

An *in vivo* genetic reversion highlights the crucial role of Myb-Like, SWIRM, and MPN domains 1 (MYSM1) in human hematopoiesis and lymphocyte differentiation

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Background: Myb-Like, SWIRM, and MPN domains 1 (MYSM1) is a metalloprotease that deubiquitinates the K119-monoubiquitinated form of histone 2A (H2A), a chromatin marker associated with gene transcription silencing. Likewise, it has been reported that murine *Mysm1* participates in transcription derepression of genes, among which are transcription factors involved in hematopoietic stem cell homeostasis, hematopoiesis, and lymphocyte differentiation. However, whether MYSM1 has a similar function in human subjects remains unclear. Here we describe a patient presenting with a complete lack of B lymphocytes, T-cell lymphopenia, defective hematopoiesis, and developmental abnormalities. **Objectives:** We sought to characterize the underlying genetic cause of this syndrome.

Methods: We performed genome-wide homozygosity mapping, followed by whole-exome sequencing.

Results: Genetic analysis revealed that this novel disorder is caused by a homozygous *MYSM1* missense mutation affecting the catalytic site within the deubiquitinase JAB1/MPN/Mov34 (JAMM)/MPN domain. Remarkably, during the course of our

study, the patient recovered a normal immunohematologic phenotype. Genetic analysis indicated that this improvement originated from a spontaneous genetic reversion of the *MYSM1* mutation in a hematopoietic stem cell.

Conclusions: We here define a novel human immunodeficiency and provide evidence that MYSM1 is essential for proper immunohematopoietic development in human subjects. In addition, we describe one of the few examples of spontaneous *in vivo* genetic cure of a human immunodeficiency. (J Allergy Clin Immunol 2015;■■■■:■■■■-■■■■.)

Key words: Hematopoiesis, immunodeficiency, *MYSM1*, genetic reversion, histone deubiquitinase

Acetylation, methylation, sumoylation, phosphorylation, and ubiquitination are some of the posttranslational modifications that can occur on histones and modify the epigenetic landscape. These histone modifications participate in the regulation of key biological processes, including DNA repair, DNA replication, and transcription.¹ Myb-Like, SWIRM, and MPN domains 1 (MYSM1) is a histone deubiquitinase that specifically deubiquitinates the histone 2A (H2A) monoubiquitinated at position K119, a marker of gene transcription silencing. Thus MYSM1 reverses gene expression repression at specific loci through its epigenetic regulatory role of transcription. The recent description of *Mysm1* knockout (KO) mice highlighted its peculiar role in the immune and hematopoietic system. Indeed, in addition to developmental abnormalities (eg, growth defect, abnormal development of hind limb, and tail malformation), *Mysm1* KO mice mainly exhibit lymphopenia, anemia, and thrombocytosis. The participation of *Mysm1* in the maintenance of hematopoietic stem cell (HSC) quiescence, proper hematopoiesis, and lymphocyte and dendritic cell differentiation relies on its control of expression of specific transcription factors (including Ebf1, Fli1, Id2, and Gfi1) important for efficient immunohematologic development.²⁻⁷ In addition, analysis of the *Mysm1* KO mice revealed an important role for *Mysm1* in the suppression of p53-dependent apoptosis during early lymphoid development and other developmental processes.⁸ Of note, a brief letter recently reported 2 siblings with immunodeficiency and anemia carrying nonsense homozygous mutations in the *MYSM1* gene.⁹ However, in that report the authors were not able to categorically assign pathogenicity to this variant.^{9,10} We here describe a patient presenting with a syndrome characterized by B lymphocytopenia, T-cell lymphopenia, bone marrow failure, and developmental abnormalities. Combination of whole-genome homozygosity

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Supported by institutional grants from INSERM, Ligue Nationale contre le Cancer (Equipe Labellisée La Ligue), GIS-Institut des maladies rares, ARC, INCa/Cancéropôle Ile de France, Institut Imagine, and the European Research Council (PIDIMMUN grant no. 249816). This program has received a state subsidy managed by the National Research Agency under the "Investments for the Future" program bearing the reference ANR-10-IAHU-01. T.L.G. received fellowships from La Ligue contre le cancer, and B.F. received fellowships from Cancéropôle. P.R. is a scientist from Centre National de la Recherche Scientifique (CNRS).

Disclosure of potential conflict of interest: P. Revy received research support from ARC, La Ligue, INCa, ANR, GIS-Institut des European Research Council. The rest of the other authors declare that they have no relevant conflicts of interest.

Received for publication February 24, 2015; revised May 28, 2015; accepted for publication June 8, 2015.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2015.06.008>

Abbreviations used

APC:	Allophycocyanin
H2A:	Histone 2A
HSC:	Hematopoietic stem cell
JAMM:	JAB1/MPN/Mov34
KO:	Knockout
MYSM1:	Myb-Like, SWIRM, and MPN domains 1
NK:	Natural killer
PE:	Phycoerythrin
WGHM:	Whole-genome homozygosity mapping
WT:	Wild-type

mapping and whole-exome sequencing led to the identification of a homozygous mutation in *MYSM1* as responsible for this disorder. Strikingly, we show that a spontaneous *in vivo* genetic reversion of the *MYSM1* mutation that occurred in a patient's HSC led to complete recovery of the immunohematologic system. This observation demonstrates a causal link between *MYSM1* deficiency and the phenotype observed in this patient and provides the first evidence that *MYSM1* is absolutely required for proper lymphocyte differentiation and hematopoiesis in human subjects.

METHODS**Cells**

Informed consent for our study was obtained from the family in accordance with the Helsinki Declaration. The INSERM Institutional Review Board also approved this study. SV40-transformed cell lines were obtained as described by Buck et al.¹¹

Genetic analysis

The DNA mutation numbering system used is based on cDNA sequence. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. The initiation codon is codon 1.

Whole-genome homozygosity mapping

Genotyping with the Illumina Human Linkage-24 BeadChip was carried out according to standard methodology. In brief, each sample (200 ng) was whole genome amplified, fragmented, precipitated, and resuspended in appropriate hybridization buffer. Denatured samples were hybridized on prepared Human Linkage-24 BeadChips for a minimum of 16 hours at 48°C. After hybridization, the BeadChips were processed for the single-base extension reaction, as well as staining and imaging on an iScan (Illumina, San Diego, Calif) and an Autoloader 2 (Illumina). Normalized bead intensity data obtained for each sample were loaded into the Illumina GenomeStudio software 2011.1, which converted fluorescence intensities into single nucleotide polymorphism genotypes.

Whole-exome sequencing

Standard manufacturer protocols were used to perform target capture with the Illumina TruSeq exome enrichment kit and sequencing of 100 bp paired-end reads on Illumina HiSeq. Approximately 10 Gb of sequence was generated for each subject, such that 90% of the coding bases of the exome defined by the consensus coding sequence project were covered by at least 10 reads. Adaptor sequences were removed, and quality trimmed reads were created with the Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Then a custom script was used to ensure that only read pairs with both mates present were subsequently used. Reads were aligned to hg19 with BWA31, and duplicate reads were marked with Picard (<http://picard.sourceforge.net/>) and excluded from downstream analyses. Single nucleotide variants and short insertions and

deletions were called by using SAMtools (<http://samtools.sourceforge.net/>) pileup and varFilter32 with the base alignment quality adjustment disabled and were then quality filtered to require at least 20% of reads supporting the variant call. Variants were annotated with both ANNOVAR33 and custom scripts to identify whether they affected the protein coding sequence and whether they had previously been seen in dbSNP or the 1000 Genomes data set or in approximately 160 exomes previously sequenced at our center. A variant called in the patient was considered a candidate mutation if it was not reported in dbSNP or the 1000 Genomes data set or in our in-house database.

