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Supplemental Data

VPS4A Mutations in Humans Cause Syndromic Congenital Dyserythropoietic Anemia due to Cytokinesis and Trafficking Defects

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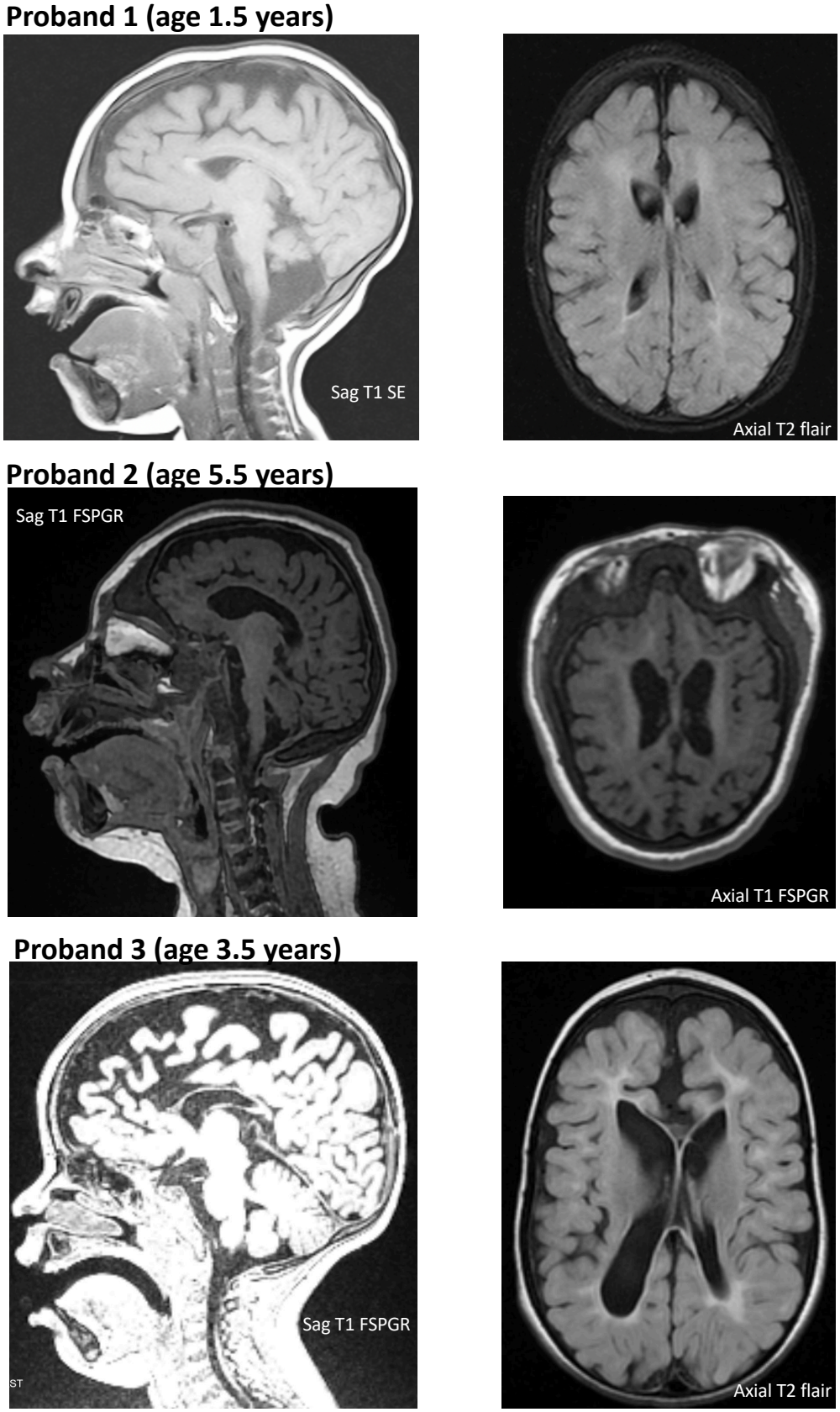

Figure S1. Sagittal and axial views of brain MRI. Probands 1 and 2 have microcephaly, as evidenced by craniofacial disproportion as well as atrophy of the cerebellum and the pons. The cerebellum and pons are less affected in proband 3, but she shares the diffuse gray/white matter loss and delayed myelination of the supratentorial brain with probands 1 and 2, the thinning of the corpus callosum, and *ex vacuo* enlargement of the extra-axial spaces. Enlarged lateral ventricles are noted in probands 2 and 3 due to the loss of periventricular white matter.

