Supplementary Material:

Introduction:

Primary immunodeficiency diseases (PIDs) are highly heterogeneous conditions that would benefit from the application of NGS in clinical testing. In most cases PIDs are monogenic and follow simple Mendelian inheritance. However, disease penetrance and expression variability as well as interactions between genetic and environmental factors can contribute to the wide range of phenotypic diversity observed across PIDs.[1] More than 220 PIDs have been described in the scientific literature to date and new PIDs continue to be reported.[2,3]

Previous attempts to identify the causal mutations underlying PIDs primarily entailed positional cloning or candidate gene sequencing based on known signaling pathways and phenotypic similarity of disease in patients and available murine models. Investigators have also used genome-wide association (GWA) approaches, which led to the discovery of multiple novel common variable immunodeficiency (CVID) susceptibility loci.[4] The use of high throughput NGS technology has helped explain novel causes of some PIDs. Over the last few years, a number of publications have reported newer molecular defects in PID by next generation sequencing technology. Recent examples include the use of WES in identifying mutations in *IKBKB* as a cause of combined immunodeficiency, *CSF3R* as a cause of congenital neutropenia, and *CTPS1* associated with defective lymphocyte proliferation and severe EBV infection. [5,6,7]

With the continued discovery of newer molecular defects in PID, keeping up with the growing literature on inherited mutations in PID can be tedious and time consuming. However, several useful online databases are available to researchers. The Human Gene Mutation Database (http://www.hgmd.org) is a comprehensive collection of mutations in nuclear genes that underlie or are associated with human inherited disease. A valuable database for primary

24 immunodeficiency is the Resource of Asian Primary Immunodeficiency Disease (RAPID). This
25 is a freely accessible database that contains information on sequence variation, as well as
26 expression at the mRNA and protein levels, in genes reported from PID patients.

27 Severe combined immunodeficiency (SCID) is a fatal PID syndrome characterized by profound
28 deficiencies of T and B cell function. It is known to be caused by mutations in at least 13
29 different genes,[8,9] but there are still SCID patients whose mutations remain unknown. Less
30 severe combined immunodeficiency (CID) is characterized by impaired but not absent T and B
31 cell function. While some CID patients have hypomorphic mutations in known SCID-associated
32 genes, the causal mutations for many CID patients remain elusive. Similarly, although causative
33 mutations for hyper IgM syndrome (HIGM) have been found in five different genes,[10,110] many
34 patients still lack identification of the causal gene mutation. Chronic granulomatous disease
35 (CGD) is caused by mutations in five NADPH oxidase structural genes (*CYBB, CYBA, NCF1,*
36 *NCF2,* and *NCF4*).[12] Mutations in *CYBB* (gp91phox) cause X-linked CGD and account for
37 nearly two thirds of cases. All other described CGD cases have an autosomal recessive pattern of
38 inheritance.

39 **Methods:**

40 All patients were followed at the Immune Deficiency Foundation's Duke University Primary
41 Immunodeficiency Center of Excellence. All studies were performed with the approval of the
42 Duke University Medical Center's Institutional Review Board and with the written informed
43 consent of the patients or their parents.

44 *Whole Genome Sequencing:*

WGS was accomplished using paired-end sequencing on the Illumina HiSeq2000 with average coverage 39.5 (SD 5.8, range: 33.6-50.0). Reads were aligned to the Human Reference Genome (NCBI36) using BWA software.[13] Single nucleotide variants (SNVs) and insertions/deletions (INDELs) were called and genotypes assigned using SAMtools.[14] Control samples (n>160) were sequenced contemporaneously in the same laboratory, and variants were called and annotated in a manner similar to the patient genomes.