Antibodies and reagents

Rabbit polyclonal anti-MYSM1 was from Millipore (Temecula, Calif). Anti-actin was from Sigma-Aldrich (St Louis, Mo), and anti-KU70 was from Neomarkers (Fremont, CA). For immunofluorescence, primary antibodies were used at a 1:200 dilution. Alexa Fluor 488-labeled secondary antibody was from Molecular Probes (Grand Island, NY) and used at a 1:1000 dilution.

Western blotting and immunofluorescence

Cells were lysed for 20 minutes on ice in lysis buffer containing 50 mmol/L Tris (pH 8.0), 2 mmol/L EDTA, 0.5% Nonidet P40, 1% phosphatase inhibitor cocktails (Sigma), and protease inhibitor (Roche Applied Science, Indianapolis, Ind) and centrifuged; supernatant was harvested and protein concentration was quantified by using the Bradford assay. Whole-cell lysates were analyzed by means of Western blotting with appropriate antibodies (1:1000 dilution). Immunofluorescence study was performed as previously described by Touzot et al.¹²

Genetic reversion analysis

Mononuclear bone marrow cells were isolated by means of gradient centrifugation on Ficoll-Hypaque ($d = 1.077 \text{ g/mL}$; Lymphoprep, Axis-Shield, Oslo, Norway). Part of the mononuclear cells were stained with anti-CD45 VioGreen, anti-CD11b allophycocyanin (APC), anti-CD19 phycoerythrin (PE), and anti-CD34 APC-Cy7 and directly sorted on an Aria II system (BD Biosciences, San Jose, Calif) into myeloid ($\text{CD45}^+\text{CD11b}^+$), pre-BI ($\text{CD45}^+\text{CD34}^+\text{CD19}^+$), and pre-BII/immature B ($\text{CD45}^+\text{CD34}^-\text{CD19}^+$) cell fractions. CD34^+ hematopoietic progenitor cells were positively selected from the other part of mononuclear cells by using the human Miltenyi indirect CD34 Microbead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and a separator (VarioMACS, Miltenyi Biotec). CD34^+ selected cells were further stained with anti- CD34 APC-Cy7, anti- CD45RA fluorescein isothiocyanate, anti-lineage (CD2, CD3, CD4, CD8, CD13, CD14, CD15, CD19, CD20, CD33, CD56, and CD235a) PE, anti- CD38 PE-Cy7, and anti- CD90 PE-Cy5 and sorted on an Aria II into HSC ($\text{CD34}^+\text{CD38}^-\text{CD45RA}^-\text{CD90}^+$), multipotent progenitor ($\text{CD34}^+\text{CD38}^-\text{CD45RA}^-\text{CD90}^-$), multilymphoid progenitor ($\text{CD34}^+\text{CD38}^-\text{CD45RA}^+\text{CD90}^-$), and common myeloid progenitor ($\text{CD34}^+\text{CD38}^+\text{CD45RA}^-$) fractions, as described elsewhere.¹³ DNA was extracted with the DNA isolation kit from Roche (Mannheim, Germany). *MYSM1* PCR products were gel purified and processed for single-molecule sequencing by using an Ion Personal Genome Machine (Ion Torrent; Life Technologies, Grand Island NY), according to the manufacturer's recommendations.

Three-dimensional model

The *MYSM1* JAB1/MPN/Mov34 (JAMM) domain has an isopeptidase activity, allowing removal of ubiquitin from conjugated substrates.⁴ The sequences of proteins from the JAMM family are highly divergent, but portions of the core, including amino acids from the catalytic site, are more conserved.¹⁴ Modeling was made by using as a template the experimental 3-dimensional structure of the Mov34 MPN domain, with which *MYSM1* is aligned with significant low E-values (3×10^{-7}) by using the Phyre fold recognition program.¹⁵ The modeled catalytic site, which is not present in the Mov34 template, is similar to those observed in the 3-dimensional

