CD8⁺ T cells^{1,3}. Interestingly, Chan
109 et al. described a compound heterozygous for loss-and gain-of-function mutations,
110 disrupting the autoinhibition of the protein, constituting a third form of mutations in
111 ZAP70 deficiency. This atypical sibling pair displayed early-onset autoimmune
112 diseases (bullous pemphigoid, colitis, hemophilia, and nephrotic syndrome) without
113 infectious complications⁴. Our patient was found to have a novel homozygous
114 missense mutation on ZAP70, resulting in loss of protein expression and function. The
115 mutation is located in the N-terminal SH2 domain and to date, there are only four
116 described patients with mutations involving this domain^{1,2}. Three were compound
117 heterozygous, which included the N-SH2 and kinase domains, while the third was a
118 single homozygous mutation found on the N-SH2 domain. Dermatitis was described in
119 all of these patients, but apart from our report, other patients also presented respiratory
120 and gastrointestinal infections^{2,5-7}.

121

122 The majority of described patients with ZAP70 mutations had a clinical diagnosis of
123 CID. Furthermore, some patients were also reported to have EBV-related
124 lymphoproliferative disease, autoimmunity (mostly autoimmune cytopenia),
125 hemophagocytic lymphohistiocytosis, and lymphoma¹. The cutaneous lesions in
126 patients with ZAP-70 deficiency have consisted of an erythematous urticarial rash,
127 bullous pemphigoid, eczema perineal ulcers, and widespread xerosis. Notably, in
128 previously reported patients, these dermatological problems have been accompanied
129 by more prominent clinical findings such as respiratory tract and gastrointestinal
130 infections¹. Similar to our case, one infection-naïve patient showing eczematous skin
131 lesions with infiltrating CD4⁺ T cells has been described in the literature⁵. The survival
132 and infiltration of CD4⁺ T cells may be attributed to an increased expression of the
133 ZAP-70 related Syk protein tyrosine kinase, leading to an abnormal activation and
134 differentiation of CD4⁺ T lymphocytes^{5,8}.

135 Immunological findings of our patient were compatible with the findings of reported
136 patients showing eosinophilia and elevated IgE levels, resembling Omenn syndrome
137 ^{5,9}. A unique feature of our patient was the decrease in the percentage of CD8⁺ T cells
138 in the 2nd flow cytometry analysis, reflecting a loss of CD8⁺ T cells during the postnatal
139 period (between 6 and 9 months of age). This phenomenon has not been described in
140 other reported patients. However, normal to low newborn T cell receptor excision circle
141 levels in the same patient were reported previously and our patient exhibited an initial
142 low percentage of recent thymic emigrants (Table 1), indicating that the immunological
143 findings of disease can exacerbate over time ¹. The other possibility of decrease CD8⁺
144 T cells can be explained by a potential loss of maternal T cells with age ¹⁰, but this was
145 excluded in our patients. These data strongly suggest that serial measurements of
146 CD8⁺ T cells in the setting of clinical suspicion of ZAP70 deficiency would be helpful
147 for appropriate diagnosis.

148

149 In conclusion, ZAP70 deficiency is characterized by a broad spectrum of clinical
150 presentations. Even in patients with subtle clinical findings, progressive reduction in
151 CD8⁺ T cell counts should evoke the possibility of ZAP70 deficiency, improving early
152 diagnosis and promoting a better outcome for these patients.

153

154 **Author Contributions:** S.B. conceptualized and supervised the study. R.B., C.M.,
155 M.C.C., A.B., Y.K.D, and M.E. performed the experiments. R.B., I.S.K., S.B.E., E.K.A.,
156 and A.O. provided patient care, collected samples and clinical data. S.B., R.B., and
157 I.S.K. wrote the paper. V.Z. and N.T. supervised and edited the paper. All authors
158 reviewed and approved the final version of the manuscript.

159

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162

163 **Conflict of interest disclosure:** No.

164

165 **Availability of data and material:** The data generated during the study are included
166 in this published article and its supplementary file.

167 **Code availability:** Not applicable.

168 **Compliance with Ethical Standards:** The study was approved by the Ethics
169 Committee of Marmara University, School of Medicine.

170

171 **Consent to participate:** Informed consent for participation was obtained from the
172 family.

173 **Consent for publication:** Informed publication consent was obtained from the family.

