1 ## Supplemental Data

2

3 ## Detailed clinical descriptions of the patients

4 **Patient 1**

5 ***Congenital anaemia***

6 A Japanese female patient followed up for intrauterine growth restriction (IUGR) was born at 29
7 weeks of gestation with a weight of 726 g (−3.3 standard deviation [SD]) and a height of 32 cm (−2.9
8 SD). Her Apgar score at 5 min was 6. She did not require surfactant replacement therapy, but was on a
9 ventilator for 9 days. Her haematological characteristics are shown in Supplemental Table 1 and
10 Supplemental Figure 1. At birth, she had severe macrocytic anaemia without reticulocytopenia (red
11 blood cells [RBC] 0.95 × 10^{12}/L, haemoglobin [Hgb] level 43 g/L, mean corpuscular volume [MCV]
12 123 fL, reticulocyte counts 158 × 10^9/L, white blood cells [WBC] 2.3 × 10^9/L, and platelets 396 ×
13 10^9/L). Although she was treated with oral iron supplementation and erythropoietin administration
14 with intermittent transfusion of packed RBC considering anaemia of prematurity, her anaemia did not
15 improve. Oral iron supplementation and erythropoietin administration were discontinued in early
16 infancy. She required repetitive transfusions of RBC once or twice a month. WBC counts (including
17 differential counts) and platelets were normal (Supplemental Figure 1). Reticulocyte counts were
18 mostly within the reference interval, but without marked reticulocytopenia in contrast to those
19 observed in Diamond-Blackfan anaemia (DBA). She was referred to the National Center for Child
20 Health and Development (Tokyo, Japan) during infancy for the evaluation and treatment of anaemia
21 and bloody diarrhoea (details of gastrointestinal symptoms are described below). The examination of
22 bone marrow aspirate revealed erythroid dysplasia, but without marked erythroid hypoplasia (M/E
23 ratio 1.5) (Supplemental Table 1). These findings did not fit the diagnosis of DBA. Immunostaining
24 for TP53 of bone marrow cells showed enhanced signals (Figure 1). The panel sequencing for known
25 DBA-associated genes, including *RPS10, RPS14, RPS17, RPS19, RPS24, RPS26, RPL5, RPL11,*
26 *RPL35A,* and *TP53*, did not detect any pathogenic variant. In toddlerhood, the follow-up bone marrow
27 test demonstrated hypercellularity and revealed dysplasia of trilineage cells with excess blasts (7.5%)
28 that fit myelodysplastic syndrome (MDS), classified into refractory anaemia with excess blasts

29 (RAEB)-1 using the World Health Organization (WHO) classification. G-banding karyotyping of the
30 bone marrow aspirate showed a normal female karyotype as 46,XX.
31
32 ***Inflammatory bowel disease***
33 The patient developed chronic bloody diarrhoea and underwent lower gastrointestinal endoscopy
34 during infancy. Inflammatory bowel disease (IBD) (unclassified) was diagnosed based on the
35 endoscopic and pathological findings. Her IBD was intractable to 5-aminosalicylate and exclusive
36 enteral nutrition. Although it responded to corticosteroids, bloody diarrhoea relapsed soon after the
37 discontinuation of corticosteroids. She was dependent on corticosteroids and required a central
38 intravenous catheter for intravenous hyperalimentation to treat malnutrition caused by IBD.
39
40 ***Other clinical features***
41 She had facial dysmorphism with epicanthic folds. She had no nail dystrophy, skin pigmentation,
42 genitourinary anomalies, or café-au-lait patches. Despite adequate caloric intake, she showed no catch-
43 up growth, and her height SD score was –3.1 SD (height 74 cm) in toddlerhood. At this point,
44 hormone-secreting capacities of the anterior pituitary gland and adrenal cortex were investigated, but
45 no abnormalities were found. Magnetic resonance imaging (MRI) showed microencephaly and skull
46 thickening, and a computed tomography (CT) scan detected calcification at the right brachiocephalic
47 artery. Her development was mildly delayed; she began to roll, sat steady, and crawled in late infancy,
48 and walked in late toddlerhood. In early childhood, she could run, use several words, and imitate
49 actions, but could not use two-word phrases, build a tower of two bricks, use a spoon, or express wants
50 with pointing. Clinical features compared to those of FILS and IMAGE-I syndromes are shown in
51 Supplemental Table 2.
52
53 **Patient 2**
54 ***Congenital anaemia***
55 Patient 2, the younger female sibling of Patient 1, was born at 37 weeks of gestation with a weight of
56 2,124 g (–1.6 SD) and height of 43 cm (–1.9 SD). She had a low Apgar score (4 at 5 min) and required