Identification of likely causal variants:

The WGS screens were designed to interrogate highly penetrant genotypes that might account for each patient's PID. Patients were screened for putatively functional rare variants that were absent in a cohort of >160 unrelated control subjects and were absent or at very low frequencies in the Exome Variant Server (EVS, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA [URL: http://evs.gs.washington.edu/EVS/]) public database. High-quality variants were annotated using the Sequence Variant Analyzer (SVA [URL: http://www.svaproject.org/]) .[15] Standard filtering criteria were applied (SNV quality, SNV consensus score, INDEL consensus score ≥20, INDEL quality ≥50, number of reads supporting SNV or INDEL ≥3). As variants with known or predicted functional consequences are more likely to be causal of such deleterious phenotypes, only functional variants were further considered: missense and nonsense SNVs, stop loss SNVs, frameshift INDELs, and splice site mutations, or structural variants that overlapped genes. Functional variants present in the proband were prioritized as potentially causal as follows: (1) a homozygous (including hemizygous X variants) genotype lacking homozygosity in controls (recessive and X-linked variants) (MAF<0.02); (2) a compound heterozygous (MAF<0.03 for each participating variant) genotype that was not observed together in any controls. Known PID genes were included in the initial evaluation of candidate variants lists

68 generated from these tests. Candidate variants were then further prioritized by gene ontology,
69 with higher weight given to variants in genes with a known role in immune function or known
70 expression in relevant cell types. When no interesting genes were identified by these criteria,
71 heterozygous genotypes in patients with very low frequencies in controls (MAF<0.02) were also
72 included. This served as an indirect screen for compound heterozygosity that could have been
73 missed on our initial screen, either due to low coverage of one of the two causal mutations, or
74 because one of the two causal variants was a copy number variant (CNV).

75 *Identification of structural variation:*

76 Structural variations (including INDELs, deletions, duplications, and CNVs) were identified
77 using ERDS (Estimation by Read Depth with SNVs, version 1.06 [URL:
78 http://people.duke.edu/~mz34/erds.htm]) software.[16] ERDS primarily uses a paired Hidden
79 Markov Model to analyze high-coverage WGS data combining read depth, paired-end,
80 polymorphism, and structural variant signature information with GC corrections. CNVs were
81 detected with default ERDS parameters.[16]

82 *DCLRE1C splicing studies:*

83 Plasmids:

84 Each human *DCLRE1C* exon, including ~300 bp of flanking intronic sequence, was amplified
85 from genomic DNA derived from peripheral blood mononuclear cells (PBMCs) of healthy
86 volunteers using PrimeSTAR GXL DNA Polymerase (Takara). *DCLRE1C* exons were
87 subcloned into the pCR-Blunt II-TOPO vector (Life Technologies) and sequenced. Using pCR-
88 Blunt II-Exon1 and pCR-Blunt II-*DCLRE1C*-Exon5 as templates, the IVS1+1 G>T and IVS5+2
89 T>A mutants (c.109+1G>T and c.362+2T>A, respectively) were made by PCR-directed

90 mutagenesis using PfuUltra II Fusion HS DNA Polymerase. The products were phosphorylated
91 by T4 polynucleotide kinase (New England BioLabs), self-ligated using T4 DNA ligase
92 (Promega), and sequenced. Genomic DNA encoding exons 1 to 3 and exons 3 to 6 were
93 subcloned into pcDNA3.1(+) vector generating the minigene 1 and minigene 5 wild type (WT)
94 and mutant constructs respectively.

95 The 5' and 3' ends of the human *DCLRE1C* cDNA sequence were amplified with PrimeSTAR
96 GXL DNA Polymerase. These were subcloned into the pCR-Blunt II-TOPO vector and
97 subjected to sequence analysis (pCR-Blunt II-*DCLRE1C*-A-WT and pCR-Blunt II-*DCLRE1C*-B-
98 WT). Using pCR-Blunt II-*DCLRE1C*-A-WT, the Δexon5 isoform of *DCLRE1C* was made by
99 PCR-directed mutagenesis to remove exon 5 as described above. A FLAG-tag was also added to
100 each isoform by PCR-directed mutagenesis. FLAG-tagged full length cDNAs encoding
101 *DCLRE1C* were subcloned into pcDNA3.1(+) vector creating pcDNA3.1(+)-*DCLRE1C*-WT-
102 FLAG, and pcDNA3.1(+)-*DCLRE1C*-Δexon5-FLAG constructs.