174

175 **Figure and Table Legends**

176 **Figure 1. Clinical features and functional consequence of the ZAP70 mutation.**

177 **A.** Cutaneous findings in the patient. A malar rash and erythematous papular lesions
178 on the dorsum of arms, and livedo reticularis formation on the legs are shown. **B.**
179 Mutation segregation in the family depicting the 37G>A change. **C.** Schematic diagram
180 of the ZAP-70 protein showing the position of the Gly13Ser missense mutation. The
181 domain architecture of the ZAP-70 protein consists of tandem Src homology 2 (SH2)
182 domains, an interdomain A (I-A) between the SH2 domains, an interdomain B (I-B),
183 and a kinase domain **D.** Western blot analysis of the ZAP-70 protein in the patient (Pt)
184 and healthy control (HC) T cells in unstimulated and anti-CD3/anti-CD4 stimulated
185 conditions. Tubulin staining is shown as a loading control (top). ZAP-70 levels in CD4⁺
186 T cells were almost monitored by intracellular staining with a monoclonal anti-ZAP-70
187 Ab (solid lines) versus isotype controls (grey filled) and representative histograms are
188 shown (bottom). **E.** The PCR-amplified 169bp genomic product harboring the 37G>A
189 mutation from the patient pre- and post-transplant, as well as from his brother can be
190 distinguished by high-resolution melting analysis (HRM) (top). Allele-specific restriction
191 profile (*SfcI*) of the *ZAP70* gene in the patient before (pre) and post HSCT as well as
192 in a HC (bottom).

193

194 **Figure S1. Impact of the ZAP70 mutation on PHA and CD3/CD28-stimulated T cell proliferation.**

195 **A.** High-resolution melting analysis of the PCR-amplified 169bp
196 genomic product harboring the 37G>A mutation allows the heterozygote parents and
197 homozygote patient to be distinguished from the sibling donor and a healthy control
198 (HC). **B.** T cells from the patient, the two parents, and a sibling donor were labeled with
199 VPD450 and activated with phytohaemagglutinin (PHA, 1µg/ml) plus IL-2 (100IU/ml).
200 Five days post stimulation, the relative percentages of CD4 and CD8 T cells were
201 monitored as shown in the representative dot plots. The VPD450 proliferation profiles
202 of gated CD4⁺ and CD8⁺ T cells are presented. **C.** T cells, as described in panel B,
203 were stimulated for 3 days with anti-CD3/anti-CD28 monoclonal antibodies and
204 CD4/CD8 plots as well as proliferation profiles are presented.

205

206 **Table 1.** Immunological evaluations of the patient before and after HSC
207 transplantation.

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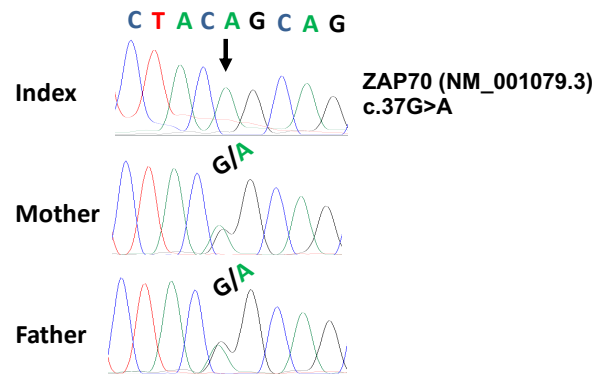
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Figure 1

