Supplemental Data:

X-Linked Macrocytic Dyserythropoietic Anemia in Females with an *ALAS2* Mutation

Vijay G. Sankaran, Jacob C. Ulirsch, Vassili Tchaikovskii, Leif S. Ludwig, Aoi Wakabayashi, Senkottuvelan Kadirvel, R. Coleman Lindsley, Rafael Bejar, Jiahai Shi, Scott B. Lovitch, David F. Bishop, David P. Steensma

Address correspondence to:
Vijay G. Sankaran, Boston Children's Hospital, 3 Blackfan Circle, CLS 03001, Boston, MA 02115; Phone: 617-919-6270; E-mail: sankaran@broadinstitute.org.
Or to: David P. Steensma, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215, Phone: 617-632-3712; E-mail: David_Steensma@dfci.harvard.edu

Supplemental Methods:

Whole-Exome Sequencing of Family Members

DNA was extracted and libraries were prepared for whole-exome sequencing (1). Sequencing reads were analyzed with an automated pipeline built around the Genome Analysis Toolkit. Mutation counts both before and after filtration of common variants are shown in Supplemental Table 2, and this analysis was performed as described previously (2). All data on polymorphisms from these individuals was deposited in the database of Genotypes and Phenotypes (dbGaP) from the National Center for Biotechnology Information.

HUMARA Assay

Analysis of X-inactivation was performed on genomic DNA extracted from peripheral blood mononuclear cells. Some DNA was digested with the methylation-sensitive enzyme HpaII and then PCR amplification of the androgen receptor (*AR*) locus was performed. The amount of each allele present in either sample with or without treatment with HpaII was quantitated on a DNA Analyzer 3730 (Applied Biosystems Inc.) using the fluorescein channel with analysis performed on GeneMapper software. The assay was carried out in a manner similar to that described previously (3).

Sanger Sequencing

Variants of interest were confirmed using PCR of regions of interest in the *ALAS2* gene. For analysis of samples after HpaII digestion, a similar PCR amplification of the regions of interest in the *ALAS2* gene was performed and subjected to Sanger sequencing as noted above. HpaII digestion results in cuts in the *ALAS2* gene between the mutation site and the PCR amplification sites, thus affecting the Sanger signal from DNA that is unmethylated.

ALAS2 Expression and Purification

The wild-type human ALAS2 expression construct, pMALc2-AE2 (4) was modified to introduce the Y365C mutation by site-directed mutagenesis using the Stratagene XL Site-directed mutagenesis kit. Transformation, expression, and purification to homogeneity were performed as previously described (5). For purification by gel filtration chromatography, a Superdex 200 (GE Healthcare) column was used. For size exclusion, proteins were separated by chromatography on two tandem HiLoad 16/600 Superdex 200 size-exclusion columns (GE Healthcare) at a flow rate of 0.2 ml/min.

Enzyme and Protein Assays

ALAS2 enzymatic activity was determined using succinyl CoA, glycine, and pyridoxal-5' phosphate in five minute endpoint assays and quantitated by chemical condensation with ethylacetoacetate followed by reaction with Ehrlich's reagent as described previously (5). Protein was assayed by the Fluorescamine method as previously described (6). For heat stability experiments, the enzymes were preincubated at 50 °C for up to 30 min in 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, and 10 or 100 uM PLP, and then assayed with the addition of 0.5 mg/ml BSA to protect against inactivation upon dilution in the assay.

Protein Modeling

Modeling was performed using the crystal structure of the *Rhodobacter capsulatus* ALAS2 homologue (7). Modeling was performed using PyMOL (http://www.pymol.org/).

Mononuclear Cell Erythroid Cultures

Peripheral blood mononuclear cells were isolated using Ficoll-Paque Plus (GE Healthcare) and cultured in IMDM media containing human AB serum, human AB plasma, holo-transferrin, insulin, erythropoietin, stem cell factor, and IL-3 as previously described (8).

