



Hyper-IgE Syndrome due to an Elusive Novel Intronic Homozygous Variant in *DOCK8*

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Abstract

Rare, biallelic loss-of-function mutations in *DOCK8* result in a combined immune deficiency characterized by severe and recurrent cutaneous infections, eczema, allergies, and susceptibility to malignancy, as well as impaired humoral and cellular immunity and hyper-IgE. The advent of next-generation sequencing technologies has enabled the rapid molecular diagnosis of rare monogenic diseases, including inborn errors of immunity. These advances have resulted in the implementation of gene-guided treatments, such as hematopoietic stem cell transplant for *DOCK8* deficiency. However, putative disease-causing variants revealed by next-generation sequencing need rigorous validation to demonstrate pathogenicity. Here, we report the eventual diagnosis of *DOCK8* deficiency in a consanguineous family due to a novel homozygous intronic deletion variant that caused aberrant exon splicing and subsequent loss of expression of *DOCK8* protein. Remarkably, the causative variant was not initially detected by clinical whole-genome sequencing but was subsequently identified and validated by combining advanced genomic analysis, RNA-seq, and flow cytometry. This case highlights the need to adopt multipronged confirmatory approaches to definitively solve complex genetic cases that result from variants outside protein-coding exons and conventional splice sites.

Keywords *DOCK8* deficiency · whole-genome sequencing · intronic variant · RNA sequencing · flow cytometry

Introduction

Dedicator of cytokinesis 8 (*DOCK8*) is a guanine nucleotide exchange factor that regulates actin polymerization, cytoskeletal rearrangements, and immunological synapse formation to induce activation, proliferation, differentiation,

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migration, and survival of immune cells [1, 2]. Its fundamental role in immunity and host defense was established following the identification of individuals with a combined immunodeficiency resulting from biallelic inactivating mutations in *DOCK8* [3, 4]. Pathogenic mutations include large deletions across *DOCK8* or nonsense or missense variants in exons or splice sites that introduce premature stop codons, aberrant splicing and/or protein instability, and subsequent loss of expression of DOCK8 protein [3–8]. Clinical manifestations of DOCK8 deficiency include severe, recurrent, and life-threatening sinopulmonary and mucocutaneous viral (molluscum contagiosum virus, HSV, HPV), bacterial (*Staphylococcus aureus*), and fungal (*Candida albicans*) infections. Patients also develop hypereosinophilia, severe atopic dermatitis/eczema, extremely elevated levels of serum IgE (10^3 – 10^5 IU/ml; normal range 0–100 IU/ml), food allergies, and increased susceptibility to malignancy [3–8]. Hematopoietic stem cell transplant (HSCT) remains the standard of care for DOCK8-deficient individuals [7, 9–12]. Currently, > 200 DOCK8-deficient patients have been reported globally.

Because of the dire prognosis for untreated DOCK8-deficient patients, it is crucial to identify affected individuals early so treatment options can be implemented prior to the onset of severe infections and irreversible tissue damage [5]. Identification of pathogenic variants is also important for genetic testing of potentially affected siblings, carrier screening, and reproductive planning [13]. Here, we present a consanguineous family where the index case exhibited clinical manifestations consistent with DOCK8 deficiency. However, initial extensive genetic analyses failed to identify a causative variant in any exons of the *DOCK8* gene. Using multiple approaches, we finally identified a novel intronic homozygous deletion upstream of exon 38 of *DOCK8*. This enabled definitive genetic diagnosis of DOCK8 deficiency in the index case, as well as diagnosing a clinically mild homozygous sibling who subsequently presented with severe disease and rapid genetic screening of two siblings born after diagnosis of the index case. This case is a salient example of the need to adopt a multi-omics approach to identify, functionally validate, and classify candidate intronic and exonic variants as pathogenic to enable the definitive molecular diagnosis of inborn errors of immunity.

Materials and Methods

Human Subjects

PBMCs were isolated from healthy donors (Australian Red Cross) and patients, as well as their siblings and parents. This study was approved by the Sydney Local Health

District RPAH Zone Human Research Ethics Committee and Research Governance Office, Royal Prince Alfred Hospital, Camperdown, NSW, Australia (Protocol X16-0210/LNR/16/RPAH/257); the South Eastern Sydney Local Health District Human Research Ethics Committee, Prince of Wales/Sydney Children's Hospital, Randwick, NSW, Australia (Protocol HREC/11/POWH/152); and the NIAID (Protocol 06-I-0015). Written informed consent was obtained from participants or their guardians.

Flow Cytometry

Immune Cell Phenotyping and Function

To investigate CD4⁺ and CD8⁺ T cell populations, PBMCs were incubated with mAbs against CD4, CD8, CCR7, CD45RA, CD28, CD57, CD95, and CD127. Naïve (CCR7⁺CD45RA⁺), central memory (TCM; CCR7⁺CD45RA⁺), effector memory (TEM; CCR7⁺CD45RA⁺), CD45RA⁺ effector memory (TEMRA; CCR7⁺CD45RA⁺), and CD4⁺ and CD8⁺ T cell subsets were then identified and enumerated. To investigate T cell exhaustion, the expression of CD28, CD57, CD95, and CD127 was determined on naïve, TCM, and TEMRA CD8⁺ T cell subsets [11, 14, 15]. B cell subsets were also assessed, as previously described [16].

DOCK8 Expression

Expression of DOCK8 in PBMCs from healthy donors and patients was determined by flow cytometry, as previously described [17]. Briefly, PBMCs were fixed with formaldehyde and stained with an unconjugated mouse anti-DOCK8 Ab or an isotype control mouse IgG1 mAb, followed by a secondary PE-conjugated rat anti-mouse IgG1. Saponin was used as the permeabilizing agent. Lymphocyte and monocyte populations were established based on FSC and SSC FACs profiles and DOCK8 expression and then investigated in these leukocyte populations [11, 14].