103 Minigene assays

104 Empty pcDNA3.1 (+) vector, minigene-1WT, minigene-1mutant, minigene-5WT and minigene-
105 5mutant were transfected into COS-7 using Lipofectamine 2000 according to standard protocol.
106 Total RNA was extracted from transfectants after 24 hours and first-strand cDNA encoding the
107 human *DCLRE1C* minigene was synthesized. DCLRE1C minigene transcript expression levels
108 were detected by semi-quantitative RT-PCR and sequenced. The transcripts of minigene-1WT
109 minigene-1mutant were also detected by quantitative RT-PCR using Taqman one-step PCR
110 Master Mix (Life Technologies) (primer list Table E1).

111 Immunoblotting

112 Empty pcDNA3.1 (+) vector, pcDNA3.1(+)-*DCLRE1C*-WT-FLAG, or pcDNA3.1(+)-
113 *DCLRE1C*-Δexon5-FLAG were transfected into COS-7 cells. Cells were lysed with a solution of
114 RIPA Buffer (Sigma Aldrich), 1X 0.5M EDTA and 1X protease inhibitor (ThermoScientific)
115 48h after transfection. Lysates were subjected to SDS–PAGE gel and transferred to a
116 polyvinylidene difluoride membrane (Millipore). The membranes were incubated with anti-
117 FLAG antibody (1:2000; Sigma Aldrich) or anti-β-actin (1:10,000; Cell Signaling Technology).
118 Proteins were visualized with the ECL Plus Western Blotting Detection System (GE Healthcare).

119 Verification of variants and communication of results to families

120 All families underwent genetic counselling at our Immunology clinic at the time of participation.
121 The identified variants were confirmed in a CLIA certified laboratory prior to communication to
122 the families.

123 **Supplementary Discussion:**

124 To date, we have performed WGS on 12 PIDD cases. We identified the causal mutation in 6
125 (discussed here). Hence we had a success rate of 50% in identifying the disease causing
126 mutation.

127 The use of NGS is accepted for investigating undiagnosed genetic conditions. NGS adds
128 considerable value through its ability to both identify novel and rare mutations in known genes
129 and to investigate a broader range of genes than by targeted gene testing.

130 Commercial laboratories are limited by the nature and cost of targeted gene testing; and
131 sometimes only gene regions that harbor the majority of previously identified mutations are
132 screened. For patients 1 and 2, the causal mutation in exon 7 of *NCF1* was missed by targeted

133 gene testing because the commercial *NCF1* screen only examined mutations in exon 2, which
134 harbors the 2GT deletion that causes most reported cases of *NCF1*-related CGD.[17] It remains
135 unclear why the missense mutation in *CD40LG* we identified in patient 3 was missed by the
136 CLIA certified laboratory. Of note, measurement of CD40L function by assessing the binding of
137 CD40L to the soluble receptor, CD40-muIg is available as a clinical test.

138 Patient 4 was initially only screened for mutations in *RAG1* and *RAG2* , the only known causes
139 of NK phenotype SCID at the time of her diagnosis.[18] The Artemis encoding *DCLRE1C* gene
140 was subsequently also found to cause NK phenotype SCID.[19] Hypomorphic *DCLRE1C*
141 mutations have also been reported as a cause of partial T and B lymphocyte immunodeficiency.
142 [20] The *DCLRE1C* gene is prone to deletions involving one or more of its 14 exons.[21] One study
143 analyzing *DCLRE1C* mutations in SCID patients found that 60% of alleles had a large deletion,
144 mostly involving exons 1-3.[22] Patient 4 in our study was found to be homozygous for the 82kb
145 deletion involving exons 1-4 in *DCLRE1C* (Figure E 1A) .

146 The defect in patient 5 was also missed by a CLIA-certified laboratory when she was tested for
147 *DCLRE1C* mutations, as targeted gene testing at that time lacked the ability to identify gross
148 deletions and was only able to discern non-diagnostic heterozygosity. Patient 6 was the only
149 patient initially screened via WGS. It is worth noting that, even if patient 6 had undergone full
150 SCID candidate gene testing (at significant expense), the cause may have been missed unless an
151 exon array or a multiplex ligation-dependent probe amplification was performed to determine the
152 copy number of the *DCLRE1C* gene, as with patient 5.