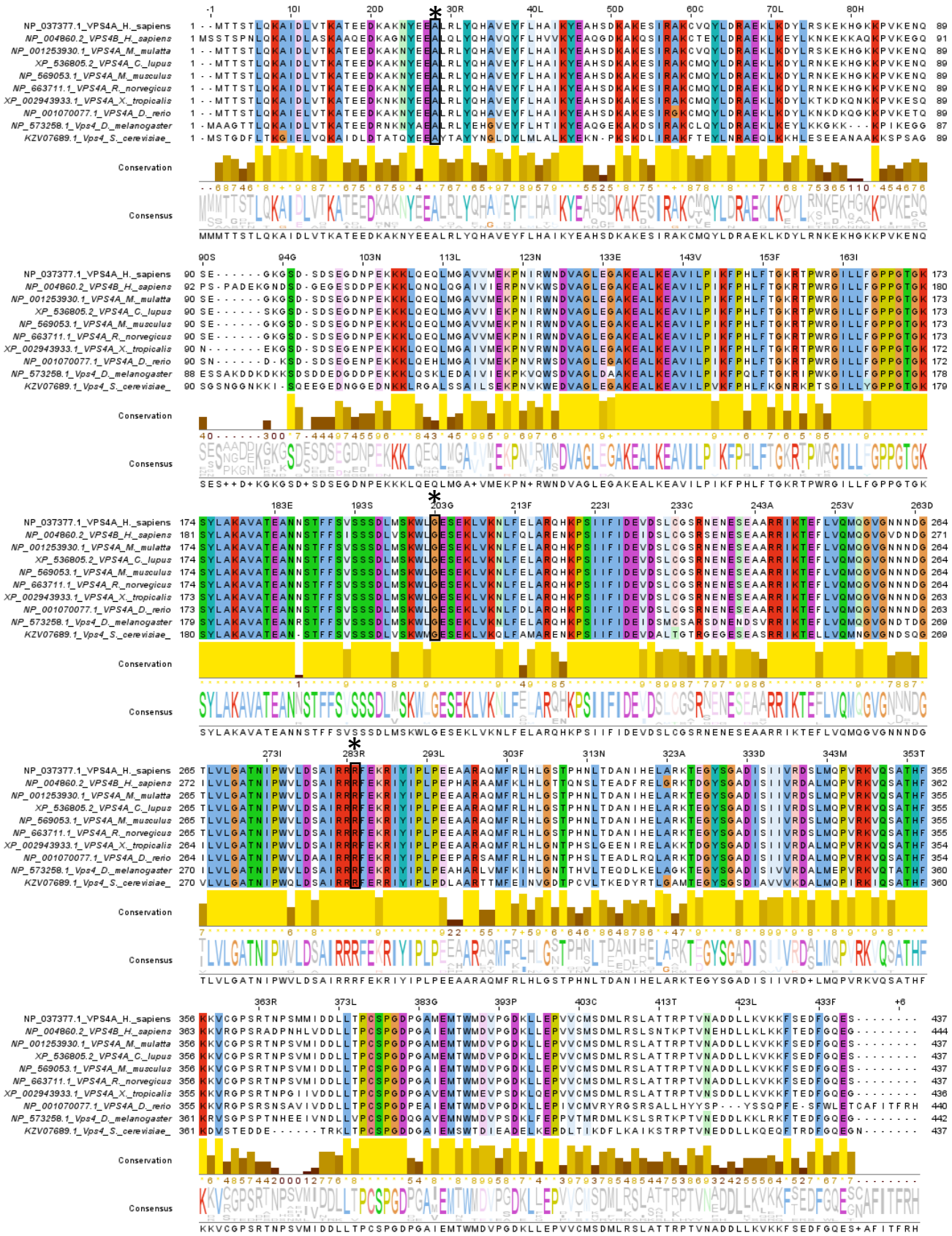

Figure S2. Multiple sequence alignment of human VPS4A and VPS4 from other species. Multiple sequence alignment for VPS4A and B shows the high degree of conservation of VPS4A across species down to yeast. Residues are numbered based on the human VPS4A as the reference sequence and colored columns indicate a residue is >75% conserved; full conservation histogram and the consensus sequence appear below. The amino acids A28, G203, and R284 mutated in the probands presented are signified by an asterisk and are conserved down to yeast. Alignment was performed with Clustal Omega accessed from European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) (https://www.ebi.ac.uk/Tools/msa/clustalo/) and analyzed using JalView.