57 ventilator support for 2 days. She also had epicanthic folds resembling her sibling's. Her
58 haematological characteristics are shown in Supplemental Table 1 and Supplemental Figure 2. Her
59 Hgb level was 54 g/L at birth, there were no decreases in reticulocyte counts, WBC counts, or platelet
60 counts (RBC 1.71 × 10^{12}/L, MCV 117 fL, reticulocyte counts 30 × 10^{9}/L, platelets 306 × 10^{9}/L, and
61 WBC 12.8 × 10^{9}/L). Bone marrow examination in infancy showed erythroid dysplasia, but without
62 marked erythroid hypoplasia (M/E ratio 1.5) (Supplemental Table 1). Immunostaining for TP53 of
63 bone marrow cells showed enhanced signals (Figure 1). She required repetitive transfusions of packed
64 RBC once or twice a month. In early toddlerhood, the follow-up examination of the bone marrow
65 aspirate demonstrated hypercellularity and dysplasia of trilineage cells with excess blasts (8.0%) that
66 fit MDS, classified into RAEB-1. G-banding karyotyping showed a normal female karyotype as
67 46,XX.
68
69 ***Other clinical features***
70 She had no nail dystrophy, skin pigmentation, genitourinary anomalies, or café-au-lait patches. She
71 showed growth failure with height 71 cm (–2.6 SD) in toddlerhood. At this point, hormone-secreting
72 capacities of the anterior pituitary gland and adrenal cortex were investigated, but no abnormalities
73 were found. She showed the following typical developmental milestone achievements: began to roll,
74 sat alone, walked, and used words during infancy. In toddlerhood, considering the abnormal
75 intracranial findings of Patient 1, brain MRI, MR angiography, and CT scans of head, chest, and
76 abdomen and pelvis were performed, showing no abnormal findings. Clinical features compared to
77 those of FILS and IMAGE-I syndromes are shown in Supplemental Table 2.
78 She had not developed bloody diarrhoea by the follow-up period. She underwent a colonoscopy
79 for her mild diarrhoea during infancy, but endoscopic and pathological findings of IBD were not
80 detected.
81
82 **Supplemental methods:**
83 ***Immunostaining of bone marrow samples***
84 Clot sections of the bone marrow obtained from Patient 1, Patient 2, and controls (a patient with

85 immune thrombocytopenia in early childhood and a patient with autoimmune neutropenia in infancy)
86 were treated with high pH target retrieval solution (Dako, Santa Clara, CA) at 98 °C for 40 min.
87 Immunostaining was performed with mouse anti-TP53 antibody (DO-7, Dako Nichirei Bioscience,
88 Tokyo, Japan) and a polymer reagent Simple Stain MAX-PO (MULTI; Nichirei Bioscience) in an
89 autostainer, i.e., Histostainer (48A, Nichirei Bioscience), for 30 min at room temperature.
90