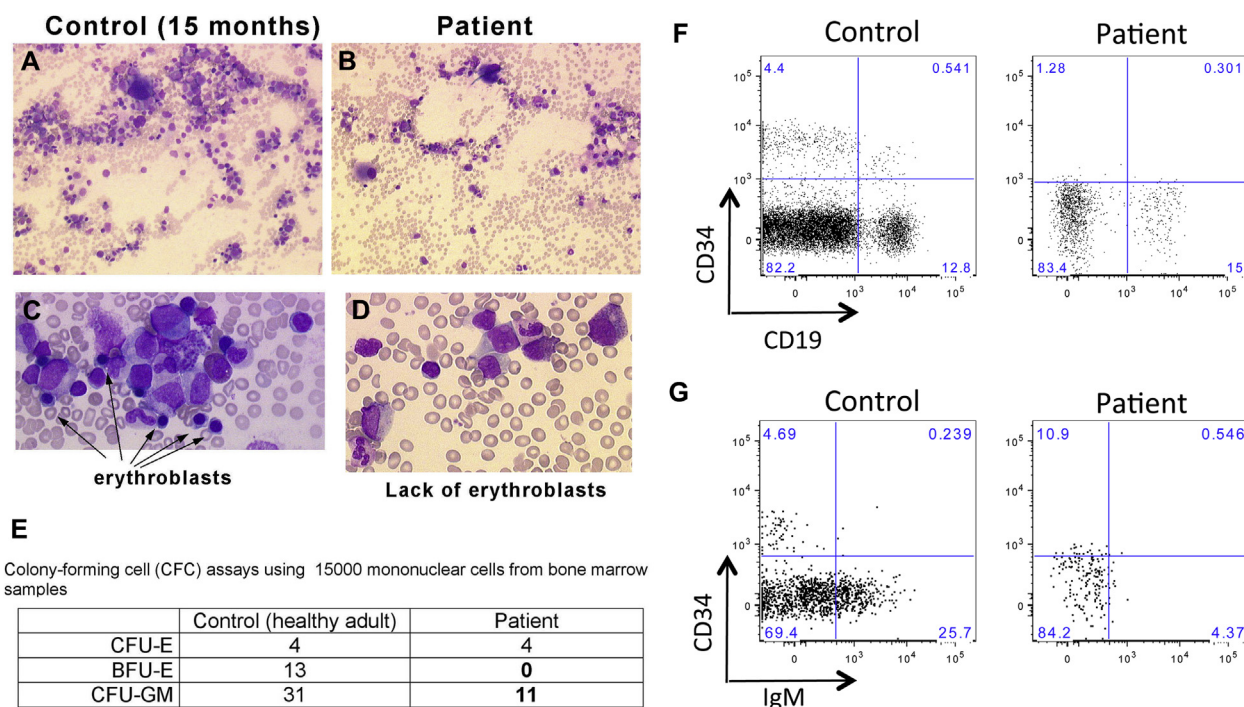


FIG 1. Phenotypic analysis of bone marrow cells. **A** and **B**, Bone marrow smear reveals hypocellular bone marrow with a paucity of erythroid precursors (erythroblasts) in the patient at 5 months of age (Fig 1, **B**) compared with a 15 month-old-control subject (Fig 1, **A**) by using May-Grünwald Giemsa staining captured with a Leica DM LB microscope with a $\times 40$ objective. The cellularity and cellular composition of the granulocyte series are normal. **C** and **D**, Absence of erythroid precursor at $\times 100$ magnification in the patient (Fig 1, **D**). **E**, Bone marrow mononuclear cells (15,000/well in duplicates) from a healthy adult control subject or the patient were cultured with Erythropoietin during 14 days. Erythroid progenitors (CFU-E and BFU-E) and granulocyte/macrophage progenitors (CFU-GM) were quantified. **F** and **G**, Bone marrow mononuclear cells from the patient and a healthy adult control subject were stained with anti-CD34 and anti-CD19 (Fig 1, **F**) or anti-CD34 and anti-IgM (gated on CD19⁺ cells; Fig 1, **G**). Numbers represent the percentage of cells within each quadrant or gate.

structures of “active” enzymes, such as AMSH-LP. In this site Zn²⁺ is coordinated by 2 histidines (His656 and His658), an aspartic acid (Asp669), and a water molecule hydrogen bonded to a glutamic acid (Glu597). An Ins-1 insertion, also found in AMSH family members and including 2 extended sequences, might provide the β -sheet interaction with the C-terminal tail of the distal ubiquitin or ubiquitin-like proteins.¹⁶

RESULTS

Patient's clinical features

The patient, a boy, is the first child of Turkish consanguineous parents. He was born at term with a weight of 2620 g (25th percentile), a height of 47 cm (25th percentile), and overt microcephaly (head circumference of 32 cm, <2nd percentile). He presented at birth with respiratory distress caused by choanal atresia that required surgical therapy and bilateral deafness caused by agenesis of the cochleovestibular nerves. Except for the absence of auditory nerves, cerebral magnetic resonance imaging was normal, with detection of both internal auditory canals. The hemogram performed at birth revealed profound aregenerative anemia (hemoglobin at 5 g/dL with reticulocytes at 15,000/ μ L) and marked leukopenia (polymorphonuclear neutrophils at 960/ μ L and lymphocytes at 1,800/ μ L). Bone marrow aspirate revealed an absence of erythroid precursors with normal granulocyte maturation (Fig 1). A colony-forming assay revealed a major reduction in both erythroid and granulomonocyte

progenitors in the patient's bone marrow compared with that seen in a healthy control subject (Fig 1, **E**). Phenotypic analysis of bone marrow cells by using flow cytometry revealed 3-fold less CD34⁺ hematopoietic progenitors compared with the bone marrow of a healthy adult donor (Fig 1, **F**). Cells from the CD19⁺ compartment are also altered in the patient's bone marrow because they do not express surface IgM and did not correspond to fully differentiated pre-BII/immature B-cell stages (Fig 1, **G**). Altogether, this analysis revealed a blockade between the pre-BI and pre-BII/immature stages in the B-cell differentiation process. At the periphery, the patient presented with agammaglobulinemia with B lymphocytosis, T-cell lymphopenia predominant on CD4⁺, and moderate natural killer (NK) cell lymphopenia (Table I). In addition, a sharp reduction in numbers of naive (CD45RA⁺CD31⁺) CD4⁺ T cells with a nevertheless normal polyclonal T-cell repertoire was noticed in the patient's blood (Table I and see Fig E1 in this article's Online Repository at www.jacionline.org). The patient was promptly started on intravenous immunoglobulin replacement and co-trimoxazole prophylaxis. Under this prophylactic therapy, the patient remained free of infection.

Identification of a MYSM1 mutation

The consanguineous status of the patient suggested an autosomal recessive disease (Fig 2, **A**). We searched for candidate

TABLE I. Immunohematologic features of the patient at 4 months of age

White cell count	2,900/ μ L (6,000-17,500/ μ L)
Polymorphonuclear neutrophils	700/ μ L (1,000-8,500/ μ L)
Lymphocytes	1,300/ μ L (2,700-13,500/ μ L)
Hemoglobin	7.5 g/dL (11.5-13.5 g/dL)
Platelets/ μ L	24,900/ μ L (150,000-500,000/ μ L)
Lymphocyte phenotype	
T cells	
CD3 ⁺	1,183/ μ L (2,500-5,600/ μ L)
CD4 ⁺	585/ μ L (1,800-4,000/ μ L)
CD8 ⁺	481/ μ L (590-1,600)
CD4 ⁺ CD45RA ⁺ CD31 ⁺ (%)	17 (60-72)
B cells	
CD19 ⁺	0/ μ L (430-3,000/ μ L)
NK cells	
CD56 ⁺ CD16 ⁺	130/ μ L (170-830/ μ L)
T-cell proliferation ($\times 10^3$ cpm)	
PHA	38 (>50)
Serum immunoglobulin levels	
IgG	1.94 g/L (2.35-4.37 g/L)
IgA	<0.06 g/L (0.2-0.62 g/L)
IgM	<0.04 g/L (0.34-0.95 g/L)

For all values, normal ranges or thresholds are indicated in parentheses.

mutations by combining whole-genome homozygosity mapping (WGHM) and whole-exome sequencing in patient. Among the identified homozygous substitutions and insertions/deletions found by using whole-exome sequencing, only 6 were located in a genetic region compatible with the WGHM analysis and absent from the 1000 Genomes, EVS, and dbSNP data sets and our in-house databases. Remarkably, only one was predicted to be “probably damaging” by Polyphen-2 (see Table E1 in this article’s Online Repository at www.jacionline.org). This variant affects the coding sequence of the histone deubiquitinase *MYSM1* (also known as 2A-DUB⁴) at position 1967 (c.1967A>G), leading to the amino acid substitution p.H656R. In addition, the analysis of this variant by the combined annotation-dependent depletion method gives rise to a scaled C-score of 27.9, meaning that it is predicted to be one of the 0.17% most deleterious substitutions that can be found in the human genome.¹⁷ Inheritance of the *MYSM1* mutation was confirmed by means of direct sequencing of the *MYSM1* gene in the patient and his parents (Fig 2, B). The p.H656R substitution is located in the highly conserved catalytic MYSM1 JAMM domain, which has an isopeptidase activity, allowing removal of ubiquitin from conjugated substrates (Fig 2, C).⁴ *In silico* analysis predicts that the mutation disrupts interaction with the zinc ion and therefore compromises the MYSM1 protease activity (Fig 2, D). Moreover, immunofluorescence study indicated that the endogenous mutant MYSM1, although correctly located in the nucleus, was weakly detected in the patient’s fibroblasts compared with that seen in control fibroblasts (Fig 2, E). Western blot analysis confirmed the reduced amount of endogenous MYSM1 in the patient’s fibroblast whole-cell lysates (Fig 2, F). Thus the p.H656R MYSM1 mutation is located in the catalytic domain and affects the stability of the protein.