Figure S3

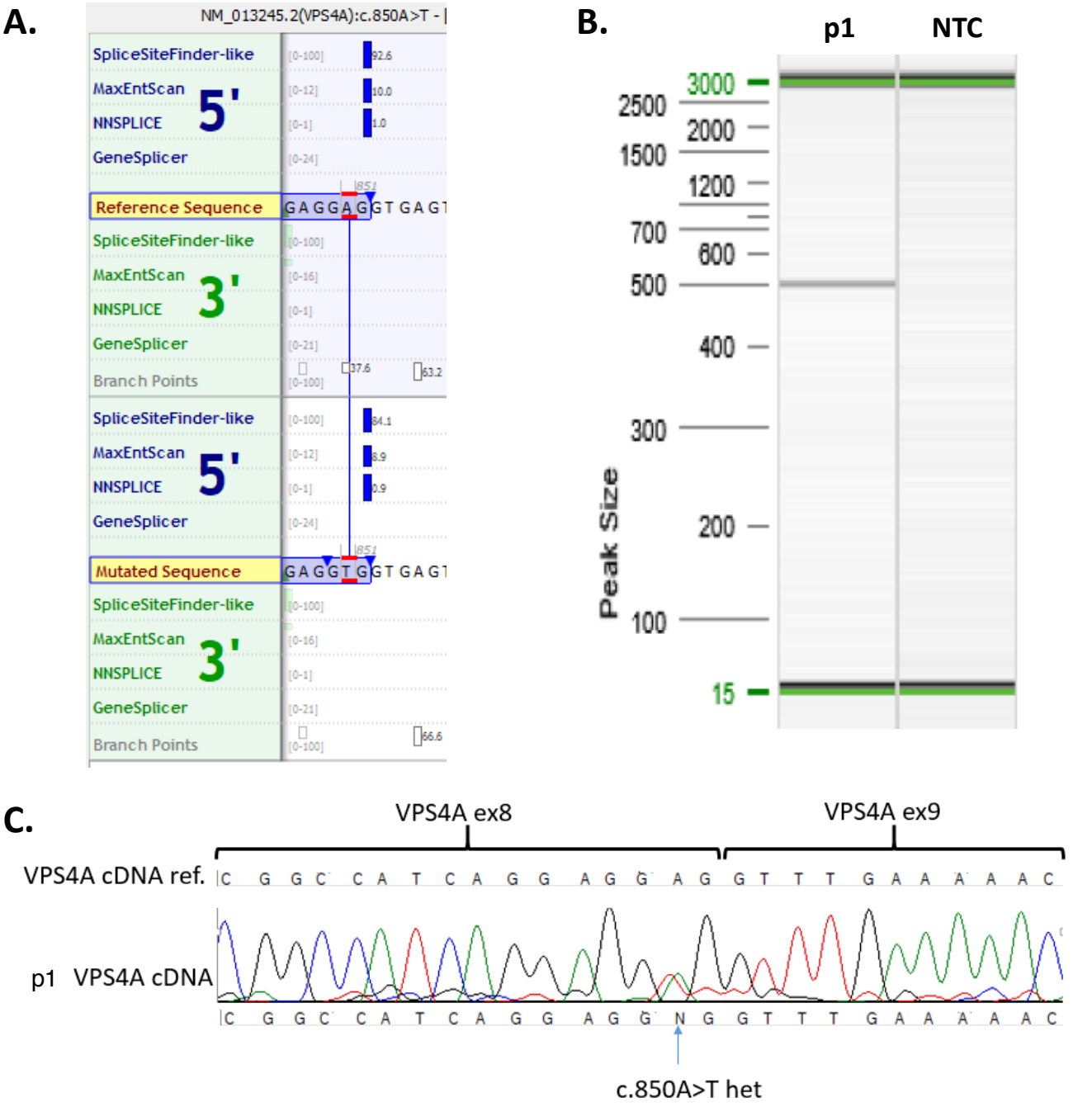

Figure S3. cDNA sequencing analysis of *VPS4A* c.850A>T variant. (A) *VPS4A* c.850A>T (p.Arg284Trp) is a mutation of the penultimate base of exon 8. Multiple splice prediction programs suggested it may affect the splice donor site of intron 8, indicated by score reductions over 10%.[1] **(B)** Gel image of *VPS4A* fragment containing c.850A>T after amplification with reverse transcription polymerase chain reaction (RT-PCR) showing the cDNA product at ~500 bp as expected for the wild type allele. **(C)** Sequence chromatogram showing that this variant is a missense mutation rather than having alternative splicing effect.

