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Supplemental information

De novo missense variants in exon 9 of *SEPHS1* cause a neurodevelopmental condition with developmental delay, poor growth, hypotonia, and dysmorphic features

Sureni V. Mullegama, Kaitlyn A. Kiernan, Erin Torti, Ethan Pavlovsky, Nicholas Tilton, Austin Sekula, Hua Gao, Joseph T. Alaimo, Kendra Engleman, Eric T. Rush, Karli Blocker, Katrina M. Dipple, Veronica M. Fettig, Heather Hare, Ian Glass, Dorothy K. Grange, Michael Griffin, Chanika Phornphutkul, Lauren Massingham, Lakshmi Mehta, Danny E. Miller, Jenny Thies, J Lawrence Merritt II, Eric Muller II, Matthew Osmond, Sarah L. Sawyer, Rachel Slaugh, Rachel E. Hickey, Barry Wolf, Care4Rare Canada Consortium, Undiagnosed Diseases Network, Sanjeev Choudhary, Miljan Simonović, Yueqing Zhang, Timothy Blake Palculict, Aida Telegrafi, Deanna Alexis Carere, Ingrid M. Wentzensen, Michelle M. Morrow, Kristin G. Monaghan, Jun Yang, and Jane Juusola

Supplemental Information

Supplement Note: Considerations for care of patients with *SEPHS1* variants

Individuals with pathogenic variants in *SEPHS1* appear to have a neurodevelopmental and growth disorder, with variable effects on other body systems. Considerations for immediate and long-term care of these individuals might include:

1. Frequent assessment of developmental milestones, with targeted interventions as needed
2. Psychoeducational evaluation in school-age children
3. Screening for neurobehavioral concerns, including attention-deficit/hyperactivity disorder and obsessive-compulsive disorder.
4. Screening for neurologic and muscular concerns with low threshold for referral to a specialist
5. Monitoring of height and weight
6. Feeding evaluation
7. Screening for endocrine abnormalities, including hypocalcemia, Hasimoto's thyroiditis, elevated B6, growth hormone deficiency. It is unknown if this needs to be followed over time
8. Eye examination
9. Sleep study