Supplemental Table 1: Hematological And Other Laboratory Parameters In Affected Family Members

Parameter	Proband (II-2)	Proband's Sister (II-4)	Proband's Mother (I-1)
Hemoglobin (g/dL) [normal, 12.0-15.0 g/dL]	11.0*	11.2*	12.0
Hematocrit (%) [normal, 37.0-47.0%]	32.1*	32.5*	36.1*
Mean cell volume (fL) [normal, 81-95 fL]	114*	108*	106*
Mean cell hemoglobin (pg) [normal, 27.6-33.9 pg]	38.9*	37.4*	35.0*
Red blood cell count ($x10^9$/L) [normal, 4.2-5.5 $x10^9$/L]	2.81*	3.04*	3.72*
Red cell distribution width [normal, 11-13.5%]	12.7%	11.8%	12.4%
Lactate dehydrogenase (U/L) [normal, 107-231 U/L]	143	ND	ND
Absolute and proportional reticulocyte count (M/µL; %) [normal, 0.043-0.095 M/µL; 0.6-2.8%]	0.0520; 1.8	ND	ND
Haptoglobin (mg/dL) [normal, 40-200 mg/dL]	75	ND	ND
Serum erythropoietin (U/L) [normal, 7-20 U/L]	86*	ND	ND
Serum ferritin (ng/mL) [normal, 10-170 ng/mL]	598-1030*	104	230*
Transferrin saturation (%) [normal, 12-45%]	96*, 83* on repeat testing	28	39
MRI LIC estimate (µmol/g dry weight) [normal, <36 µmol/g dry weight]	>350*	ND	ND
MRI cardiac T2* (ms) [normal, >20 ms]	49	ND	ND
Liver biopsy iron index [normal, <1.0]	11*	ND	ND
Serum hepcidin (ng/mL) [normal, 17-286 ng/ml]	32.3	ND	ND
HFE genotype	Wild-type	Wild-type	Wild-type
Other features	Horseshoe kidney; atrial septal defect (secundum type); placenta accreta; neonatal febrile seizures; recurrent childhood UTIs; adenocarcinoma of the uterine cervix	None	Recurrent miscarriages; brittle fingernails (telomere assay WNL)

Asterisks denote values outside the normal range. Proband's leukocyte count and differential, platelet count, hemoglobin electropheresis, routine coagulation tests (prothrombin time, activated partial thromboplastin time), vitamin B12 and folate levels, uric acid and general chemistry group (including total and direct bilirubin, transaminases, creatinine and blood urea nitrogen) were all within normal limits. Family is of mixed Irish, Polish, and German ancestry. Abbreviations: MRI=magnetic resonance imaging. LIC = liver iron concentration. WNL = within normal limits. UTI = urinary tract infection. ND = not done.

Supplemental Table 2: Classes of variants in a family with macrocytic anemia and dyserythropoiesis

	Proband (Individual II-2)	Proband's Mother (Individual I-1)	Proband's Sister (Individual II-4)	Proband's Daughter (Individual III-1) (Unaffected)
Novel				
CODON CHANGE PLUS CODON DELETION	28	19	15	23
CODON CHANGE PLUS CODON INSERTION	10	10	12	13
CODON DELETION	49	43	42	51
CODON INSERTION	6	10	7	11
DOWNSTREAM	215	192	202	226
FRAMESHIFT	24	21	28	31
INTRAGENIC	5	3	3	6
INTRON	403	403	371	382
NONSYNONYMOUS CODING	172	176	182	216
NONSYNONYMOUS START	1	0	0	0
SPLICE SITE ACCEPTOR	8	8	4	6
SPLICE SITE DONOR	2	4	4	3
START GAINED	1	1	1	3
START LOST	2	0	0	3
STOP GAINED	2	2	1	3
STOP LOST	0	1	0	0
SYNONYMOUS CODING	114	106	101	126
Total	1095	1044	1026	1146
TRANSCRIPT	15	15	15	14
UPSTREAM	18	6	15	7
UTR 3-PRIME	4	10	9	5
UTR 5-PRIME	16	14	14	17
Known				
CODON CHANGE PLUS CODON DELETION	93	87	82	89
CODON CHANGE PLUS CODON INSERTION	33	31	42	32
CODON DELETION	130	125	124	132
CODON INSERTION	88	88	91	90
DOWNSTREAM	8685	8284	8510	8515
FRAMESHIFT	282	268	280	267
INTERGENIC	52	61	55	51
INTRAGENIC	86	90	78	89
INTRON	15350	14982	15368	15297