Figure S4

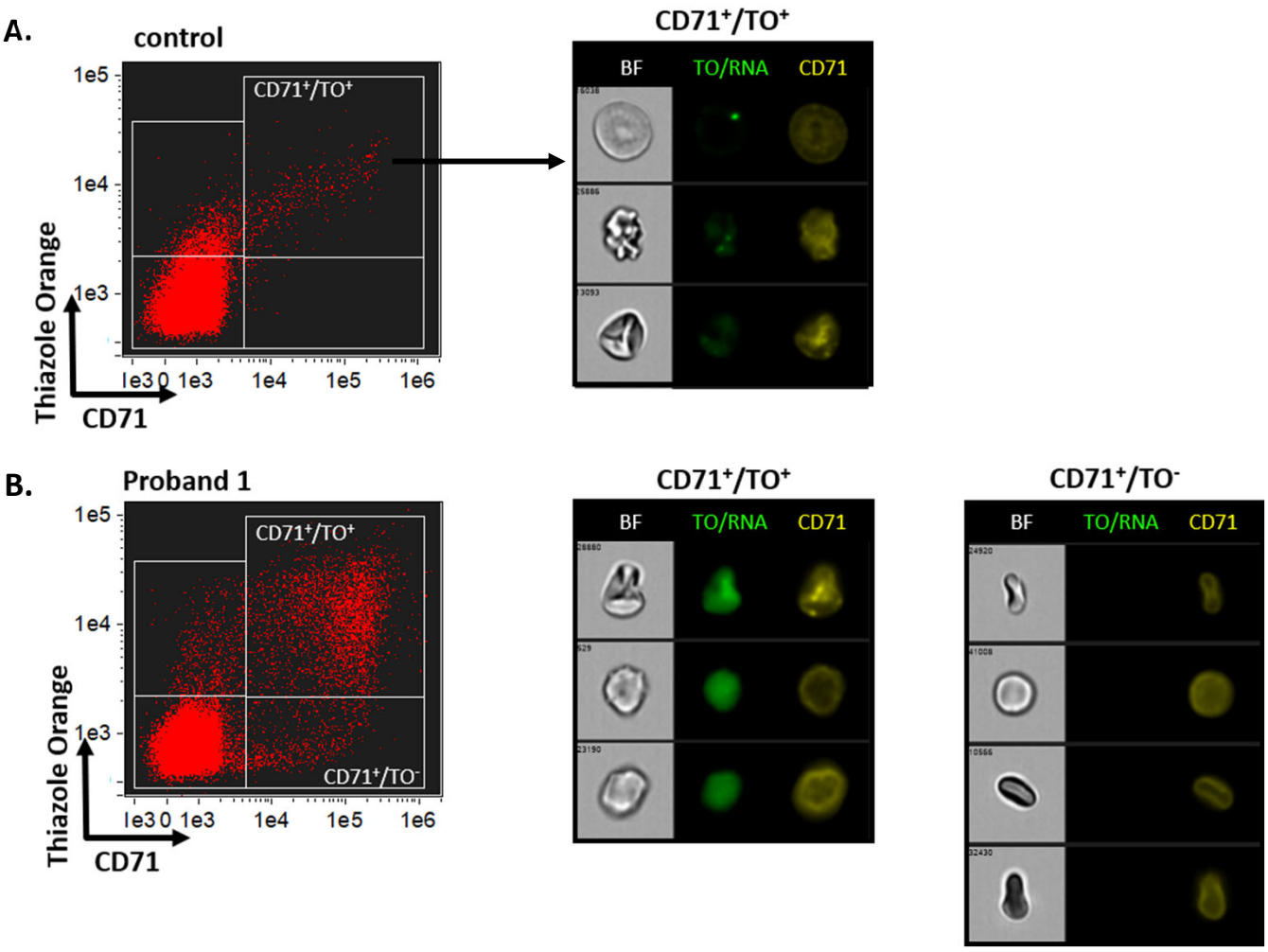

Figure S4. Imaging flow cytometry analysis of peripheral blood reticulocytes. (A) Flow cytogram and representative images from the $CD71^{+}/TO^{+}$ reticulocyte population of an SCD control sample. (**B)** Flow cytogram and representative images from the $CD71^{+}/TO^{+}$ reticulocyte (middle) and unique $CD71^{+}/TO^{-}$ populations (right) in whole blood from proband 1. The $CD71^{+}/TO^{-}$ population contains mature RBCs (not reticulocytes) still retaining CD71 in their membrane. Similar results were obtained for all three probands.