The patient experienced spontaneous *in vivo* genetic reversion

Remarkably, during the course of our study, we noticed a spontaneous improvement of the patient’s immunohematologic

features, including a correction in the number of circulating T and NK lymphocytes, reticulocytes, and neutrophils (Fig 3, A). More strikingly, circulating B lymphocytes (CD19⁺), which were completely absent in the patient at age 4 months (Table I), were detected at a normal level at 33 and 39 months of age (Fig 3, A) and exhibited a polyclonal and diversified repertoire (see Fig E2 in this article’s Online Repository at www.jacionline.org). In addition, the hemoglobin level normalized in the absence of blood transfusions (Fig 3, A). We extracted genomic DNA from the patient’s purified circulating B lymphocytes and performed micro-satellite genotyping to test whether this immunohematologic restoration could originate from maternal cells, as observed in other situations.¹⁸ This analysis indicated that the circulating B cells were of the patient’s own origin and not from his mother (see Fig E3 in this article’s Online Repository at www.jacionline.org). Then we amplified and directly sequenced the *MYSM1* gene in the patient’s B cells to seek after a possible genetic reversion. Unlike the patient’s fibroblasts that carried the homozygous *MYSM1* mutation (Figs 2, B, and 3, B), the patient’s B lymphocytes were heterozygous for the mutation (Fig 3, B), indicating a spontaneous *in vivo* reversion to normal of inherited mutation on one allele, a rare event known as “back mutation.”^{19,20} Next, to determine precisely what hematopoietic cell lineage experienced the genetic reversion, we purified the major populations of stem, progenitor, and precursor cells from the patient’s bone marrow aspirate (the patient was 39 months old)¹³; extracted the genomic DNA; PCR amplified the *MYSM1* region harboring the mutation; and quantified the presence of the wild-type (WT; c.1967A) and mutated (c.1967G) *MYSM1* sequences through single-molecule sequencing. To validate the quantitative reliability of this method, we first analyzed DNA from 2 WT control subjects, the patient’s parents, the patient’s primary fibroblasts, and the patient’s blood cells before reversion (at 4 months of age). In both WT control subjects, 99.8% of reads carried the WT *MYSM1* sequence (the remaining 0.2% likely represented sequence on PCR errors), whereas, as expected for a heterozygous status, the patient’s parents exhibited virtually 50% of WT and 50% of mutated *MYSM1* sequences (Fig 3, C and D). In accordance with a homozygous mutated status, the *MYSM1* mutation was recovered in 99.86% and 96.8% of sequences obtained from the patient’s fibroblasts and blood cells before reversion, respectively. Similar analysis revealed that the back mutation (ie, the WT *MYSM1* sequence in the patient) was detected with a frequency ranging from 41.3% to 50.4% of sequence reads among the various sorted bone marrow populations, meaning that between 82.6% and 100% of these cells carried the genetic reversion (Fig 3, C and D). Importantly, the genetic reversion was found in 100% of the HSCs, suggesting that the back mutation took place in this cell type and ultimately led to correction of all the patient’s immunohematologic defects, including B-lymphocyte development in bone marrow (Fig 3, E).

To further analyze the effect of the genetic reversion in circulating PBMCs at a later time point, we cell sorted the patient’s T lymphocytes (CD3⁺), B lymphocytes (CD19⁺), monocytes (CD14⁺), and NK cells (CD56⁺) when the patient was 65 months of age and assessed the *MYSM1* mutation frequency by using the same approach as described above. DNA from a healthy control subject, the patient’s mother, and the patient’s fibroblasts were used as quantitative controls (Fig 4, A and B). Virtually 50% of sequence reads from the patient’s B lymphocytes, NK cells, and monocytes carry WT *MYSM1* sequences