Intracellular Cytokine Production

To investigate cytokine production, CD45RA⁺ memory CD4⁺ T cells were isolated from healthy donors and patients by cell sorting and then cultured with T cell activation and expansion (TAE) beads (Miltenyi Biotech) at a cell: bead ratio of 2:1. After 5 days of culture, cells were restimulated with PMA (100 ng/ml) and ionomycin (750 ng/ml) for 6 h with brefeldin A (10 µg/ml) added after 2 h. Cells were fixed and stained intracellularly for IFNγ (B27) and IL-4 (8D4-8) expression using 0.5% saponin as the permeabilizing agent. The expression of these cytokines was then determined by flow cytometric analysis [11, 18].

Genome Sequencing

Whole-genome sequencing was performed by the Kinghorn Centre for Clinical Genomics Sequencing Laboratory, which was clinically accredited to ISO 15189 by the National Association of Testing Authorities, Australia (NATA, www.nata.com.au). Genomes were sequenced on a HiSeq X platform (Illumina) using TruSeq Nano DNA Library Preparation Kit and Illumina 150 bp paired-end sequencing to a mean coverage of $\geq 30\times$, with 97.7% of canonical protein-coding transcripts and splice sites (± 8 intronic nucleotides) covered at $\geq 15\times$ (historical median). Paired-end reads were initially aligned to the human genome reference sequence (GRCh37) using the Burrows–Wheeler Aligner (BWA-MEM), and variant calls were made using the Genomic Analysis Tool Kit (GATK). Over the reportable range (protein-coding exons, exon–intron boundaries ± 8 nucleotides, and untranslated regions), sensitivity was $> 99\%$ for single nucleotide variants and $> 95\%$ for small indels < 20 bp, with this analytical validity established using the variant location relative to GiaB v2.19 NA12878, Certified Reference Material. The reportable range of coverage for clinical genetic testing was based on the “National Pathology Accreditation Advisory Council’s Requirements for Human Medical Genome Testing Utilising Massively Parallel Sequencing Technologies” (<https://www1.health.gov.au/internet/main/publishing.nsf/Content/npaac-pub-mps>) and guidelines published by the European Society of Human Genetics [19].

Sanger Sequencing

PCR amplification and Sanger sequencing of *DOCK8* exons, with their immediately adjacent intronic or untranslated regions, were performed using genomic DNA from the patient’s PBMC as described previously [3]. To determine segregation of the candidate deletion variant in *DOCK8*, Sanger sequencing was performed by Garvan Molecular Genetics, which is also accredited by NATA to ISO/IEC 15,189, using genomic DNA isolated from PBMCs from various family members using the following primers: *DOCK8_F* tgtaaacgacggccagtGTTTTCTTCTGTCTCGTTTCTG; *DOCK8_R* caggaaacagctatgaccCCTCCAGCATGCTCAGATAC.

RNA Sequencing (RNA-seq)

PBMCs from I.2 (heterozygous carrier) and three healthy donors were stimulated in vitro with PMA/ionomycin for 16 h; mRNA was then purified using an RNeasy kit (Qiagen). cDNA libraries were prepared using a TruSeq Stranded mRNA Library Preparation Kit (Illumina), and sequencing was carried out on a NextSeq platform (Illumina) to obtain 150 bp paired-end reads. RNA-seq data are publicly

available from the Sequence Read Archive (SRA; GEO). Read ends were trimmed with Trimmomatic (v0.39) using a sliding window quality filter [20]. Trimmed reads were mapped to the human reference genome (GRCh38) using HISAT2 (v2.2.1) [21], and the GENCODE release v38 of the human genome was used to annotate genes [22]. Splicing of *DOCK8* was explored manually using the Integrative Genomics Viewer (IGV; v2.9.2) [23]. The National Center for Biotechnology Information (NCBI) database isoform 3 (NM_001193536.1) was used as the reference transcript for *DOCK8* exon coordinates in this study, as it was the major isoform in our PBMC samples, consistent with previous studies in T cells [6]. Protein domain diagrams were generated using the drawProteins R package [24]. The *DOCK8* protein structure prediction from AlphaFold [25] was visualized using PyMOL software (v. 2.3.2, Schrodinger).

Results

A Novel Case of Hyper-IgE Syndrome

The index case, a male, was the first-born child to consanguineous parents of Afghan origin (II.1, Fig. 1A). When he presented in 2013 (age 3.5 years) for immunological assessment, he had a clinical history of multiple food allergies, severe eczema, recurrent skin abscesses, and chest and ear infections. On examination, he had severe failure to thrive (weight: 9.8 kg, $< < 3$ rd centile; height: 95 cm, 5–10th centile), significant respiratory distress with widespread coarse crepitations, hepatosplenomegaly, chronic lichenified skin with multiple areas of inflammation, vesicles on his face, alopecia and ulceration of his scalp, dystrophic nails, and clubbing. A CT scan confirmed bronchiectasis. Baseline investigations showed microcytic hypochromic anemia, thrombocytosis, hypereosinophilia, elevated ESR and CRP, low serum IgM but increased IgA and IgG, and extremely high IgE (17,300 kU/ml). He had $CD4^+$ T cell lymphopenia ($0.35 \times 10^9/L$); however, $CD8^+$ T cells, B cells, and NK cells were within reference ranges of aged-matched healthy controls. Other relevant clinical features included a history of recurrent bacterial (*Streptococcus pyogenes*, *Hemophilus influenzae*), fungal (Candida, Aspergillus), and cutaneous (HSV, VZV) and non-cutaneous (chronic active EBV with viraemia, CMV, HHV6, norovirus, adenovirus) viral infections.