Table S1. Flow cytometry analysis of hematopoietic progenitors.

		control iPSCs, n=6		proband 1 iPSCs, n=5		p value
		mean	SD	mean	SD	
day 10	CD43+	65.1	13.5	63.7	6.3	n.s.
	GPA+/CD41-	4.5	2.8	1.5	0.7	n.s.
	GPA+/CD41+	66.3	12.2	66.7	13.3	n.s.
	GPA-/CD41+	17.9	13.4	23.0	10.7	n.s.
	GPA-/CD41-	11.8	2.2	9.2	3.3	n.s.
day 10+5	CD43+	76.2	3.5	69.1	4.0	p<0.05
	GPA+/CD41-	44.7	10.3	38.9	15.9	n.s.
	GPA+/CD41+	4.9	1.9	7.8	1.6	p<0.05
	GPA-/CD41+	47.5	11.4	52.1	15.7	n.s.
	GPA-/CD41-	3.0	1.1	1.4	0.4	p<0.05

Hematopoietic progenitors produced from iPSCs were identified at day 10 of hematopoietic differentiation culture (**Figure 3**) as $CD43^+/CD235a(GPA)^+/CD41^+$.[2-4] At day 10, control cells were typically 60-70% $CD43^+$ and 60-70% of those were $GPA^+/CD41^+$. After 5 days of *ex vivo* erythropoiesis (day 10+5), control cells were typically still 70% $CD43^+$ with very few being $GPA^+/CD41^+$ (<5%); ~50% of the $CD43^+$ cells had lost CD41 and were now only GPA+, while the remainder of the cells were $CD41^+/GPA^-$. Data presented are mean ± SD of n=6 cultures from control iPSCs and n=5 cultures from proband 1-derived iPSCs; p values determined by Student's t-test.

Table S2. Flow cytometry analysis of ex vivo erythropoiesis cultures.

		control iPSCs, n=7		proband 1 iPSCs, n=6		p value
		mean	SD	mean	SD	
day 10+5	GPA+	51.1	7.7	52.7	8.3	n.s.
	E1	65.2	8.8	47.5	6.4	p<0.05
	E2	15.1	6.9	19.5	4.6	n.s.
	E3	8.3	2.0	16.2	2.3	p<0.001
	E4	0.9	0.5	0.8	0.3	n.s.
	E5	0.3	0.2	0.5	0.3	n.s.
day 10+8	GPA+	76.4	6.4	69.0	15.9	n.s.
	E1	0.3	0.3	0.7	0.5	n.s.
	E2	1.3	0.7	3.0	4.8	n.s.
	E3	74.8	2.4	60.6	6.8	p<0.001
	E4	14.9	2.4	19.1	1.3	p<0.05
	E5	4.8	1.7	9.7	2.9	p<0.05

Gating of α_4 integrin vs band 3 to monitor erythroblast differentiation was done as described previously.[5] Gates are numbered here as E1-E5 going clockwise from the top left corner (most immature erythroblasts), to bottom right (most mature erythroblasts), as shown in **Figure 3C&D**. At day 10+5, 50% of the control cells were typically GPA^+; 70% of those fell into the E1 gate corresponding to proerythroblasts (top left gate, α_4 $integrin^{high}$/Band 3^-). By day 10+8, approximately 75% of the cells were GPA^+; approximately 70% of those appeared in the E3 gate corresponding to late basophilic erythroblasts (α_4 $integrin^{int}$/Band 3^+) and some (~15%) polychromatophilic erythroblasts (E4, α_4 $integrin^{low}$/Band 3^+). In the proband line, the percent of GPA^+ cells was similar to the control; however, erythroblast differentiation progressed faster in the proband-derived line versus the control. At day 10+5, only 48% on average of the *VPS4A*-mutant GPA^+ cells fell into the E1 gate versus 65% for the control GPA^+ cells ($p<0.05$), with the rest progressing to more mature stages of differentiation, particularly in E3. Similarly, at day 10+8, just 60% fell into the E3 gate ($p<0.001$), the rest of the cells being more advanced already than the control cells with significant increases in the subsequent E4 and E5 populations ($p<0.05$). Data presented are mean ± SD of n=7 cultures of control iPSCs and n=6 cultures of proband 1 iPSCs; p values determined by Student's t-test.