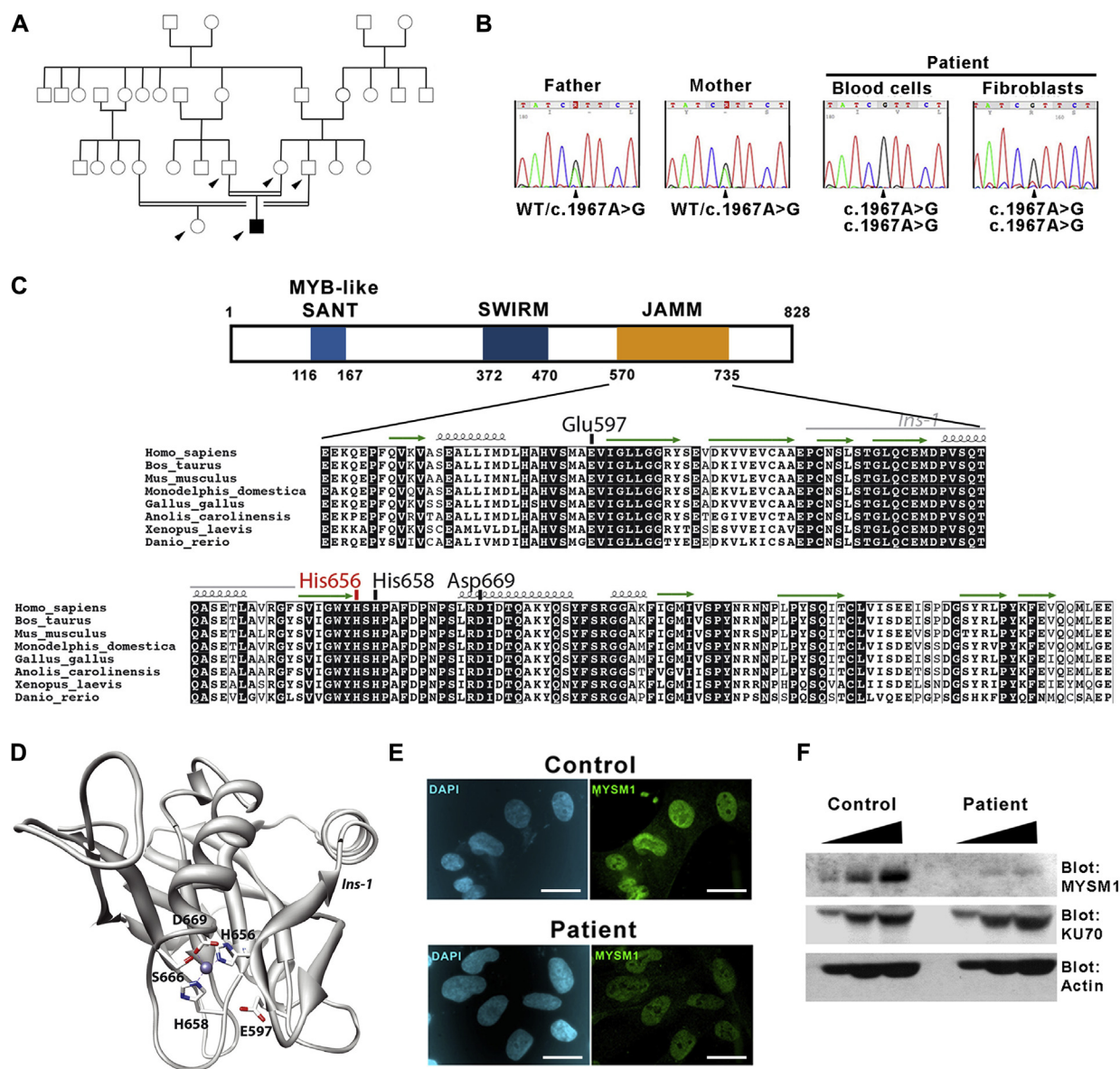


FIG 2. Mutation identified in the catalytic domain of MYSM1. **A**, Pedigree of the patient's family. WGHM was performed with the subjects indicated by arrows. **B**, Direct sequencing of the *MYSM1* gene in the patient and his parents. **C**, The mutation identified in the patient (p.H656R) is localized in the MPN domain and affects a histidine (H656 in red) highly conserved among species. **D**, Model of the 3-dimensional structure of the MYSM1 JAMM/MPN domain. **E**, Immunofluorescence with an anti-MYSM1 antibody revealed lower detection of MYSM1 in the patient's cells with that seen in a control subject. Identical parameters of image acquisition were applied in both conditions. Scale bars = 20 μm. **F**, Immunoblot of MYSM1 with lysates from a healthy control subject and the patient. KU70 and actin immunoblots were used as loading controls.

(Fig 4, A and B), indicating that approximately 100% of these cells originated from reverted precursors (Fig 4, C). In contrast, WT *MYSM1* sequence reads only represented 31.7% of total reads in the patient's T lymphocytes (Fig 4, A and B), meaning that reverted circulating T lymphocytes represent 63.5% of the patient's total T lymphocytes (Fig 4). Overall, these observations indicate that a WT *MYSM1* allele provides a strong selective advantage for the differentiation of NK cells, monocytes, and B cells and, to a lesser extent, T cells. Additionally, the lower representation of reverted circulating T cells might reflect a longer lifespan of the cells already present before the reversion compared with the other cell types analyzed.

DISCUSSION

Our study defines a novel human disorder mainly characterized by B lymphocytosis, T lymphopenia, and hematopoiesis impairment caused by MYSM1 deficiency. The complete restoration of an immunohematologic system upon genetic reversion in the patient's HSCs highlights the strong *in vivo* selective advantage provided by WT MYSM1, reinforcing the critical role for this factor in human hematopoiesis and lymphopoiesis. This genetic reversion also corroborates the causal link between the immunohematopoietic impairment and the MYSM1 defect. Similar genetic reversions in the immunohematopoietic system have been reported in rare situations of DNA

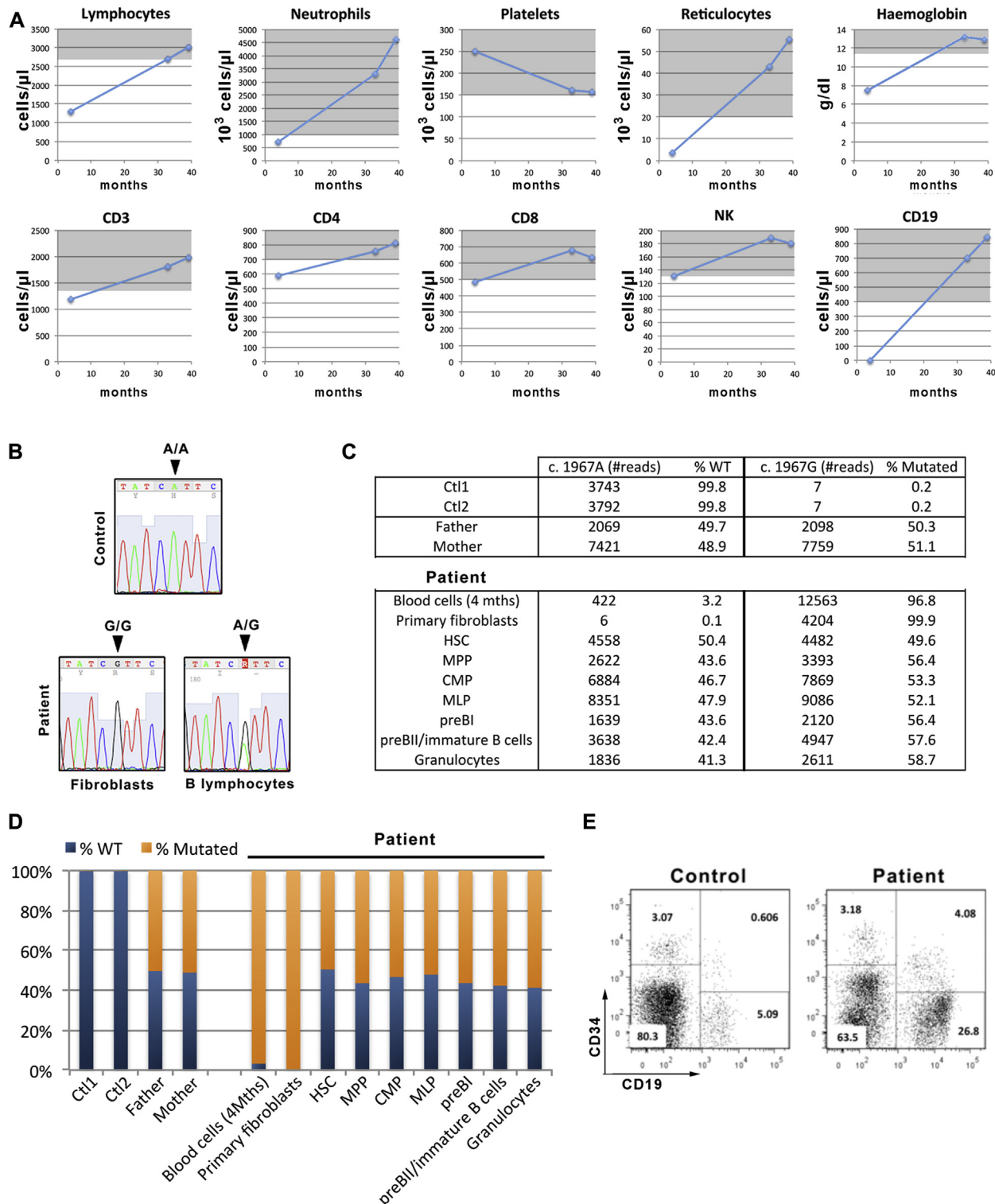
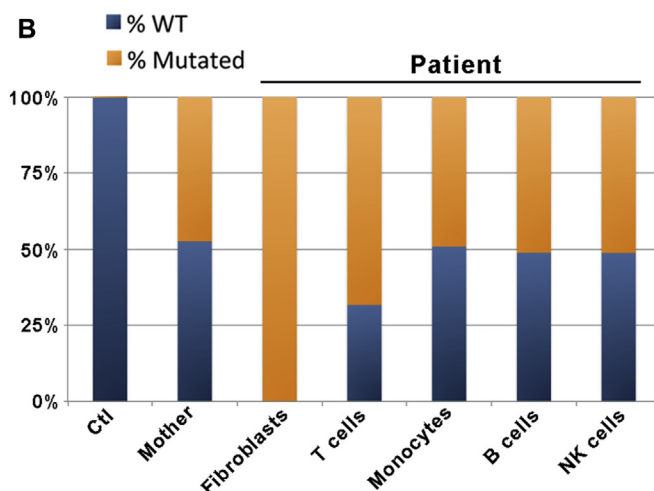