Patient II.1 underwent haploidentical HSCT in 2014 using mobilized peripheral blood stem cells from his father (I.1). He developed central venous line infections with *Staphylococcus aureus* and *Corynebacterium urealyticum* and had CMV reactivation and low-level adenoviremia post-HSCT, necessitating infusion of allogeneic trivirus (CMV, EBV,

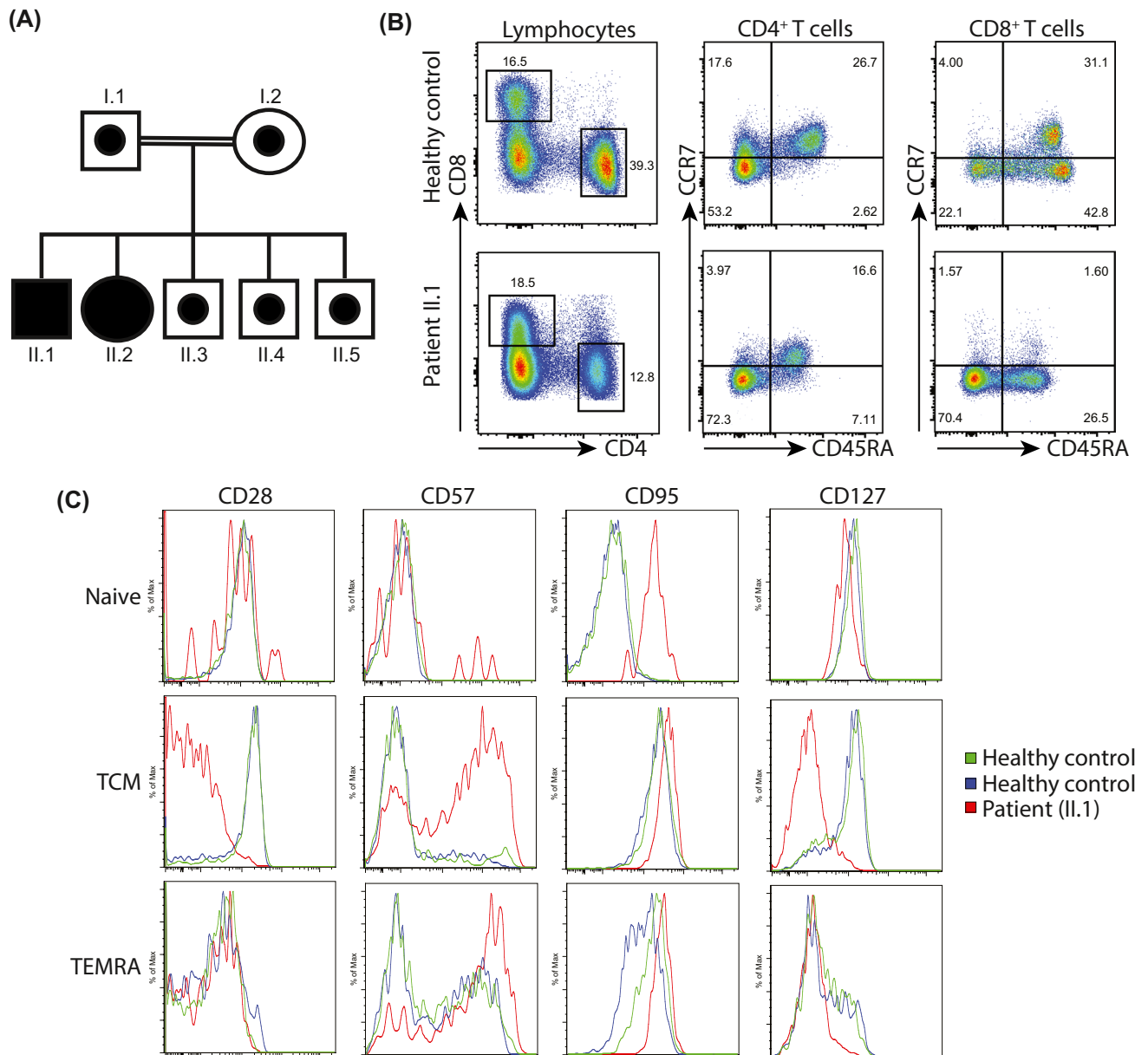


Fig. 1 T cell exhaustion and DOCK8 deficiency in a novel kindred with hyper-IgE syndrome. **A** Pedigree showing the segregation of mutant *DOCK8* in a consanguineous family. Solid symbols represent homozygous individuals, and open symbols with circles in the center represent heterozygous carriers. **B**, **C** PBMCs from healthy controls and patient II.1 were labeled with mAbs against CD4, CD8, CCR7, CD45RA, CD28, CD57, CD95, and CD127. **B** Pro-

portions of naïve (CCR7⁺CD45RA⁺), TCM, (CCR7⁺CD45RA⁻), TEM (CCR7⁻CD45RA⁻), and TEMRA (CCR7⁻CD45RA⁺) CD4⁺ and CD8⁺ T cell populations in a representative healthy control (top panel) and patient II.1 (bottom panel). **C** Expression of CD27, CD57, CD95, and CD127 on gated naïve, TCM, and TEMRA CD8⁺ T cell subsets from healthy controls and patient II.1

adenovirus)-specific CTLs. The post-transplant lymphoproliferative disease was managed by rituximab. Although he ultimately had immune reconstitution with 100% donor cells and normal B cell number, he remained hypogammaglobulinemic, requiring ongoing immunoglobulin replacement therapy.