NONSYNONYMOUS CODING	10075	9941	10017	10054
NONSYNONYMOUS START	1	3	2	1
SPLICE SITE ACCEPTOR	87	82	84	85
SPLICE SITE DONOR	63	57	61	56
START GAINED	149	147	160	156
START LOST	21	21	23	22
STOP GAINED	72	73	72	73
STOP LOST	16	18	18	16
SYNONYMOUS CODING	10988	10905	10927	11006
SYNONYMOUS STOP	12	10	9	8
Total	48133	47024	47794	47875
TRANSCRIPT	956	899	918	930
UPSTREAM	361	342	359	368
UTR 3-PRIME	324	319	318	340
UTR 5-PRIME	209	191	196	198

Supplemental References:

1. Sankaran, V.G., and Gallagher, P.G. 2013. Applications of high-throughput DNA sequencing to benign hematology. *Blood* 122:3575-3582.

2. Sankaran, V.G., Ghazvinian, R., Do, R., Thiru, P., Vergilio, J.A., Beggs, A.H., Sieff, C.A., Orkin, S.H., Nathan, D.G., Lander, E.S., et al. 2012. Exome sequencing identifies GATA1 mutations resulting in Diamond-Blackfan anemia. *The Journal of clinical investigation* 122:2439-2443.

3. Thouin, M.M., Giron, J.M., and Hoffman, E.P. 2003. Detection of nonrandom X chromosome inactivation. *Current protocols in human genetics / editorial board, Jonathan L. Haines ... [et al.]* Chapter 9:Unit9 7.

4. Cotter, P.D., Rucknagel, D.L., and Bishop, D.F. 1994. X-linked sideroblastic anemia: identification of the mutation in the erythroid-specific delta-aminolevulinate synthase gene (ALAS2) in the original family described by Cooley. *Blood* 84:3915-3924.

5. Bishop, D.F., Tchaikovskii, V., Hoffbrand, A.V., Fraser, M.E., and Margolis, S. 2012. X-linked sideroblastic anemia due to carboxyl-terminal ALAS2 mutations that cause loss of binding to the beta-subunit of succinyl-CoA synthetase (SUCLA2). *The Journal of biological chemistry* 287:28943-28955.

6. Bishop, D.F., Wampler, D.E., Sgouris, J.T., Bonefeld, R.J., Anderson, D.K., Hawley, M.C., and Sweeley, C.C. 1978. Pilot scale purification of alpha-galactosidase A from Cohn fraction IV-1 of human plasma. *Biochimica et biophysica acta* 524:109-120.

7. Astner, I., Schulze, J.O., van den Heuvel, J., Jahn, D., Schubert, W.D., and Heinz, D.W. 2005. Crystal structure of 5-aminolevulinate synthase, the first enzyme of heme biosynthesis, and its link to XLSA in humans. *The EMBO journal* 24:3166-3177.