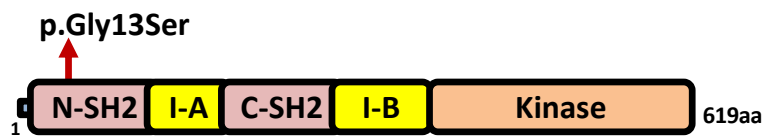
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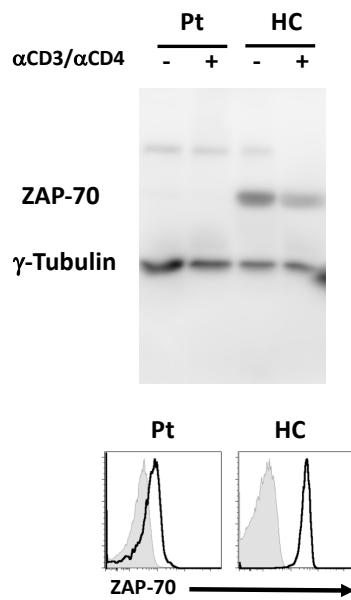
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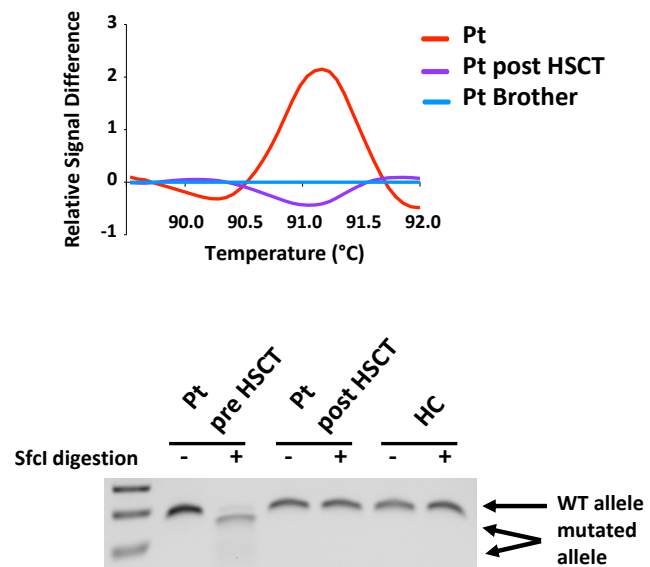
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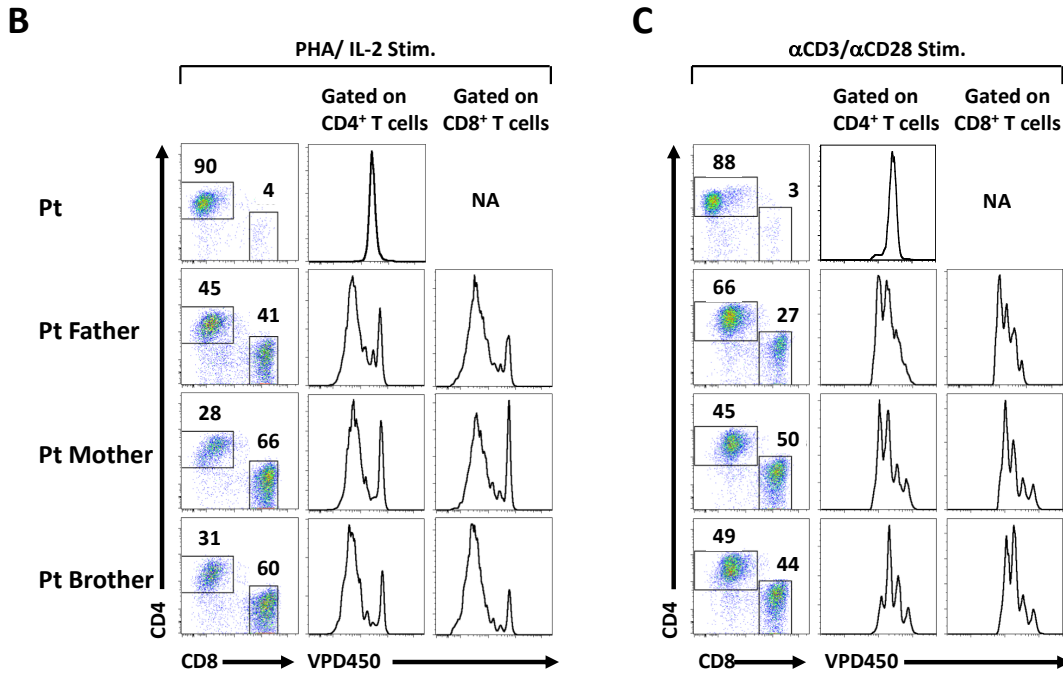
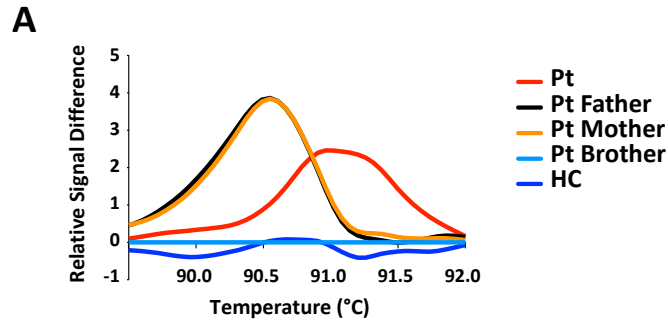
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Supplementary Figure 1