153 Defects involving *DCLRE1C* gross deletions as we observed in patients 4-6 would likely have
154 been missed by targeted gene testing technology generating ambiguous results due to PCR

155 failure in Sanger sequencing. Even WES may have missed this causal mutation, since inferring
156 CNVs is much more challenging and less reliable with exome data.[23]

157 The cost of clinical genetic testing in these patients is important to address. For patients 1 and 2,
158 the cost for testing *NCF1*, *NCF2* and *CYBA* at a CLIA-certified commercial laboratory was
159 $3200 per patient.

160 Of note, the current cost of sequencing in our research laboratory is around $3200 for WGS and
161 $650 for WES. The current standard turnaround time for WES and WGS at the CHGV (research
162 lab) is 49 days. For urgent cases, rapid sequencing can be done at the CHGV in about 2 weeks.
163 Standard turnaround time will vary by research or clinical laboratory. Interestingly, one group
164 reported a system that permits WGS with bioinformatics analysis of suspected genetic disorders
165 within 50 hours. This time frame is very promising for emergency use when rapid diagnosis is
166 needed in an emergency or critical care setting. [24]

167 NGS panels for a selected group of genes have become commercially available and are proving
168 to be more popular and economical than sequencing individual genes. One recent report suggests
169 that NGS-based evaluation may be used as a first line genetic test for cases of PID .[25]
170 Furthermore, NGS may be clinically and economically beneficial in patients who remain
171 undiagnosed despite traditional genetic diagnostic evaluations.[26]

172 "Next-generation sequencing (NGS) has been clearly shown to be a successful approach in
173 identifying causes of Mendelian diseases, even when the condition is seen in a single patient.
174 Variations in data generation across platforms and methods of data interpretation can be
175 challenging in this context, particularly when disease causing variation is very rare and present in
176 only a single family. However, a set of criteria has recently been proposed for deciding if the

177 clinical and experimental data are sufficient to establish a causal relationship with only one
178 affected individual.[27]There are challenges associated with NGS technology, including the
179 complexity of data analysis and the potential for the mapping and variant calling algorithms to
180 miss variants. Reliability of NGS data analysis is highly dependent on the choice of a reliable
181 control cohort of high quality and depth. In addition, this technology requires extensive interplay
182 between geneticists, clinicians and bioinformaticians and the analysis can be very complex.

183 It is also likely that a small proportion of the genome will remain refractory to NGS. WES and
184 WGS offer the advantage of interrogating the entire genome, rather than being limited to only
185 likely gene candidates, and WGS may prove to be faster and less expensive than targeted gene
186 approaches in many cases.

187 Though the patients studied already had a clinical diagnosis, determining the underlying genetic
188 causes of their diseases is important for several reasons. First, it may impact clinical care. For
189 example, patients with Artemis deficiency are at an increased risk for deleterious effects from
190 ionizing radiation. In addition, we have found that patients and families lacking a definitive
191 genetic diagnosis have emotional distress from lack of information regarding genetic counseling
192 and uncertainty regarding potential future impact on subsequent pregnancies.

193 The choice between performing WGS or WES is not an easy one. WES is less expensive and
194 more readily available, but can miss disease causing mutations in noncoding regions. Examples
195 include intronic *GATA2* mutations in patients with MonoMAC syndrome [28] and a mutation in the
196 5' untranslated region of *IKBKG* (NEMO) in X-linked Ectodermal Dysplasia with
197 immunodeficiency.[29] Identification of large structural variations such as deletions was formerly

problematic with WES. However, recent new analytical approaches make it possible to screen for clinically relevant CNVs using existing exome-based CNV detection methods.[30]

In summary, this work suggests that the application of NGS should be strongly considered in all PID cases where the initial studies have not determined the molecular etiology of disease.

Supplementary Figure Legends:

Supplementary Figure E1: Detection of CNVs by ERDS. A read depth (RD) of 40 indicates that both copies of the gene are present; RD of 20 suggests that 1 gene copy is missing; RD of 0 suggests that both gene copies are missing. Detection of 82kb deletion on chromosome 10 including exons 1-4 of *DCLRE1C* in patient 4 (A), 5 (B) and 6 (C). The deletion is homozygous in patient 4.