Flow Cytometric Analysis Confirms DOCK8 Deficiency in II.1

DOCK8 deficiency was suspected from the clinical presentation and consistent immunological investigations of II.1. Our previous flow cytometric-based analyses of inborn errors of immunity identified a unique “lymphocyte signature” for DOCK8-deficient patients typified by a reduced CD4/

CD8 ratio and enhanced/premature CD8⁺ T cell exhaustion/senescence [11, 15, 18, 26]. This phenotype has also been reported for individuals with biallelic pathogenic variants in *RHOH* [27] or *STK4* [28], heterozygous deleterious mutations in *CDC42* [29], or gain of function (GOF) heterozygous mutations in *PIK3CD* [30]. However, the intact frequency of transitional B cells and paucity of memory B cells differentiate DOCK8 deficiency from *PIK3CD* GOF and *RHOH* deficiency [11, 16, 26, 27], elevated expression of CD95 on naïve CD8⁺ T cells distinguishes DOCK8 deficiency from *STK4* deficiency [11, 15, 28], and absence of phenotypic features of CD8⁺ T cell exhaustion delineates DOCK8 deficiency from *CDC42* dysregulation [29]. Thus, to provide correlative support for a potential diagnosis of DOCK8 deficiency, we performed extensive flow cytometric analysis of PBMCs from II.1. Using this approach, his lymphocytes were found to exhibit features of this canonical DOCK8-associated immune cell phenotype, including reductions in the frequency of memory B cells (not shown) and CD4⁺ T cells, and a subsequent reduced CD4:CD8 ratio (Fig. 1B) [11, 15, 18, 26]. While the distribution of CD45RA⁺CCR7⁺ naïve, CD45RA⁻CCR7⁺ TCM, and CD45RA⁻CCR7⁻ TEM CD4⁺ T cells in II.1 was similar to healthy controls (Fig. 1B), his CD8⁺ T cells showed signs of accelerated differentiation in vivo, evidenced by a severe reduction in proportions of naïve and a dramatic accumulation of TEM and TEMRA cells compared to healthy controls (Fig. 1B) [15, 18, 26]. Further analysis of CD8⁺ T cell from II.1 revealed signature features of exhaustion. Thus, all naïve CD8⁺ T cells expressed CD95, which is typically absent from naïve CD8⁺ T cells in healthy donors, and greater proportions of TCM and TEM cells from II.1 concomitantly expressed higher levels of the senescence/activation markers CD57 and CD95 and had downregulated CD28 and CD127 compared to these CD8⁺ T cell subsets in healthy controls (Fig. 1C) [11, 15, 26].

Subsequently, a flow cytometric assay to detect DOCK8 protein became available [17, 31]. Analysis of PBMCs from healthy controls revealed abundant DOCK8 expression in lymphocytes and monocytes (Fig. 2A). In contrast, DOCK8 was undetectable in monocytes and expressed at very low/negligible levels in lymphocytes from II.1 (Fig. 2A). There was also no evidence of somatic reversion, which can result in the expression of DOCK8 in some lymphocyte subsets [14, 31], in the patients' leukocytes (Fig. 2A). Surprisingly, while these cellular phenotypes were consistent with a diagnosis of DOCK8 deficiency, targeted sequencing of *DOCK8*, as well as other SCID/CID genes [32], failed to detect any candidate variants in protein-coding regions of these genes (not shown). Taken together, II.1 clinically, phenotypically, and biochemically resembled DOCK8 deficiency, despite being unable to identify a genetic variant to provide a definitive diagnosis.

Discovery of a Second Case of DOCK8 Deficiency in This Kindred

To extend investigations of this family, we assessed the parents (I.1, I.2) and younger siblings of II.1. Expression of DOCK8 in PBMCs from his younger brother II.3 was similar to that observed for healthy controls (Fig. 2A). However, despite being informed that his younger sister II.2 was relatively healthy, her lymphocytes and monocytes also lacked DOCK8 expression (Fig. 2A). Detailed clinical history at age 2.5 years revealed II.2 actually had experienced mild clinical features of DOCK8 deficiency, including eczema, food allergy, and corneal scarring suggesting HSV keratitis in early infancy. Consistent with these observations, T and B cells from II.2 exhibited the signature "DOCK8 phenotype" (Fig. 2B, not shown) [11, 15, 26], suggesting she carried the same genetic defect as II.1 and was thus also clinically DOCK8 deficient. Notably, the CD4/CD8 ratio in II.2 was similar to healthy controls (Fig. 2B), suggesting skewing of the T cell compartment to CD8⁺ T cells reflects disease progression rather than intrinsic DOCK8 deficiency.

In addition to a unique cellular phenotype [26], DOCK8-deficient lymphocytes exhibit characteristic functional defects, including skewing of their CD4⁺ T cells to the Th2 lineage and a reduction in Th1-type cells [11, 18]. To provide further evidence that these siblings had DOCK8 deficiency, we assessed cytokine expression by their memory CD4⁺ T cells following in vitro activation. Consistent with our previous findings, the frequencies of memory CD4⁺ T cells from II.1 and II.2 that expressed IFN γ were reduced, while those expressing IL-4 were increased compared to memory CD4⁺ T cells from healthy donors (Fig. 2C). The net effect of this was a reduction in the ratio of IFN γ /IL-4⁺ memory CD4⁺ T cells in both siblings relative to healthy donors (Fig. 2D), consistent with DOCK8 deficiency [11, 18].