278 **A boy with a novel homozygous *ZAP70* mutation presenting with**
279 **dermatological phenotype and vanishing CD8⁺ T cells in the follow-up**

280

281 **Babayeva et al.**

282 **Supplementary File**

283 **Materials and Methods**

284 **Antibodies and flow cytometry**

285 Lymphocyte subset analyses were evaluated using the following monoclonal
286 antibodies (mAbs): Fluorescein isothiocyanate (FITC)-conjugated CD3 (UCHT1, BC,
287 FRA), Allophycocyanin (APC)-Alexa Fluor 700 (APC-A700) CD4 (13B8.2, BC), Krome
288 Orange (KO) CD45 (J33, BC), Alexa Fluor 750 (APC-A750) CD45RA
289 (2H4DH11LDB9, BC), Phycoerythrin (PE) CD197 (CCR7) (G043H7, BC),
290 Pycoerythrin-Cyanin 7 (PC7) CD8 (SFC121Thy2D3, BC), APC-A700 CD14 (RMO52,
291 BC), PE CD16 (3G8, BC), Pycoerythrin-Cyanin 5.5 (PC5.5) CD56 (N901, BC), APC-
292 A750 CD19 (J3-119, BC), PB CD20 (B9E9, BC), PB CD21 (BL13, BC), PB CD31
293 (5.6E, BC), PC5.5 CD38 (LS198-4-3 BC), Phycoerythrin-Texas Red-x (ECD) CD45RO
294 (UCHL1, BC), FITC IgD (IA6-2, BC), PE PD1 (PD1.3, BC), APC CXCR5 (J252D4,
295 BC), PC5.5 CD25 (B1.49.9, BC), Alexa Fluor 647 FOXP3 (259D, BD Biosciences,
296 Calif, USA), PE CTLA4 (BNI3, BC). For lymphocyte subset analysis, 100 µl of whole
297 blood was incubated with mAbs against surface markers for 20 minutes in the dark at
298 room temperature. Red cells were then lysed and washed before acquisition.
299 Peripheral T cells were divided into subpopulations as follows: CD4⁺ naive T cells
300 (CD4⁺ CD45RA⁺ CCR7⁺), central memory CD4⁺ T cells (CD4⁺ CD45RA⁻ CCR7⁺),
301 effector memory CD4⁺ T cells (CD4⁺ CD45RA⁻ CCR7⁻), terminally differentiated
302 effector memory CD4⁺ T cells (TEMRA, CD4⁺ CD45RA⁺ CCR7⁻), CD8⁺ naive T cells
303 (CD8⁺ CD45RA⁺ CCR7⁺), central memory CD8⁺ T cells (CD8⁺ CD45RA⁻ CCR7⁺),
304 effector memory CD8⁺T cells (CD8⁺ CD45RA⁻ CCR7⁻), and TEMRA CD8⁺ T cells
305 (CD8⁺ CD45RA⁺ CCR7⁻). Peripheral B cells were classified into four distinct
306 populations: naive mature B cells (CD19⁺ CD27⁻ IgD⁺), CD21^{low}CD38^{low} B cells, non-
307 class-switched memory B cells (CD19⁺ CD27⁺ IgD⁺) and class-switched memory B
308 cells (CD19⁺ CD27⁺ IgD⁻). All stained cells were acquired on a Navios EX cytometer

309 (Beckman Coulter) and analyzed with FlowJo™ (Single Cell Analysis Software v10 –
310 10.6.2) and Kaluza Analysis Software (Version 2.1).

311 **Detection of ZAP70 expression**

312 1×10^6 T cells were stimulated in 100 μ l serum-free RPMI 1640 at 37°C with anti-CD3
313 (clone OKT3 (1 μ g/ml) and anti-CD4 (clone 13B8.2 (1 μ g/ml) mAbs for 1 min, followed
314 by addition of secondary cross-linking goat anti-mouse Ig (1:100) for 2 min. Reactions
315 were terminated by addition of ice cold PBS, and then lysed in 1% NP-40 detergent
316 buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, pH 7.4) containing 5 μ g/ml
317 aprotinin (Sigma Aldrich), 1mM sodium orthovanadate (Sigma Aldrich) and 1mM
318 sodium fluoride (Sigma Aldrich). After incubation on ice for 15 min, lysates were
319 centrifuged at 14,000 rpm, reduced in SDS containing loading buffer and boiled for 5
320 min. Samples were separated on a 4-10% precast SDS-PAGE (NuPage,
321 ThermoScientific), and transferred electrophoretically to PVDF. Membranes were
322 blocked for 1h with 5% milk in Tris-buffered saline-0.05% Tween and primary
323 antibodies (clone 2F3.2 for ZAP-70) were incubated overnight at 4°C. Primary
324 antibodies were washed twice with Tris-Tween solution and secondary antibodies
325 coupled to HRP were incubated at room temperature for 1h (1:10.000 in Tris-Tween
326 1% milk). Membranes were washed twice and immunoreactive bands were visualized
327 by enhanced chemiluminescence (Pierce ECL Western, ThermoScientific) following
328 the manufacturer's instructions and detected using Amersham Imager 680.