Supplementary Figure E2: Sanger confirmation of IVS1+G>T and IVS5+2T>A *DCLRE1C* variant constructs.

Supplementary Table E1: Primer list; Abbreviations “F” and R” are forward and reverse primers respectively.

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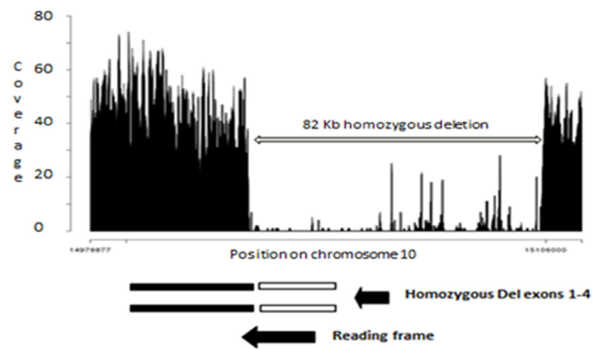
	Primers	Sequence (5'-3')	Restriction Site incorporated
Plasmid construction and sequencing			
Minigene 1	DCLRE1C-Exon1-F	GCTAGCTTGGCTTCAGCTGCGGTTTT	NheI
	DCLRE1C-Exon1-R	CTTAAGCACCAGCAAAGCTACCAAGA	AflII
	DCLRE1C-Exon2-F	CTTAAGTCTCATTCTTCTGTGGCTGC	AflII
	DCLRE1C-Exon2-R	AAGCTTCAAGTTCACAAACAGCCAAAGC	HindIII
	DCLRE1C-Exon3A-F	AAGCTTGCTCTTGGTGGCACTGAAAT	HindIII
	DCLRE1C-Exon3A-R	CTCGAGTTCGTTTCTTCCAAAATCTGTATTTCG	XhoI
Minigene 5	DCLRE1C-Exon3B-F	GCTAGCCTGTTCACCTGTGACTAAGG	NheI
	DCLRE1C-Exon3B-R	CTTAAGTACAAGTGTGTGCCACGACA	AflII
	DCLRE1C-Exon4-F	CTTAAGCATGGAAACAGAATTGTGTCAGAG	AflII
	DCLRE1C-Exon4-R	AAGCTTGTAGTTTTGTGAGTCCAGCC	HindIII
	DCLRE1C-Exon5-F	AAGCTTTGTGAACAGTCAGGCACACA	HindIII
	DCLRE1C-Exon5-R	GGATCCAAACTCACTGCAGCCTCCAA	BamHI
	DCLRE1C-Exon6-F	GGATCCCAAACTGGGTAGCATCTCCA	BamHI
	DCLRE1C-Exon6-R	CTCGAGTCACCTGAAGTCAGGAGTTC	XhoI
WT-DCLRE1C	DCLRE1C-cDNA-A-F	CTTAAGTTGGCTTCAGCTGCGGTTTT	NheI
	DCLRE1C-cDNA-A-R	TGCTCCTTTCTCCAAACCAC	
	DCLRE1C-cDNA-B-F	AGGAGTCCAGGTTCATGTGA	
	DCLRE1C-cDNA-B-R	CTCGAGGTTGCTCTAGGTTGAAACGC	XhoI
Mutagenesis of Minigene 1 and Minigene 5			
	DCLRE1C-IVS1-mutagenesis-F	TTGAGTGAGGGCTGCG	
	DCLRE1C-IVS1-mutagenesis-R	CTTTGTGGCAGTGGGACA	
	DCLRE1C-IVS5-mutagenesis-F	GAAAGGGGGTCATTTATTTTGTCATTT	
	DCLRE1C-IVS5-mutagenesis-R	ATAACTGATCCCGGACAGTG	
FLAG introduction			
	DCLRE1C-WT-FLAG-F1	GACAAGTAAGAATTCAAAGCGTTTCAACCT	
	DCLRE1C-WT-FLAG-R1	GTAGTCGGTATCTAAGAGTGAGCATT	
	DCLRE1C-WT-FLAG-F2	GACGATGACAAGTAAGAATTCAAAGCGTTT	
	DCLRE1C-WT-FLAG-R2	GTCCTTGTAGTCGGTATCTAAGAGT	
	DCLRE1C-IVS1-FLAG-F1	GACAAGTGAGGGCTGCGCGT	
	DCLRE1C-IVS1-FLAG-R1	GTAGTCCTCAACTTTGTGGCAGTG	
	DCLRE1C-IVS1-FLAG-F2	GACGATGACAAGTGAGGGCTG	
	DCLRE1C-IVS1-FLAG-R2	GTCCTTGTAGTCCTCAACTTTGTGG	
	DCLRE1C-IVS5-FLAG-F1	GACAAGTAATGGAACTGTCCTGTACAC	
	DCLRE1C-IVS5-FLAG-R1	GTAGTCTTGCCCTGAAATAAAAACCTCTC	
	DCLRE1C-IVS5-FLAG-F2	GACGATGACAAGTAATGGAACTGTCCT	
	DCLRE1C-IVS5-FLAG-R2	GTCCTTGTAGTCTTGCCCTGAAATAAAAAC	
RT-PCR primers			
Control	GAPDH-F	ACCACAGTCCATGCCATCA	
	GAPDH-R	CACCACCCTGTTGCTGTAGCC	
Minigene 1	DCLRE1C-Exon1-F	GCTAGCTTGGCTTCAGCTGCGGTTTT	
	DCLRE1C-Exon3A-R	CTCGAGTTCGTTTCTTCCAAAATCTGTATTTCG	
Minigene 5	DCLRE1C-Exon4F2	ATCGAGACTCCTACCCAGAT	
	DCLRE1C-Exon6R2	GAGTGCAGAAGCTCCATTCT	
FLAG constructs	IVS-cDNA-RT-F	CAGCTGCGGTTTGGGGTCC	
	IVS1-muta-R	CTTTGTGGCAGTGGGACA	