Based on this, II.2 and her parents were counseled for HSCT, but this was declined because she was reasonably well at this stage. In 2014, II.2 (aged 4 years) developed further symptoms of DOCK8 deficiency, including recurrent lower bacterial respiratory tract infections, cutaneous HSV infection, chronic ear infections, severe food and environmental allergies, asthma, eczema, and elevated serum IgE (8100 IU/ml). She commenced intravenous immunoglobulin replacement and underwent haploidentical HSCT using stem cells mobilized from her father (I.1) in 2020 (aged 10 years).

Identification and Confirmation of a Novel Intronic Pathogenic Variant by Combining Whole-Genome Sequencing and RNA Sequencing

In 2016, through the Clinical Immunogenomic Research Consortium of Australasia (CIRCA) [33], II.1 and II.2

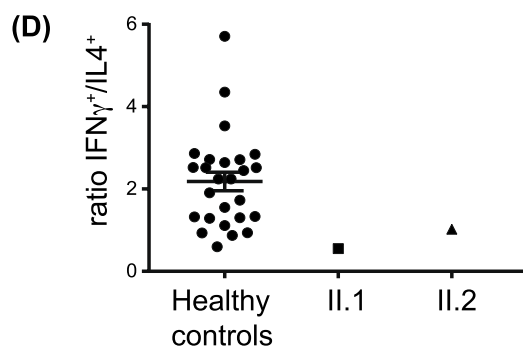
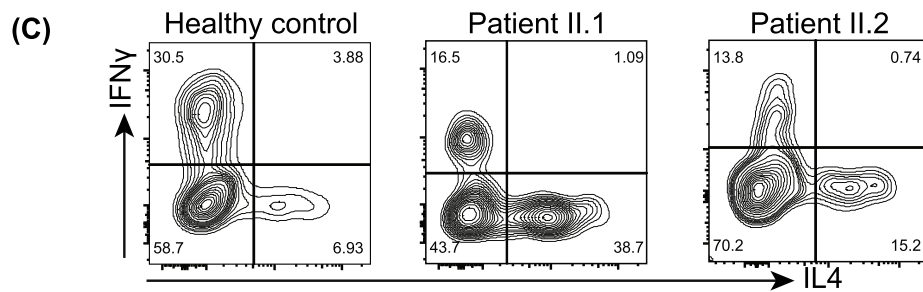
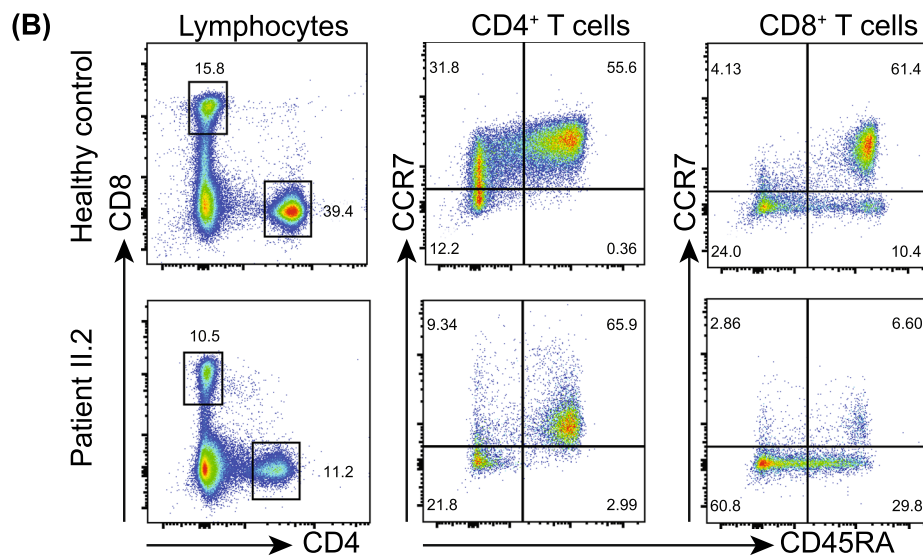
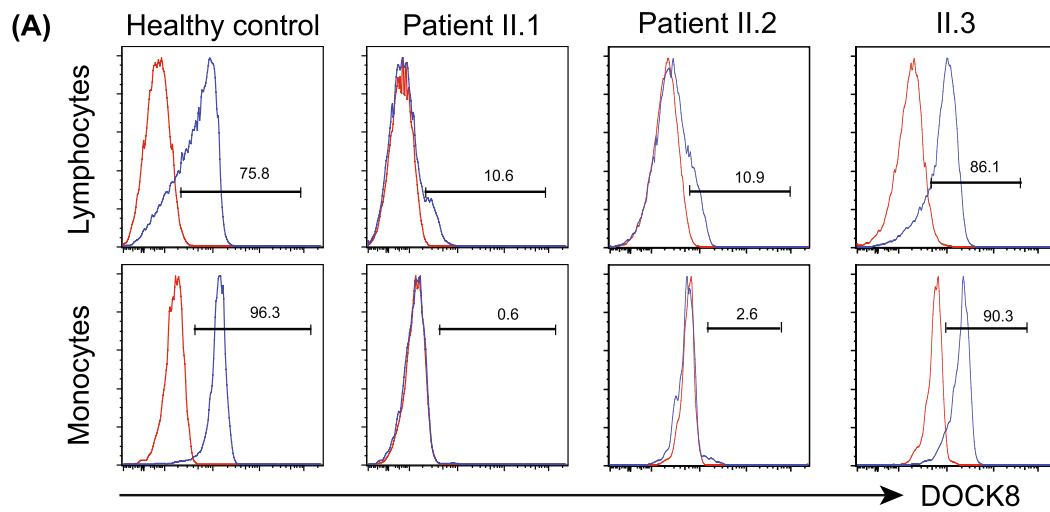