91 ***Exome sequencing***

92 Genomic screening was conducted under the "Initiative for Rare and Undiagnosed Diseases in
93 Paediatrics" program supported by the Japan Agency for Medical Research and Development after
94 obtaining written informed consent from the patients' parents. Exome sequencing of the patients and
95 their parents were performed with SureSelect Human All ExomeV6 (Agilent Technologies, Santa
96 Clara, CA) and the Illumina HiSeq 2500 system (Illumina, San Diego, CA), as previously described.[1]
97 We filtered the dataset using the Japanese variant database JPN38K
98 (https://jmorp.megabank.tohoku.ac.jp). To obtain a list of candidate pathogenic variants, the following
99 filtering criteria were applied: (1) non-synonymous or frameshift variants in coding exons or splicing
100 sites, (2) allele frequencies below 0.5% in 38KJPN, and (3) absence of the variant in our in-house
101 exome data to filter pipeline-specific systematic errors. Potential pathogenicity was assumed from
102 inheritance patterns — *de novo*, homozygous, compound heterozygous, or hemizygous — and
103 functional effects were predicted using SIFT, PolyPhen2, and CADD.

104 Direct sequencing was performed to confirm the presence of the identified *POLE* variants. The
105 genomic DNA samples from the patients were PCR-amplified for the region containing the variant
106 using AmpliTaq Gold 360 PCR Master Mix (Thermo Fisher Scientific, Waltham, MA). The PCR
107 products were sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher
108 Scientific) on an ABI 3130 sequencer (Thermo Fisher Scientific).
109

110 ***Functional characterization of POLE variants***

111 A vector containing human *POLE* cDNA (FHC24944) was purchased from Kazusa DNA Research
112 Institute (Chiba, Japan). We modified the pBQM812A-1 vector (System Biosciences, Palo Alto, CA)

113 by replacing the cumate-inducible promoter with a doxycycline-inducible promoter and the CymR
114 repressor sequence with the reverse tetracycline transactivator sequence.[2] *POLE* cDNA was cloned
115 into the modified vector by adding the haemagglutinin (HA) sequence to the N-terminus using the
116 Gibson assembly technique (NEBuilder HiFi DNA Assembly Master Mix; New England Biolabs,
117 Ipswich, MA). We introduced c.3392del and c.5672_5674del into the wildtype (WT)-POLE1-
118 expressing vector using a standard PCR-based method. HEK293 cells were maintained in Dulbecco's
119 modified Eagle's medium supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, and 10%
120 foetal bovine serum (FBS). The cells were transfected with each vector using Lipofectamine 3000
121 reagent (Thermo Fisher Scientific), and inducible stable cell lines were established according to the
122 protocol for pBQM812A-1.

123 For Western blotting, nuclear and cytoplasmic extracts were prepared from inducible stable
124 HEK293 cells expressing each HA-POLE1 (WT, Asp1131fs or Thr1891del). The cells cultured in 10-
125 cm dishes were treated with 1 μg/mL doxycycline for 24 h. The cells were harvested using 0.25%
126 trypsin-ethylene diamine tetra acetic acid (EDTA) solution and centrifuged at 2,000 rpm for 3 min.
127 Cell pellets were lysed in five times the pellet volume (PV) of hypotonic lysis buffer (10 mM Tris, pH
128 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM dithiothreitol with a protease inhibitor cocktail) and
129 allowed to swell on ice for 15 min. Triton X-100 was added to a final concentration of 0.6%, and the
130 samples were vortexed for 10 seconds. The homogenates were pelleted by centrifugation at 10,000 × *g*
131 for 30 seconds, and the supernatants were stored as cytoplasmic lysates. The nuclear pellets were
132 suspended in extraction buffer (20 mM Tris pH 8.0, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA,
133 25% glycerol, and 1 mM dithiothreitol with a protease inhibitor cocktail) at two-thirds volume of the
134 PV and rotated for 15 min at 4 °C. The homogenates were centrifuged at 20,000 × *g* for 5 min, and the
135 supernatants were stored as cytoplasmic lysates. Nuclear and cytoplasmic extracts were separated
136 using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).
137 Immunoblotting was performed with rat anti-HA antibody (clone 3F10; Sigma-Aldrich, St. Louis,
138 MO), rat anti-tubulin antibody (YL1/2; Abcam, Cambridge, UK) and rabbit anti-histone H3 antibody
139 (ab1791; Abcam) as primary antibodies, and horseradish peroxidase (HRP)-conjugated goat anti-rat
140 IgG (Sigma-Aldrich) and HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich) as secondary

141 antibodies.