FIG 3. Spontaneous genetic reversion in the patient. **A**, Immunohematologic values in the patient before (at 4 months) and after (at 33 and 39 months) reversion revealed a spontaneous correction to normal values in all immunohematopoietic cell compartments. Gray areas represent normal values obtained in healthy subjects. **B**, Sequencing analysis indicated that a spontaneous genetic reversion occurred in *MYSM1* at position c.1967 in the patient's B lymphocytes, leading to a WT sequence on 1 allele. **C**, Quantitative analysis of WT (c.1967A) and mutated (c.1967G) *MYSM1* sequences obtained by using high-throughput sequencing in the different subjects and cell types in patient at 39 months of age. **D**, Graphic representation of the results obtained in Fig 3, C. **E**, Analysis of bone marrow mononuclear cells from a healthy adult control subject and the patient after genetic reversion reveals complete restoration of B-cell development in the patient (compared with Fig 1, F).

A

	# reads WT	% WT	# reads Mut	% Mut	% reverted	total # reads
Blood Ctl	21937	99.9	15	0.01	NA	21952
Blood Mother	4277	52.6	3847	47.4	NA	8124
Fibroblasts patient	13	0.2	6332	99.8	0.06	6345
CD3 ⁺ Patient	4684	31.7	10079	68.3	63.5	14763
CD14 ⁺ Patient	12885	50.9	12433	49.1	101.8	25318
CD19 ⁺ patient	5302	48.9	5543	51.1	97.8	10845
CD56 ⁺ Patient	4131	48.8	4337	51.2	97.6	8468

B



C

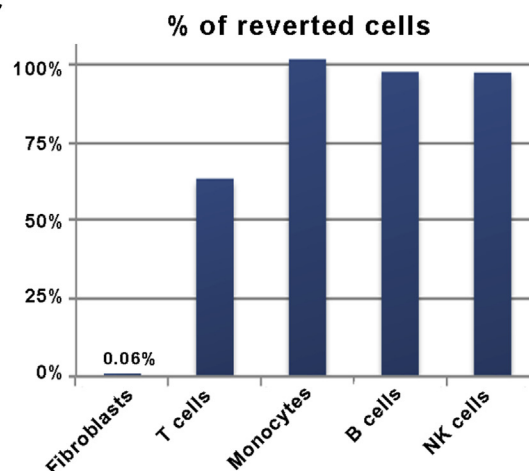


FIG 4. Frequency of reverted circulating PBMCs in the patient at 65 months of age. **A**, Quantitative analysis of WT (c.1967A) and mutated (c.1967G) *MYSM1* sequences obtained by using high-throughput sequencing in the different subjects and cell types. **B**, Graphic representation of the results obtained in Fig 4, A. **C**, Graphic representation of the percentage of reverted cells in the different cell populations of the patient.

repair defect–associated diseases, such as Fanconi anemia^{19–22} and Bloom syndromes,^{21,23} as well as other primary immunodeficiencies.^{24–26} Remarkably, the *MYSM1*-deficient patient shares several phenotypic aspects with *Mysm1* KO mice. Indeed, almost complete lack of B lymphocytes, T and NK lymphopenia, and anemia were observed in both the patient and *Mysm1* KO mice. It is likely that the immunohematologic defect and, in particular, the B lymphocytosis observed in the patient results from a transcriptional deregulation of key factors involved in B-cell development and HSC homeostasis, as found in *Mysm1*-deficient mice. However, such transcriptomic analyses were not possible in the patient because HSCs were virtually absent before the genetic reversion. Nonetheless, one cannot exclude that a part of the clinical features relies on the DNA repair defect because DNA damage accumulation in HSCs can lead to overt hematopoietic impairment^{27,28} and that most of the defect observed in *Mysm1* KO mice is restored when the P53-dependent apoptotic pathway is abolished.⁸ Moreover, recently, it has been reported that *MYSM1* is recruited to DNA damage.²⁹ Further analysis is required to test whether the *MYSM1* deficiency identified in the patient could result in a DNA repair defect.

Overall, our study reports a spontaneous *in vivo* genetic reversion in an HSC, a phenomenon described only in a few cases. In addition, our observation defines a novel immunohematologic deficiency and provides evidence that *MYSM1* is absolutely required for B-cell differentiation and proper immunohematologic development in human subjects.

We thank the patient and his family; Dr Chantal Brouzes for the myelogram analysis; and Laetitia Gaillard, Solenn Pruvost, Laure Caccavali, Sonia Luce, Jeremy Schwartzentruber, and Jacek Majewski for technical assistance.

Clinical implications: Mutation in the human histone deubiquitinase *MYSM1* leads to a syndrome characterized by a complete lack of B lymphocytes, T-cell lymphopenia, defective hematopoiesis, and developmental abnormalities.

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METHODS

T-cell repertoire analysis

Total RNA was prepared from sorted T cells by using the Total RNA Miniprep Kit (Sigma-Aldrich), and cDNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen). The different BV germline genes can be clustered in 24 families according to their level of homology (IMGT nomenclature, <http://imgt.cines.fr>).

For quantitative repertoire, PCR reactions were carried out by combining a reverse primer and a specific fluorophore-labeled probe for the constant region (MGB-TaqMan probe) with one of 24 primers covering the different BV chains. Real-time PCR reactions were subsequently carried out in a 25- μ L reaction mixture with a final concentrations of 400 nmol/L of each oligonucleotide primer, 200 nmol/L of the fluorogenic probe, and 1 $\times$ FastStart Universal Probe master Mix (Roche). Thermal cycling conditions comprised a FastStart Taq DNA polymerase activation at 95°C for 10 minutes and then subjected to 40 cycles of denaturation at 95°C for 15 seconds, annealing, and extension at 60°C for 1 minute. For all these different reactions, real-time quantitative PCR was then performed on an ABI 7300 system (Applied Biosystems, Courtaboeuf, France). For Immunoscope profiles, products were then subjected to runoff reactions with a nested fluorescent primer specific for the constant region for 3 cycles. The fluorescent products were separated and analyzed by using an ABI-PRISM 3730 DNA analyzer. The size and intensity of each band were analyzed with Immunoscope software,^{E1} which has been adapted to the capillary sequencer. Fluorescence intensities were plotted in arbitrary units on the y-axis, and CDR3 lengths (in amino acids) were plotted on the x-axis.