Fig. 2 Lack of DOCK8 protein expression in patient leukocytes. **A** PBMCs from healthy controls or family members II.1, II.2, and II.3 were fixed, permeabilized with saponin, and then stained intracellularly with an Ab against DOCK8 (blue histogram) or an isotype control Ab (red histogram). DOCK8 expression was determined in lymphocyte and monocyte populations, as defined according to forward (FSC) and side scatter (SSC) profiles, in healthy controls, II.1, II.2, and II.3. **B** PBMCs from healthy control and patient II.2 were labeled with mAbs against CD4, CD8, CCR7, and CD45RA. Proportions of naïve (CCR7⁺CD45RA⁺), TCM, (CCR7⁺CD45RA⁻), TEM (CCR7⁻CD45RA⁻), and TEMRA (CCR7⁻CD45RA⁺) CD4⁺ and CD8⁺ T cell populations. **C, D** CD45RA⁻ memory CD4⁺ T cells were isolated from healthy controls and patients II.1 and II.2 and activated in vitro. Intracellular expression of IFN γ and IL-4 was determined after 5 days, following restimulation with PMA/ionomycin. **C** Representative FACs plots of IFN γ and IL-4 expression. **D** Ratio of IFN γ ⁺/IL-4⁺ cells

underwent clinical whole-genome sequencing (WGS) with bioinformatic reduction and analysis to a virtual exome and analysis including the first and last 8 noncoding nucleotides of all introns. Surprisingly, but consistent with earlier targeted gene sequencing and despite all coding nucleotides being sequenced at > 15 depth during WGS, no variants were detected in any of the 47 protein-coding exons of *DOCK8* [6] or in any other genes associated with inborn errors of immunity [32, 34].

We also considered the possibility of structural variants [35] (with deletions > 20 bp) affecting *DOCK8*; however, none were detected. We then expanded our search to include all known Mendelian genes using broad search criteria based only on population allele frequency, in silico variant annotation criteria, and published gene–disorder relationships (www.omim.org). We identified and then evaluated in detail five homozygous variants (*COL18A1*, *KRT4*, *GAA*, *MYO7A*, and *TNXB*), five compound heterozygous variants (*ASPM*, *COG5*, *COL6A3*, *RP1L1*, and *TTN*), and 44 heterozygous variants without an accompanying *trans* allelic variant (*ABCB11*, *ADAMTS18*, *ADCY10*, *ANKRD11*, *ARID1B*, *ASAH1*, *C1R*, *CAP2*, *CDH15*, *CHRNA3*, *CISH*, *COL6A3*, *CUBN*, *FAM20C*, *FRAS1*, *GJA5*, *GYS1*, *KLHL3*, *LDB3*, *LFNG*, *MCM9*, *MFAP5*, *MMP9*, *MYT1L*, *NDUFA9*, *NFKB2*, *NNT*, *NPR3*, *PIGT*, *PKD1L1*, *PKHD1*, *PSTPIP1*, *RP1L1*, *SERAC1*, *SOD2*, *SON*, *SPG20*, *TGM5*, *TMIE*, *TRPM4*, *TTN*, *UNG*, *VPS13C*, *WDPCP*). All of these were considered for pathogenicity but were rejected as either not meeting reportable candidate variant criteria or not fitting the patient's clinical presentation.

Expanding our search further to include all rare variants, coding and noncoding, across the *DOCK8* locus subsequently revealed a novel homozygous 4 bp intronic deletion (Chr9(GRCh38): g.434771_434774del) located 9 bp upstream of exon 38 of *DOCK8* in II.1 and II.2. This intronic variant was initially undetected as our clinical genomics interpretation pipeline only included 8 bp upstream and downstream of exons. Sanger sequencing demonstrated that