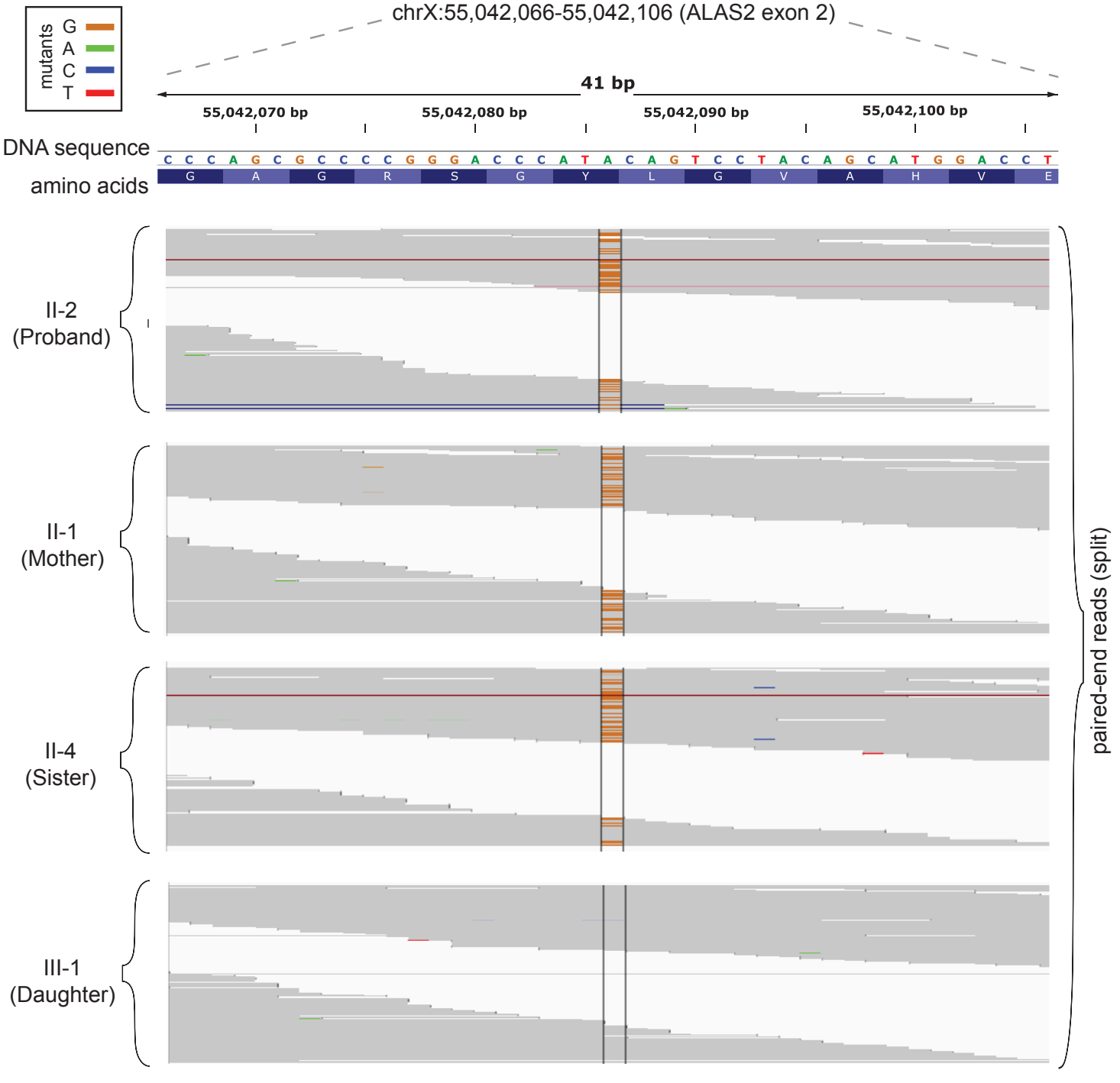
8. Ludwig, L.S., Gazda, H.T., Eng, J.C., Eichhorn, S.W., Thiru, P., Ghazvinian, R., George, T.I., Gotlib, J.R., Beggs, A.H., Sieff, C.A., et al. 2014. Altered translation of GATA1 in Diamond-Blackfan anemia. *Nature medicine* 20:748-753.

Supplemental Figure Legends:

Supplemental Figure 1. Integrated Genomics Viewer was used to visualize sequence reads from all family members who underwent whole-exome sequencing to validate number of reads at position chrX: 55042086 (hg19 coordinates) and show those reads with the variant (mutant) allele present.

Supplemental Figure 2. Sanger sequencing traces following HpaII digestion of genomic DNA showing the region around 55042086 on the X chromosome (hg19 coordinates, trace is showing the positive strand on the X chromosome).

Supplemental Figure 1



Supplemental Figure 2