B-cell repertoire analysis

VH gene use and CDR3 analysis have been performed by using the Immunoscope method coupled with real-time PCR to provide quantitative information on VH gene use. Briefly, PCR reactions were carried out by combining a primer and a specific fluorophore-labeled probe for the constant region (CH μ or CH γ) with one of 8 primers covering the different VH1-7 chains. Reactions have been carried out with the TaqMan 7300 (Applied Biosystems) and standard reagents from Applied Biosystems. The relative use of each VH family was calculated according to the following formula:

$$\begin{aligned} x &= 16 \\ U(\text{IgVH}y) &= \sum 2^{(C_t(x) - C_t(y))} \\ x &= 1, \end{aligned}$$

in which $C_t(x)$ is the fluorescent threshold cycle number measured for VH_y family. Then PCR products have been subjected to runoff reactions with a nested fluorescent primer specific for the constant region gene. The fluorescent products have been separated and analyzed on an ABI-PRISM 3730 DNA Analyzer to determine CDR3 lengths according to the procedure, as described previously.^{E2}

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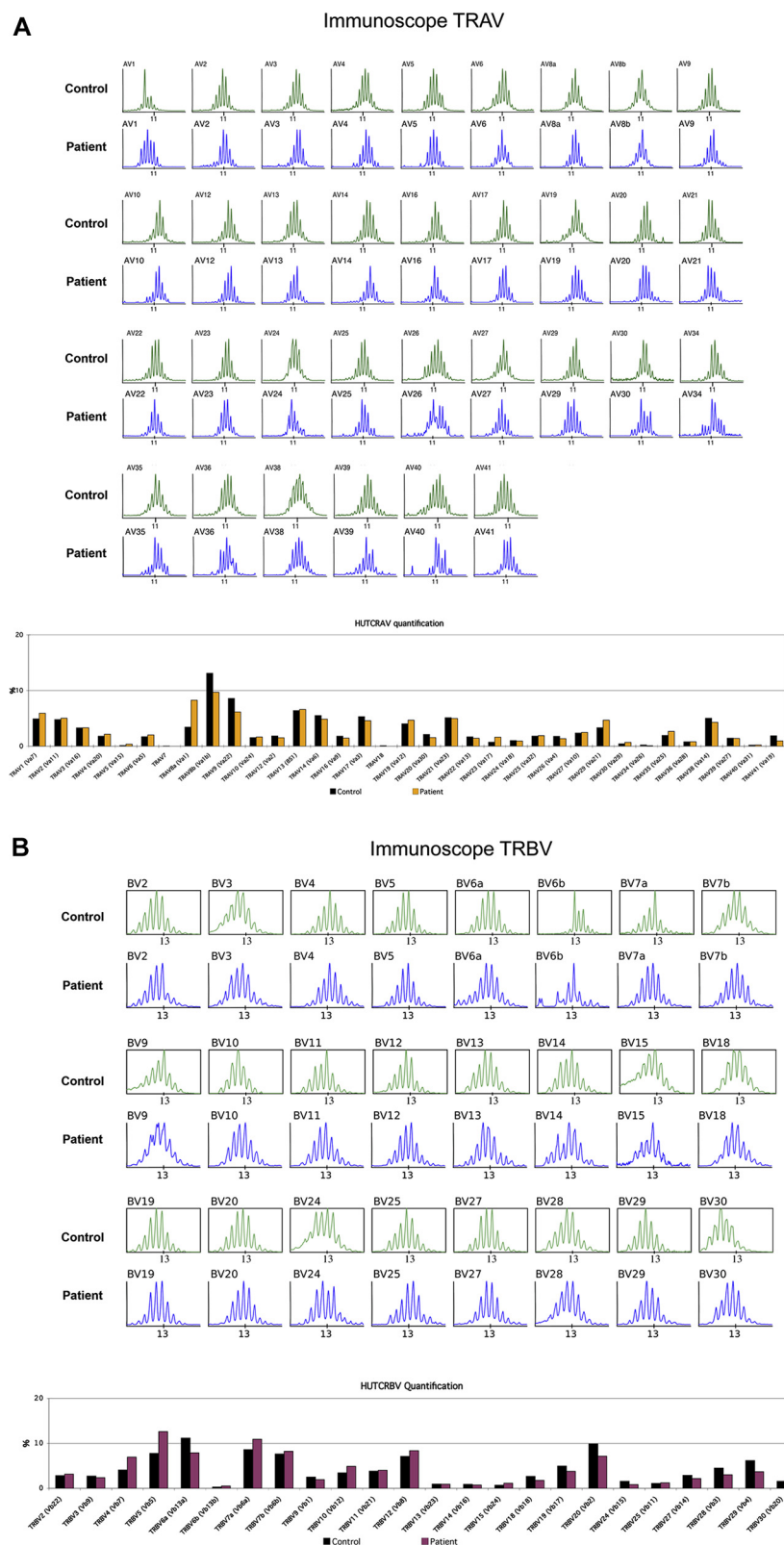
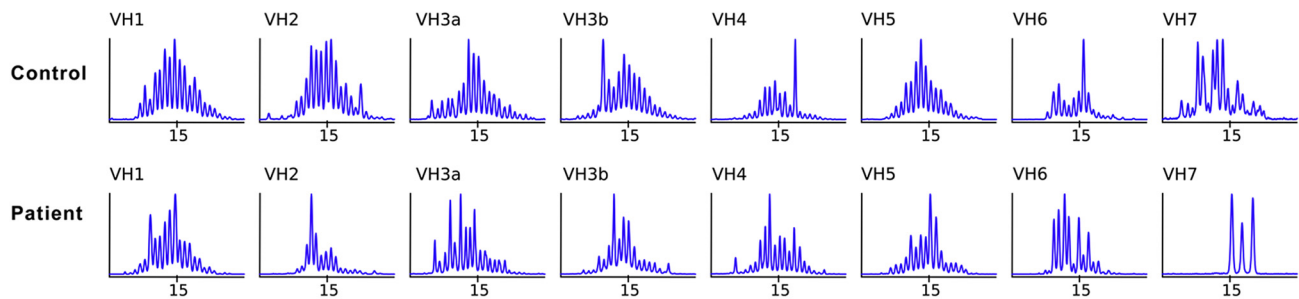


FIG E1. Analysis of the T-cell receptor α (**A**) and β (**B**) repertoires in the patient at 28 months of age. cDNA prepared from PBMCs of the patient and a control subject was amplified in multiple PCRs primed by specific primers, followed by runoff reactions with a nested fluorescent primer specific for the segment. Labeled DNA copies were analyzed, and the size and intensity of each band were recorded and analyzed with Immunoscope software. CDR3 size distribution analysis of the patient's and control subject's peripheral T cells was performed for all AV and BV segments.

A

Control		Patient	
IgM	% IgM	IgM	% IgM
VH1	3,0	VH1	0,2
VH2	0,5	VH2	0,6
VH3a	1,1	VH3a	3,0
VH3b	53,0	VH3b	65,6
VH4	26,2	VH4	18,2
VH5	1,0	VH5	0,6
VH6	0,2	VH6	0,3
VH7	0,1	VH7	0,0
% Total	85,1	% Total	88,7

IgM**B**

Control		Patient	
IgG	% IgG	IgG	% IgG
VH1	1,6	VH1	0,2
VH2	0,1	VH2	0,1
VH3a	0,3	VH3a	0,5
VH3b	9,4	VH3b	8,1
VH4	3,4	VH4	2,0
VH5	0,1	VH5	0,3
VH6	0,0	VH6	0,0
VH7	0,0	VH7	0,0
% Total	14,9	% Total	11,3