Table S3. Antibodies used for flow cytometry and immunofluorescence

Target/Type	Conjugate	Manufacturer	Catalog No.	Used for:
CD71	BV421	BD Biosciences	562995	reticulocyte flow, erythroid flow, IFC
CD45	PerCP-Cy5.5	Biolegend	304028	reticulocyte flow, progenitor flow
CD34	BV421	BD Biosciences	562577	progenitor flow
CD43	FITC	BD Biosciences	555475	progenitor flow
CD41	APC	BD Biosciences	559777	progenitor flow
CD235a/GPA	PE	BD Biosciences	555570	progenitor flow, erythroid flow, IFC
α4 integrin	APC	BD Biosciences	559881	erythroid flow
Band 3*	Dylight488	N/A	N/A	erythroid flow
a-tubulin	biotin	eBioscience/Thermo Fisher	13-4502-80	IFC
VPS4	unconjugated	Sigma-Aldrich	SAB4200025	IFC
B-tubulin	unconjugated	Invitrogen/Thermo Fisher	PA5-16863	confocal
anti-rabbit	AF488	Invitrogen/Thermo Fisher	A11034	IFC
anti-rabbit	AF633	Invitrogen/Thermo Fisher	A21071	confocal
Streptavidin	AF647	Invitrogen/Thermo Fisher	S32357	IFC
Rb isotype IgG	unconjugated	Cell Signaling Technology	3900S	IFC, confocal

*Dylight 488-conjugated Band 3 antibody was a generous gift from Dr. Mohandas Narla (New York Blood Center).

Materials and Methods

Study Approval

Individuals having clinical diagnosis of CDA and their parents were enrolled in CDAR (ClinicalTrials.gov Identifier: NCT02964494), a registry for individuals with the diagnosis of CDA in North America, under a protocol approved by the Institutional Review Board of Cincinnati Children's Hospital. The families provided written informed consent, in accordance with the Declaration of Helsinki. Normal human $CD34^+$ cells isolated from umbilical cord blood and normal control iPSCs were obtained from the Translational Research Development Support Laboratory of Cincinnati Children's Hospital under a protocol approved by this institute.

Hereditary Hemolytic Anemia Gene Panel

DNA was isolated from peripheral blood and analyzed on a Next Generation Sequencing panel of genes known to be associated with hereditary hemolytic anemias (HHA panel); the regions of interest for enrichment and DNA sequencing included the coding exons plus 20 bases of intronic boundaries for 32 genes known to be associated with RBC membrane and enzyme disorders and with congenital dyserythropoietic anemias: ABCG5, ABCG8, AK1, ALDOA, ANK1, C15orf41, CDAN1, EPB41, EPB42, G6PD, GATA1, GCLC, GPI, GPX1, GSR, GSS, HK1, KIF23, KLF1, NT5C3A, PFKM, PGK1, PIEZO1, PKLR, RHAG, SEC23B, SLC2A1(GLUT1), SLC4A1, SPTA1, SPTB, TPI1, and XK. Regulatory regions and deep intronic areas of these genes with published disease-causing mutations were included in the HHA panel design.

Whole Exome Sequencing

Samples from probands 1 and 2, as well as their parents, were submitted to GeneDx for clinical family trio-based whole exome sequencing (WES). Samples from proband 3 and the parents were submitted to Cincinnati Children's Hospital Medical Center for Integrated Genomic Analysis, which included

clinical trio-based WES and clinical chromosome microarray. As reported by the laboratories, the WES capture was performed using SureSelect Clinical Research Exome kit or SureSelect All Exon kit (Agilent). Enriched library fragments were sequenced on Illumina HiSeq system (Illumina). The reads were mapped against UCSC hg19 human reference and analyzed using custom-developed tool or Ingenuity Variant Analysis software (Qiagen).

cDNA amplification and sequencing

RNA was extracted from reticulocytes of proband 1 using QIAamp RNA Blood Mini kit (Qiagen) per manufacturer protocol. High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to generate cDNA from RNA. The following primers were used for reverse transcription polymerase chain reaction (RT PCR) to test the potential splicing effect of c.840A>T: *VPS4A* E7-10F CACAAGCCCTCCATCATCTT and *VPS4A* E7-10R GCATGGAGTCAGGAGGTCAT. The PCR program was: 1. 95° for 3 min; 2. 95° for 30sec; 3. 60° for 30sec; 4. 72° for 1 min; 5. Go to step 2 for 34 more times; 6. 72° for 5 min; 7. 12° forever. The PCR product was run on Qiaxcel Advanced system (Qiagen). The cDNA product size from wild type allele was expected to be 477 bp. According the manufactures guideline, the size variability range for fragments less than 500 bp is +/- 20bps. Sanger sequencing was performed at the Sequencing Core of Cincinnati Children's Hospital Medical Center.