Abbreviations 'F' and 'R' are forward and reverse primers respectively.

A



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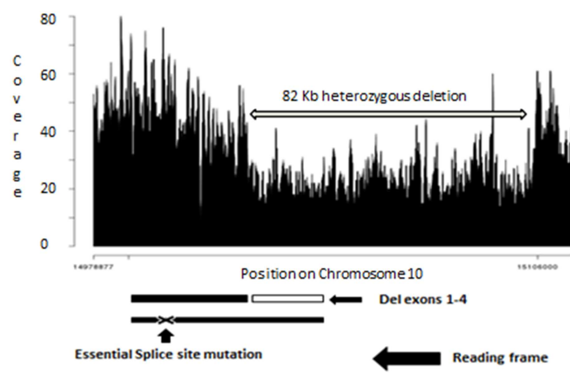

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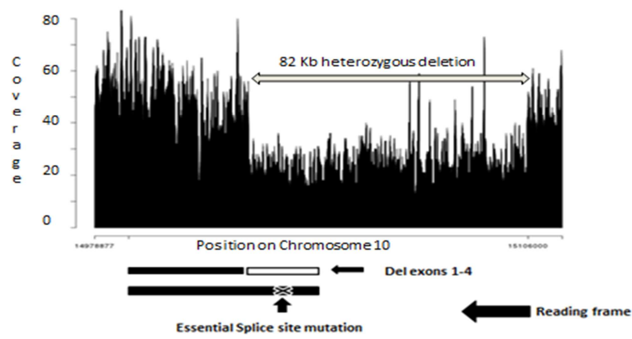
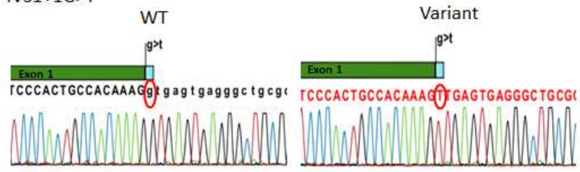


IVS1+1G>T



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