142 To visualize subcellular localization of each HA-POLE1 protein (WT, Asp1131fs or Thr1891del),
143 inducible stable HEK293 cells were seeded on glass-bottom dishes and grown with 1 μg/mL
144 doxycycline for 24 h. The cells were then fixed in 4% formaldehyde in phosphate-buffered saline
145 (PBS) at room temperature for 10 min. Blocking and plasma membrane permeabilization were
146 performed by incubating the cells with 4% Block Ace (KAC, Kyoto, Japan) and 0.1% Triton X-100 at
147 room temperature for 1 h. Immunofluorescence was performed using rat anti-HA antibody (clone
148 3F10) and rabbit anti-POLE2 antibody (HPA02755; Thermo Fisher Scientific) as primary antibodies,
149 and Alexa Fluor 488-conjugated donkey anti-rat IgG antibody (A21208, Thermo Fisher Scientific) and
150 Alexa Fluor 647-conjugated goat anti-rabbit IgG antibody (A27040; Thermo Fisher Scientific) as
151 secondary antibodies. Nuclei were stained with Hoechst 33342 (Dojindo Laboratories, Kumamoto,
152 Japan). The cells were observed under an FV3000 confocal microscope (Olympus, Tokyo, Japan).
153

154 ***Generation of patient-derived induced pluripotent stem cells (iPSCs)***

155 Human iPSCs were generated and maintained on iMatrix-511 (Nippi, Inc., Tokyo, Japan) in StemFit
156 AK02N (Reprocell, Inc., Kanagawa, Japan) or StemFlex (Thermo Fisher Scientific) media. For Patient
157 1, a small intestinal tissue biopsy specimen obtained from the patient was chopped into small pieces
158 and seeded on a dish in DMEM/F12 (Thermo Fisher Scientific) containing 20% FBS. The medium
159 was changed every 2 days until confluence. Intestinal epithelial cells were maintained in DMEM/F12
160 containing 20% FBS until reprogramming. On the first day of reprogramming, we changed the culture
161 medium to NutriStem Medium (Sartorius Stedim Biotech, Göttingen, Germany) and used the
162 StemRNA-3rd Gen Reprogramming Kit (Reprocell, Inc.) to induce six reprogramming factors,
163 including OCT4, SOX2, KLF4, c-MYC, NANOG, and LIN28, following the manufacturer's protocol.
164 From days 2-4, we replaced the fresh NutriStem medium and transfected a reprogramming cocktail
165 using Lipofectamine RNAiMAX (Thermo Fisher Scientific) every day. From day 5, the old medium
166 was changed every 2 days until colony pickup.

167 For Patient 2, peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood
168 of the patient by centrifugation on Ficoll-Paque gradient using Leucosep (Greiner Bio-One

169 International GmbH, Frickenhausen, Germany) following the manufacturer's protocol. The CytoTune-
170 iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) was used to induce four
171 reprogramming factors, including OCT4, SOX2, KLF4, and L-MYC. PBMCs were infected with
172 Sendai virus vector at 37 °C in a 5% CO_2 incubator for 2 h. Next, the infected PBMCs were seeded
173 into iMatrix-511-coated 12-well plates (AGC Techno Glass Co., Ltd., Shizuoka, Japan) in KBM 501
174 medium (Kohjin Bio Co., Ltd., Saitama, Japan) with 10% FBS (Thermo Fisher Scientific). On days 2
175 and 4, 1 mL of StemFit AK02N medium was added gently to avoid disturbing cell adhesion, after
176 which the entire medium was changed every 2 days until colony pickup.
177 From days 14-21, individual colonies were picked into iMatrix-511-coated 4-well plates (Thermo
178 Fisher Scientific) in StemFit AK02N medium supplemented with 10 μM Y-27632 (Fujifilm Wako Pure
179 Chemical Corporation, Osaka, Japan) or in StemFlex medium supplemented with 10 μM Y-27632. The
180 medium was changed every other day and cells were passaged approximately once per week using
181 enzymatic (TrypLE Select Enzyme or Accutase Cell Dissociation Reagent; Thermo Fisher Scientific)
182 or mechanical methods.
183