Protein diagram and crystal structures

The schematic of VPS4A domain structure was generated using the Illustrator for Biological Sequences (IBS, http://ibs.biocuckoo.org/).[6] Crystal structures for the yeast VPS4 hexamer (PDB ID: 6API) and human MIT domain (PDB ID: 1YXR) were accessed from the Protein Data Bank, www.pdb.org and visualized using UCSF Chimera.[7] All residue numbers labeled in the yeast VPS4 structure (6API) were identified and labeled with the corresponding residue numbers in human VPS4A based on multiple sequence alignment with Clustal (http://www.clustal.org/omega/) and as indicated by Poly-Phen II sequence alignment and predictions (http://genetics.bwh.harvard.edu/pph2/).

Reticulocyte isolation and qPCR

Proband and control reticulocytes were isolated from peripheral blood using magnetic separation. Blood cells were washed, and the buffy coat was removed. RBCs were labeled with anti-human CD71 microbeads (Miltenyi Biotec), washed, and separated using positive selection on an autoMACS® Pro Separator (Miltenyi Biotec). RNA was isolated from $CD71^+$ reticulocyte pellets with the QIAamp RNA Blood Mini Kit (Qiagen). cDNA was generated using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and qPCR was performed using TaqMan probes (*VPS4A*, Hs00203085_m1; *VPS4B*, Hs01078182_m1; and *ACTB*, Hs01060665_g1) and TaqMan Universal PCR Master Mix. Relative expression was determined using the ddCt method. Control samples used were from individuals with reticulocytosis including sickle cell disease, iron-deficiency anemia post iv iron treatment, and autoimmune hemolytic anemia.

$CD34^+$ cell cultures

Human $CD34^+$ cells were isolated from umbilical cord blood; the study protocol was reviewed by Cincinnati Children's Hospital Medical Center Institutional Review Board and was determined that this process does not meet the regulatory criteria for research involving human subjects. *Ex vivo* erythropoiesis culture from $CD34^+$ cells was performed according to the protocol of Giarratana, et al.[8] Briefly, cells are cultured in serum-free medium of StemPro-34 (Gibco), BIT 9500 (StemCell Technologies), ferrous sulfate (900 ng/ml), ferric nitrate (90 ng/ml), L-glutamine (Invitrogen), and pen/strep (Invitrogen). For days 0-5, the medium is supplemented with 10^{-6} M hydrocortisone (Sigma), 100ng/ml SCF (Peprotech), 5ng/ml IL-3 (Peprotech), and 3 IU/ml erythropoietin (Epo, Amgen). For days 6-7, the medium is supplemented only with Epo, followed by 2-3 days with medium free of cytokines. Cultures are monitored by cytospins and flow cytometry to determine erythroblast stage.

Generation of iPSC-lines from healthy donors and individuals enrolled in CDAR

Peripheral blood from healthy donors and individuals enrolled in CDAR was obtained at Cincinnati Children's Hospital Medical Center after signed informed consent, under Institutional Review Board (IRB)-approved research protocols. iPSCs were generated from peripheral blood mononuclear cells (PBMCs) as previously described.[9; 10] PBMCs were purified from peripheral blood by Ficoll separation (GE Healthcare) and plated at 1-5x10^6 cells/mL in X-Vivo 10 (Lonza) supplemented with 10% FCS, SCF (100 ng/mL), TPO (100ng/mL), IL-3 (100ng/mL), IL-6 (20 ng/mL), FLT3L (100ng/mL) and GM-CSF (10ng/mL) (all cytokines from Peprotech) in a low-attachment plate (Corning) and cultured at 37°C in 5% CO_2. After two days of culture, cells were transduced with a lentivirus containing OCT4, KLF4, MYC, and SOX2 using polybrene at MOI of 20-40.[11] Two days after lentiviral transduction, cells were plated onto irradiated mouse embryonic fibroblasts (MEF, GlobalStem) in X-Vivo 10 supplemented with 10% FCS, SCF (100 ng/mL), TPO (100 ng/mL), IL-3 (10 ng/mL), IL-6 (20 ng/mL), and FLT3L (100 ng/mL). After two days of culture on MEF, the media was changed to hESC media (DMEM F/12, 20% KnockOut Serum Replacement (KSR, Life Technologies), 1% L-glutamine, 1% NEAA, 0.1 mM β-mercaptoethanol) supplemented with 10 ng/mL bFGF (Peprotech). Once colonies with morphological characteristics of iPSCs were observed, they were plucked and passaged onto Matrigel (BD Biosciences) and cultured in mTeSR1 medium (Stem Cell Technologies). Targeted Sanger sequencing was performed on cell lines generated from probands to ensure they retained their respective mutations.