329 Intracellular ZAP-70 levels were monitored following cell fixation and permeabilization
330 (cytofix/cytoperm kit; Becton Dickinson) using a PE-conjugated anti-ZAP-70 mAb
331 (clone 1E7.2, eBiosciences). Expression was evaluated on a BD Fortessa II Flow
332 Cytometer (Becton Dickinson) and analyses were performed using FlowJo Software
333 (v10 – 10.6.2).

334

335 **Proliferation assay**

336 T cells were purified by negative selection using the RosetteSep T Cell Enrichment kit
337 (StemCells Technologies) according to manufacturer's procedures. CD3⁺ β T cells
338 (1×10^6) were stained with VioletProliferationDye 450 (VPD450, Becton Dickinson)
339 (5 μ M in 1 ml) as per the manufacturer's instructions. Stained T cells (1×10^6) were
340 plated into anti-CD3/anti-CD28 coated wells (clones OKT3 and 9.3, BioXCell) of a 24
341 well plate or in presence of phytohaemagglutinin (PHA 1 μ g/ml, Sigma) plus IL-2 (100

342 IU/ml) in 1ml RPMI1640 supplemented with 10%FCS and 1% Penicillin/Streptomycin.
343 Proliferation was evaluated at day 3 or 5 following staining with anti-CD4 and anti-CD8
344 mAbs and data acquired on a BD Fortessa II Flow Cytometer. Analyses were
345 performed using FlowJo Software (v10–10.6.2).

346

347 **Genetic analysis and Sanger sequencing**

348 Genomic DNA was extracted from EDTA-anticoagulated peripheral blood using
349 standard methods. DNA was extracted from blood samples using a semi-automated
350 robot as recommended by the manufacturer (Qiagen). The library preparation for next-
351 generation sequencing (NGS) was performed using the Clinical Exome Solution Kit by
352 Sophia Genetics, a custom panel for severe combined immunodeficiency. NextSeq
353 500 (Illumina) was used as the sequencing platform. Variant calling and data analysis
354 were performed using the Sophia DDM version5.2 software. Sanger sequencing was
355 performed on the index case and family members to confirm the mutation identified by
356 NGS. Briefly, genomic DNA was amplified by PCR and ampimers were sequenced
357 using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems; Life
358 Technologies, Darmstadt, Germany). The sequencing products were separated on an
359 Applied Biosystems 3130 I Genetic Analyzer.

360

361 **Allele specific restriction analysis**

362 T cells were harvested 5 days after TCR stimulation and genomic DNA was extracted
363 using QiaAMP DNA Blood Mini kit (Qiagen). Exon 2 of the *zap70* gene was amplified
364 by Real-Time qPCR using the LightCycler 480 SybrGreen I Master mix (Roche) on the
365 LightCycler480 machine (Roche). Briefly, 10ng of genomic extract were amplified with
366 primers specific to exon 2 (Forward: GGGAGGCCCCAGGGGCGATG; Reverse:
367 CACGAGCGACAGCACATAGC) following the program 5 sec 95°C, 10 sec 60°C, 10
368 sec 72°C, for 30 cycles. An extensive melting curve (from 62 to 95°C, rate 0.02°C/sec)
369 was performed for High-Resolution Melting analysis with LightCycler software.

370 The 169bp amplicon was then purified using the Nucleospin Gel PCR cleanup kit
371 (Machery-Nagel) and digested with 1U of the *SfcI* restriction enzyme for 30min at 37°C
372 to assess the specific restriction of the mutated allele. *SfcI*-digested DNA fragments

373 were separated electrophoretically on a 1.8% agarose gel and the image was captured
374 using an UV-illuminator (Ingenius - SnapGene).