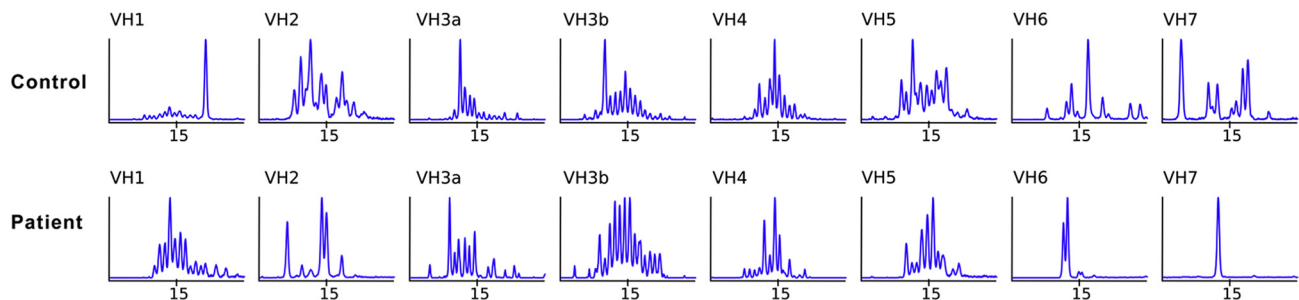
IgG

FIG E2. Analysis of B-cell receptor IgM (A) and IgG (B) repertoires by using Immunoscope software in the patient at 39 months of age (after genetic reversion) and in a control subject reveals a polyclonal repertoire in the patient.

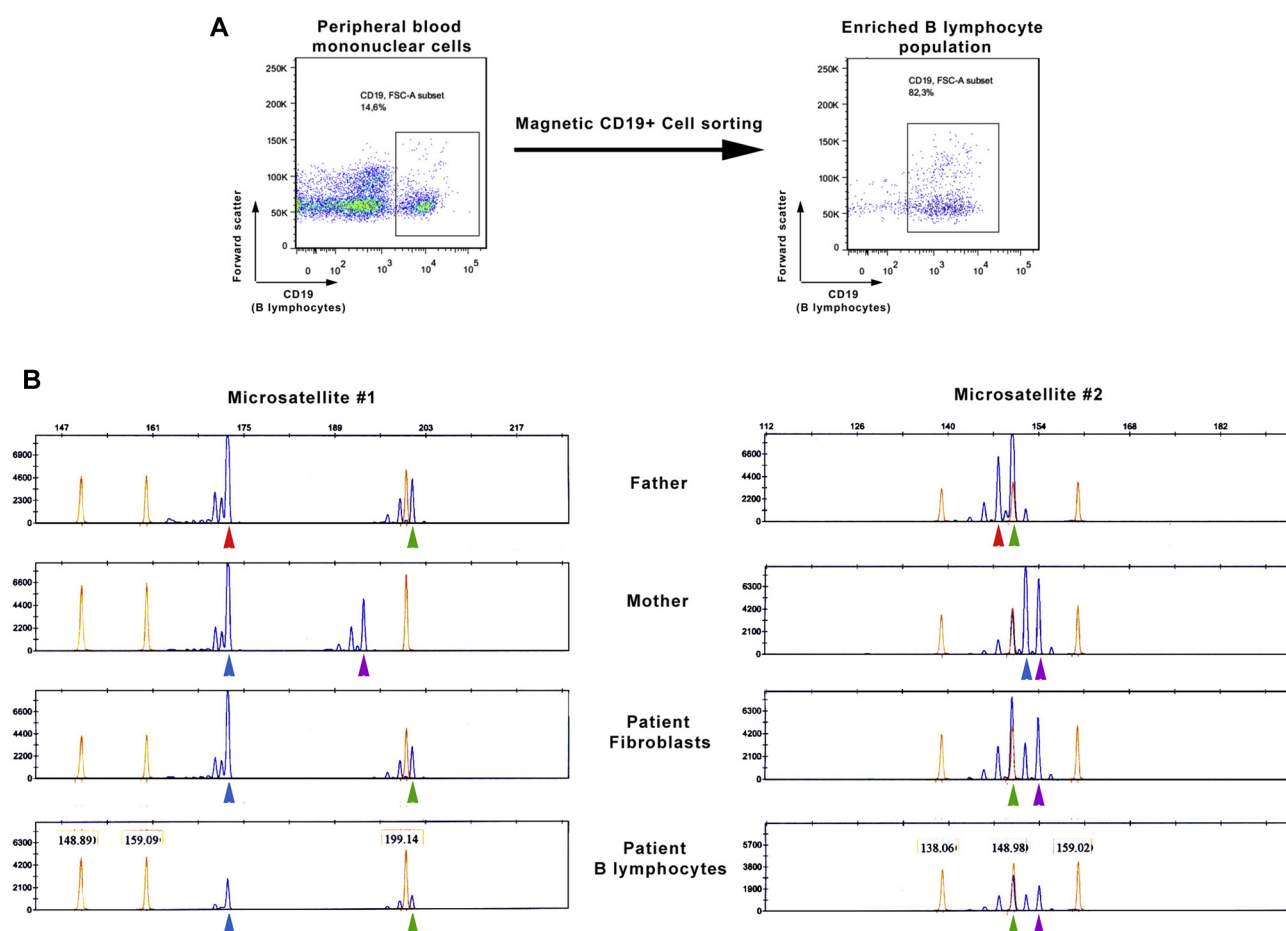


FIG E3. The patient's circulating B cells are not of maternal origin. **A**, The patient's circulating B cells were enriched from PBMCs by means of magnetic cell sorting with an anti-CD19 antibody. B-cell enrichment was confirmed by using fluorescence-activated cell sorting analysis. **B**, Genetic study of the linkage patient's family. Two microsatellite repeats were amplified with fluorescently labeled primers. Then the alleles from each subject in the family were separated by size, and the marker was tested for linkage with another. The raw family data are represented. *Colored arrows* represent the different alleles. Circulating B lymphocytes in the patient are not of maternal origin because the microsatellite profiles from the mother's blood cells and the patient's B cells are different.

TABLE E1. Candidate genes with homozygous changes found in the patient, as evidenced by WES

Gene	Gene position (GRCh37)	Mutation (cDNA)	Mutation protein	Polyphen-2 score	Gene description
NM_001085487.1 (<i>MYSM1</i>)	chr1:59.167747-59.123.590	c.1967A>G	p. H656R	0.999	Myb-like, SWIRM and MPN domains 1; histone H2A deubiquitinase
NM_015888.4 (<i>HOOK1</i>)	chr1:60.278.533-60.344.050	c.493T>A	p.L165M	0.094	Hook homolog 1 (Drosophila)
NM_001164835.1 (<i>LITDI</i>)	chr1:62.658.474-62.680.000	UTR		ND	LINE-1 type transposase domain containing 1
NM_003838.3 (<i>FPGT</i>)	chr1:74.661.896-74.676.148	Splice site		ND	Fucose-1-phosphate guanylyltransferase
NM_003393.3 (<i>WNT8B</i>)	chr10:102.242.185-102.242.367	c.759G>A	p.P253P	0	Wingless-type MMTV integration site family, member 8B
NM_001166283.1 (<i>RGMA</i>)	chr15:93.588.616-93.588.434	c.1080C>T	p.P360P	0	RGM domain family, member A

ND, Not determined; *UTR*, untranslated region.