***Ex vivo* erythropoiesis culture from iPSCs**

Hematopoietic progenitors were derived from iPSCs using the STEMdiff hematopoietic kit (Stem Cell Technologies, Vancouver, BC). By day 10, the floating cells were enriched in multipotent hematopoietic progenitors (**Table S1**), identified as $CD34^{+/-}$/$CD43^+$/CD235a$(GPA)^+$/$CD41^+$ by flow cytometry as previously described.[2-4] These were harvested and further differentiated in *ex vivo* erythropoiesis culture. The base medium (generously shared by Dr. Stella Chou in personal

communication) consisted of IMDM (Corning), Ham's F-12 (Corning), 0.05% BSA (Gibco), glutamine (Invitrogen), pen/strep (Invitrogen), N2 supplement (Gibco), B27 without retinoic acid (Gibco), 1-thioglycerol ($4x10^{-4}$ M, Sigma), and ascorbic acid (50ng/ml). For the first 5 days, the base medium was supplemented with Stem Cell Factor (SCF 50ng/ml, Peprotech), erythropoietin (Epo, 2U/ml, Amgen), and dexamethasone (0.4µg/ml). On day 10+5, cells were washed and returned to culture with medium supplemented with Epo, dexamethasone, and 5% human A/B serum (Sigma). Cells were harvested for experimental analysis on day 10+8 when control cells were primarily late basophilic and polychromatophilic erythroblasts (**Table S2**). Terminal differentiation of GPA^+ erythroid precursors in these cultures was monitored by flow cytometry of α_4 integrin and band 3 [5] and by morphology on cytospins. For cytospins, ≥50,000 cells were suspended in 150µl of FBS and spun at 400 rpm for 3 minutes onto glass coverslips in a Cytospin-4 Cytocentrifuge (Thermo Shandon). After drying, coverslips were stained with Wright stain (Harleco EMD) following the manufacturer's protocol.

Flow Cytometry

All antibodies used for flow cytometry are listed in **Table S3**. For analysis of reticulocytes by flow cytometry and imaging flow cytometry (IFC), 1µl whole blood was washed and resuspended in FACS buffer (PBS+0.5% BSA) containing fluorescently conjugated antibodies against CD45 and CD71. After 30min, cells were washed and resuspended in BD Retic-Count (BD) and incubated for 30min at room temperature following the manufacturer's protocol. For iPSC cultures, cells were resuspended in FACS buffer containing fluorescently conjugated antibodies and Fixable Viability Dye efluor780 (eBiosciences) and incubated for 30min at room temperature. Cells were then washed and analyzed on a FACSCanto II cytometer (BD Biosciences).

Imaging Flow Cytometry (IFC)

For intracellular staining, cells from $CD34^+$ and iPSC cultures were fixed by adding 16% paraformaldehyde directly to cell culture dishes to a final concentration of 4%. After 30min, cells were

washed with PBS and stored at 4°C until staining. For immunofluorescence staining, fixed cell pellets were permeabilized with acetone as described previously.[12] Briefly, cells were washed in sequence with ice-cold 50% acetone, 100% acetone, and then 50% acetone. Permeabilized cells were then incubated with primary antibodies or isotype control (see **Table S3**) for 1 hr at room temperature, followed by incubation with secondary antibodies for 30 min at RT. For analysis of binucleated events, cells were stained with Fixable Viability Dye efluor780 (eBiosciences) prior to fixation with 4% PFA. Following fixation, cells were stained with conjugated antibodies (see Table S3) and Hoechst 34580 (Sigma) and incubated for 1hr at 37°C. Labeled cells were analyzed on an Imagestream ISX-100 (Amnis) and analysis was performed in IDEAS analysis software (Amnis).

Immunofluorescence staining and confocal microscopy

Cultured cells were placed and cultured in ibidi imaging coverslip chambers at least 16hrs prior to fixation with 4% PFA for 30min. Cells were washed and then permeabilized and blocked with PBS+0.1% Tween-20 + 3% BSA. Samples were incubated with primary antibodies for 1hr at room temperature, washed, and incubated with secondary antibodies and DAPI for 30min at room temperature. See **Table S3** for all antibodies used. Stained cells were imaged on a Nikon C2 confocal microscope using 60x magnification.

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