184 ***RNA sequencing (RNA-seq) analysis***

185 Total RNA was extracted from the iPSCs generated from the *POLE* variant carriers and control
186 individuals (three lines for Patient 1, one line for Patient 2, and one line each from the two controls)
187 using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's instructions. The RNA
188 samples were treated with TruSeq Stranded Total RNA Library Prep Kit (Illumina), and the libraries
189 were sequenced on DNBSEQ-T7 (MGI Tech Co., Ltd., Shenzhen, China). Reads were aligned to the
190 reference human genome hg38, visualized with Integrative Genomics Viewer
191 (https://software.broadinstitute.org/software/igv/), and reads per gene were counted using STAR-
192 2.7.9a software.[3] Within-sample normalization was conducted using the fragments per kilobase million
193 method, and between-sample normalization was performed using the trimmed mean of the M-values
194 method.[4]
195

196 ***Western blotting of TP53 protein***

197 For Western blotting, nuclear proteins were extracted from iPSCs generated from the patients, and
198 normal controls (two iPSC lines each). First, the cells grown in 10-cm dishes were harvested using
199 0.25% trypsin-EDTA solution and centrifuged at 2,000 rpm for 3 min. Cell pellets were lysed in five
200 times the PV of hypotonic lysis buffer (10 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, and 1 mM
201 dithiothreitol with a protease inhibitor cocktail) and allowed to swell on ice for 15 min. Triton X-100
202 was added to a final concentration of 0.6%, and the samples were vortexed for 10 s. The homogenates
203 were pelleted by centrifugation at $10,000 \times g$ for 30 seconds and the supernatants were discarded. The
204 nuclear pellets were suspended in extraction buffer (20 mM Tris pH 8.0, 1.5 mM $MgCl_2$, 420 mM
205 NaCl, 0.2 mM EDTA, 25% glycerol, and 1 mM dithiothreitol with a protease inhibitor cocktail) at
206 two-thirds volume of the PV and rotated for 15 min at 4 °C. The homogenates were centrifuged at
207 $20,000 \times g$ for 5 min, and the supernatants (nuclear fraction) were separated using 10% SDS-PAGE.
208 Immunoblotting was performed with mouse anti-TP53 antibody (DO-7) and rabbit anti-histone H3
209 antibody (ab1791) as primary antibodies, and HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich)
210 and HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich) as secondary antibodies.
211
212 ***Generation of Pole-deficient medaka***
213 Supplemental Figure 3 shows the sequence alignment of human and medaka *Pole* orthologues. We
214 designed two crRNAs that recognize the sequences ACG TAG AAT ACA TCA CCA AC (site 1) and
215 GAA TAC ATC ACC AAC AGG TC (site 2) of the medaka DNA polymerase epsilon (*Pole*) gene to
216 establish a *Pole* mutant using CRISPR/Cas9. Partial sequences of medaka *Pole*, two crRNA
217 recognition sites, and PCR primers for the amplification of the target region are shown in
218 Supplemental Figure 4. The formation of duplex guide ribonucleoproteins (dgRNP) is in accordance
219 with that reported by Hoshijima *et al*.[5] The crRNA, tracrRNA, and Cas9 proteins were purchased from
220 Integrated DNA Technologies (IA). The PCR primers (CAT CTT GTG CAC CAA AAA GC and AAG
221 TCT TGG GGT TTT GAA AT) were purchased from Fasmac (Kanagawa, Japan). Microinjection and
222 establishment of *Pole* knockout strains are in accordance with "Medaka: Biology, Management, and
223 Experimental Protocol, Volume 2".[6] Briefly, microinjected with dgRNPs into embryos at the 1 cell
224 stage, eggs were incubated for 4 days at 25 °C, and the genomic DNA was extracted and subjected to