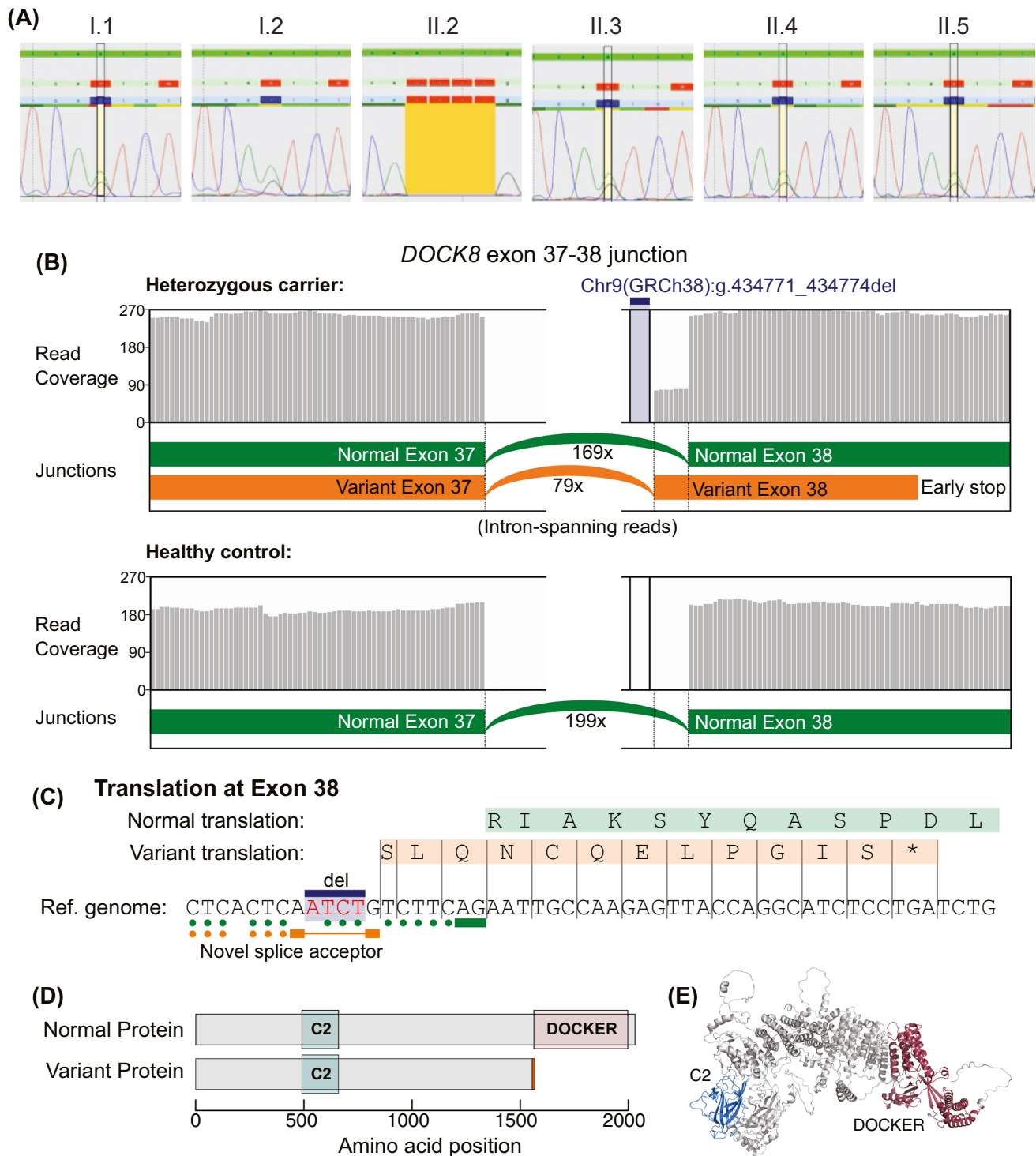
both parents, the DOCK8-sufficient brother II.3 and another sibling born in 2018 (II.4), were all heterozygous for this variant, establishing complete segregation of the homozygous variant with the clinical phenotype (Fig. 3A).

The gnomAD database lists only 4 single nucleotide substitutions between nucleotides – 16 and – 1 of exon 38 of *DOCK8*, suggesting this region is highly conserved and that the variant detected is likely deleterious in a homozygous state. Indeed, four in silico splicing prediction tools (SSF, MaxEnt, NNSPLICE, GeneSplicer) predicted degradation or loss of the nearby splicing acceptor site of intron 38 (or equivalently numbered) in all known transcripts. To explore whether the predicted loss of splicing occurred, RNA-seq was performed on PBMCs from the heterozygous mother I.2 (II.1 and II.2 had undergone HSCT). This led to the identification of a novel splice site forming 1 bp downstream of the deleted base pairs (Chr9(GRCh38):g.434771_434774del) between exons 37 and 38. This variant splice site affected 32% (79/248) of junction-spanning reads in PBMCs from I.2 (Fig. 3B, C) but was undetected in PBMCs from healthy donors ($n = 3$). The splice donor site downstream of exon 37 is preserved, but the deletion forms a novel splice-acceptor site 7 bp upstream of the normal exon 38, introducing a frameshift. The new splice-acceptor site would have one fewer pyrimidine nucleotides (at position – 6 relative to the new acceptor site), which could affect splicing efficiency (Fig. 3B) relative to the other (wild-type) allele [36, 37]. The variant exon 38 encodes 12 alternate amino acids followed by a premature termination codon (SLQNCQELP-GIS*), whereas the normal exons 38–47 encode 471 amino acids including the DOCKER domain, which is required for interaction with the GTPase CDC42 and LRCH1, which regulates CDC42 function (Fig. 3D, E) [38].

Another sibling (II.5) was born in December 2019. Our workup of this family over the preceding ~ 7 years allowed us to rapidly determine whether this child was affected by DOCK8 deficiency. Flow cytometry revealed that cord blood immune cells expressed normal levels of DOCK8 protein and targeted Sanger sequencing established that II.5 was a heterozygous carrier of the intronic variant (Figs. 1A, 3A).

Discussion

The accessibility and affordability of NGS have seen an exponential discovery of genetic causes of human diseases. Indeed, when the first inherited monogenic defect causing immune dysregulation was reported in 2010, ~ 200 known inborn errors of immunity had been identified in the preceding ~ 40 years [34, 39]. In the following decade, > 250 novel inborn errors of immunity have been reported, with almost all of them being discovered by NGS approaches [34, 39, 40]. Despite these major advances, a frequent outcome



of NGS is uncertainty, with many cases yielding candidate genes or variants of unknown significance, but remaining undiagnosed [13, 41–43]. The outcomes of NGS can also be greatly influenced by the advantages, disadvantages, and limitations of the genetic sequencing platform (targeted gene panels, whole-exome sequencing, WGS), as well as the analytical pipeline (clinical vs research) utilized [13, 39, 44].

In this study, we report the challenges in identifying a causative genetic variant by exon sequencing and clinical WGS in a kindred presenting with cardinal clinical, phenotypic, and functional features of *DOCK8* deficiency, including a complete absence of *DOCK8* protein in the patients' immune cells. The genetic lesion was eventually discovered by broadening the genomic analysis to include deeper

Fig. 3 Sanger sequencing and RNA sequencing confirmation of the deleterious intronic variant in *DOCK8*. **A** Sanger sequencing performed on DNA extracted from PBMCs of the family members revealed that I.1, I.2, II.3, II.4, and II.5 were heterozygous carriers, and II.2 was homozygous for the Chr9(GRCh38):g.434771_434774del mutant *DOCK8* variant, confirming WGS data for II.1 and II.2. **B–D** RNA sequencing was performed on PBMC from I.2 (heterozygous carrier) and three healthy controls (one representative healthy control is shown). **B** Differential splicing occurred in 32% of reads between exons 37–38 of *DOCK8* in I.2 (heterozygous for a 4 bp intronic deletion upstream of exon 38). Gray bars show the mRNA read coverage mapped at each bp position. **C** Predicted aberrant splicing of the variant transcript at exon 38 results in a frameshift and introduces a premature termination codon after 12 amino acids of exon 38. Elements of the canonical splice site motif are indicated: solid bars underline the 3' AG sequences immediately upstream of exons; dots indicate the polypyrimidine tract (C or T upstream of the splice-acceptor), which are reduced for the variant splice site. **D** Protein domains for *DOCK8* (based on Uniprot: Q8NF50-3, corresponding to the NM_001193536.1 transcript). Predicted truncation of the variant protein retains the “C2 DOCK-type” domain but lacks the C-terminal “DOCKER” domain. A short sequence of 12 different amino acids is predicted on the C-terminal end of the variant protein (orange bar). **E** *DOCK8* predicted structure from AlphaFold

intronic regions, combined with RNA-seq to confirm the in silico-predicted deleterious impact of an intronic deletion on splicing of *DOCK8* exons and subsequent protein expression. With hindsight, the discovery of the pathogenic variant could have been expedited if additional base pairs adjacent to exon boundaries had been considered in the genomic analysis pipeline. The anti-*DOCK8* mAb used in our study was raised against amino acids 119–277 of human *DOCK8* protein. This epitope would be translated from the RNA transcript encoded by the mutant *DOCK8* allele. Thus, our finding of complete lack of *DOCK8* protein in monocytes and near-complete lack of *DOCK8* protein in lymphocytes from II.1 and II.2 suggests that the pathogenic intronic variant is not only likely to alter exon splicing to introduce a premature stop codon potentially resulting in a truncated protein but also probably disrupts the stability of the entire *DOCK8* protein. The premature stop codon likely triggers nonsense-mediated decay (NMD) of the aberrant transcript, particularly since this premature stop codon is located far from the next exon–exon junction (> 55 bp distances support NMD targeting in mammals) [45]. NMD could also explain our observation that 32%, rather than 50%, of the variant splicing occurred in a heterozygous carrier, although experiments using puromycin to inhibit NMD would be one way to further test this explanation. Sequence analysis suggested that the novel splice-acceptor site would override the canonical splice-acceptor site, which remains in cis on the variant allele, although our data does not necessarily preclude the possibility of canonical splice-acceptor usage on the variant allele.

Detection of this pathogenic homozygous *DOCK8* variant in II.1 and II.2 provided a definitive molecular diagnosis of *DOCK8* deficiency, thereby ending a long diagnostic journey for this family. This finding (1) supported the clinical decision to treat II.1 and II.2 with HSCT, the standard of care for *DOCK8* deficiency [7, 9–12]; (2) enabled rapid screening of a newborn child and established that both parents and the siblings II.3 and II.4 are heterozygous carriers; and (3) will facilitate additional genetic testing for future pregnancies.

Our investigations of this family highlight the importance of investigating intronic regions for deleterious variants and that diagnostic outcomes from WGS can be enhanced by utilizing bioinformatic analyses that consider potential variants affecting exon splicing lying further intronic to the usual end points of analysis. Interestingly, recent studies reported the identification of deep intronic variants in *STAT3* [46] or *IKBKG* (NEMO; [47]) as causative of autosomal dominant hyper-IgE syndrome or X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency, respectively. To our knowledge, this is only the second example of an intronic variant in *DOCK8* lying beyond the splicing boundaries that has been formally validated as being pathogenic [48]. Interestingly, this previous case bears similarities to our study, inasmuch that the variant was also located > 8 bp upstream of a coding exon, and confirmation of the impact of the pathogenic insertion mutation was demonstrated by RNA-seq and flow cytometry [48]. Thus, the incorporation of transcriptomics, proteomics, and functional validation with WGS represents a powerful — and often necessary — approach to discover novel pathogenic variants and unequivocally diagnose complex and challenging cases of inborn errors of immunity.

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Author Contribution SGT, PG, LB, and CSM conceived and designed the study; PG, BP, JYY, JR, WAF, SK, and CSM conducted experiments; DEC, AS, and JP provided patient care and data collection; JS and GU performed Ion Torrent, and HJ and HCS performed initial *DOCK8* exon sequencing; SGT, PG, LB, and CSM supervised the study; SGT and CSM wrote drafts of the original and revised manuscripts; all authors contributed to the final version of the manuscript and approved submission of the final version.

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Availability of Data and Materials Available upon request to the corresponding author.

Code Availability Not applicable.

Declarations

Ethics Approval This study was approved by the Sydney Local Health District RPAH Zone Human Research Ethics Committee and Research Governance Office, Royal Prince Alfred Hospital, Camperdown, NSW, Australia (Protocol X16-0210/LNR/16/RPAH/257); the South East Sydney Local Health District Human Research Ethics Committee, Prince of Wales/Sydney Children's Hospital, Randwick, NSW, Australia (Protocol HREC/11/POWH/152); and NIAID (Protocol 06-I-0015). Written informed consent was obtained from participants or their guardians.

Consent to Participate Written informed consent was obtained from participants or their guardians.

Consent for Publication Patients signed informed consent regarding publishing their data.

Conflict of Interest The authors declare no competing interests.

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