225 PCR amplification. The efficiency of genome editing was evaluated using an automated
226 electrophoresis system MCE-202 with a DNA-500 reagent kit (Shimadzu, Kyoto, Japan). Two
227 dgRNPs were designed to efficiently edit the target DNA regions. Following the selection of four pairs
228 of founder fish, we observed the phenotypes of the next generation. Two out of the four pairs of
229 founder fish laid eggs with a specific phenotype. We selected four founder fish, No. 5, No. 6, No. 7,
230 and No. 8, and mated them with WT fish. Supplemental Figure 5 shows the types of insertions or
231 deletions identified in the target region of the F1 fish using amplicon sequencing. As a result, we
232 established two mutant lines, one with a 4 bp deletion mutation using one crRNA (site 2) and another
233 with a 9 bp deletion mutation using another crRNA (site 1) at the *Pole* gene. We named these two
234 mutant strains *Pole^del4* and *Pole^del9*, respectively. Two mutant strains *Pole^del4* and *Pole^del9*
235 showed essentially similar phenotypes, and thus we used the *Pole^del9* strain for further analysis. As
236 shown in Supplemental Figure 4, the *Pole^del9* strain deleted the splice-acceptor site and was
237 expected to be non-functional.
238
239 ***Morphological observation of medaka***
240 Medaka has a more extended period time from fertilization to hatching than zebrafish. For this reason,
241 the length of the body and other developmental phenotypes in pre-hatched embryos cannot be
242 accurately analysed. Therefore, we dissolved the chorion using hatching enzymes[7,8] and observed the
243 embryos. The hatching enzyme was supplied by NBRP Medaka (https://shigen.nig.ac.jp/medaka/).
244 Seven days after fertilization, the eggs were transferred to waterproof sandpaper (#1200) (AS One,
245 Osaka, Japan) placed in the lid of a 10-cm Petri dish. Breeding water was removed, and a moderate
246 amount of Iwamatsu's balanced salt solution (BSS)[9] was added to prevent the drying of the eggs. The
247 eggs were slowly rolled to remove outer surface hairs and lightly scratch the surface of the eggs. The
248 eggs were then transferred to a 24-well plate, and the BSS was removed. The hatching enzyme diluted
249 three times in BSS was added so that the eggs were immersed and incubated at 27 °C. As soon as the
250 embryos emerged from the chorion, embryos were transferred to a Petri dish containing 1 × BSS. The
251 embryos were captured using a microscope camera (Zeiss Axiocam 208 colors; Carl Zeiss Meditec,
252 Jena, Germany) attached to a stereomicroscope (model M165C; Leica, Wetzlar, Germany).

253

254 ***Blood cell counts of medaka***

255 Following anaesthesia with tricaine methane sulfonate (MS222; Sigma-Aldrich), the fish's heart was
256 exposed by dissection. Blood was collected from the bulbus arteriosus using glass capillaries (GD-1;
257 Narishige, Tokyo, Japan) coated with 10,000 U/mL heparin sodium salt (Wako, Osaka, Japan).[10] In our
258 experiments, we used Dulbecco's modified PBS (DPBS), treated to remove Mg^{2+} and Ca^{2+} ions
259 [DPBS (–)] to prevent coagulation during the dilution of whole blood when necessary. Total blood
260 cells were diluted to 1:200, stained with Shaw's diluent, and counted using a haemocytometer.[11] Blood
261 cells (2×10^5 cells) diluted in DPBS (–) containing 2.5% FBS were centrifuged (Cytopro 7620;
262 Wescor Inc., Logan, UT) at 1,000 rpm for 4 min to prepare cell centrifuge specimens and transferred
263 onto glass slides. After air drying, cells were stained with o-dianisidine (Wako) and Giemsa (Wako) as
264 previously described.[12] Blood cells were examined by light microscopy (model BX51; Olympus,
265 Tokyo, Japan).

266

267 ***Statistical analysis***

268 Data were analysed using the GraphPad Prism 9.1.2 software (GraphPad Software, Inc., San Diego,
269 CA). The two-group datasets were analysed using Student's *t*-test. Statistical significance was set at P
270 < 0.05.

271

272

273 **References**

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