A

Individual 1

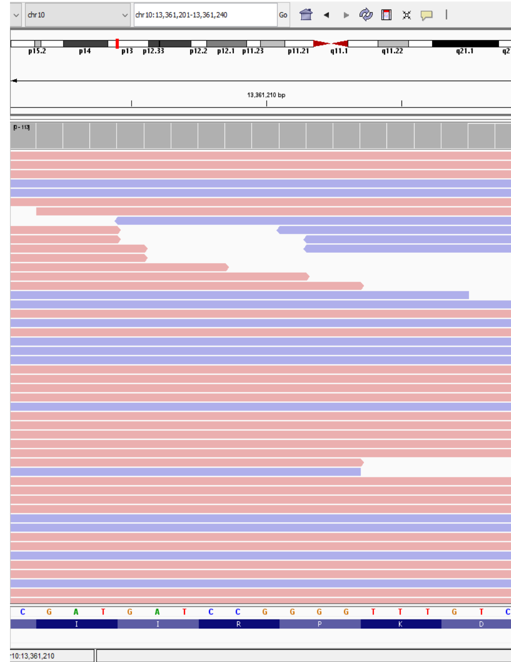
Mother

Father

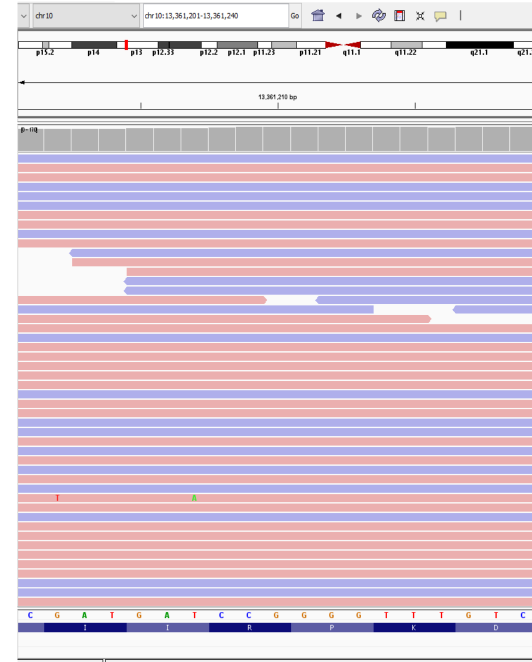
B

Individual 2

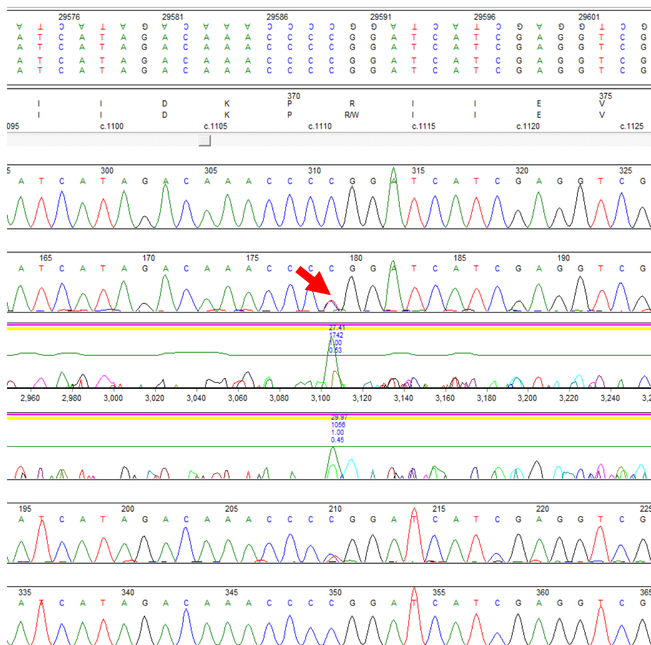
Mother



Father



Individual 2

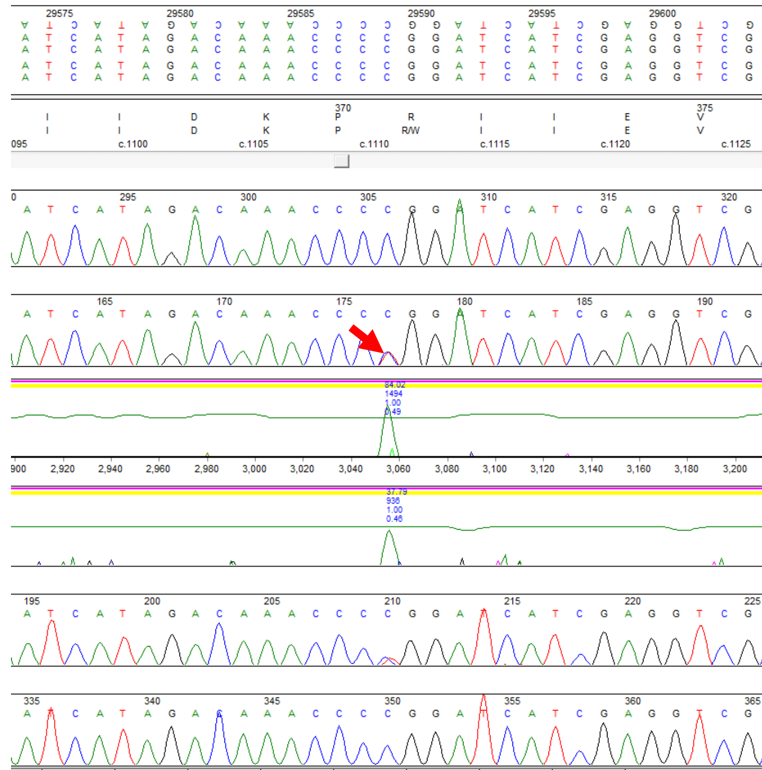


Mother & Father

No ABI data (high quality NGS, no confirmation)

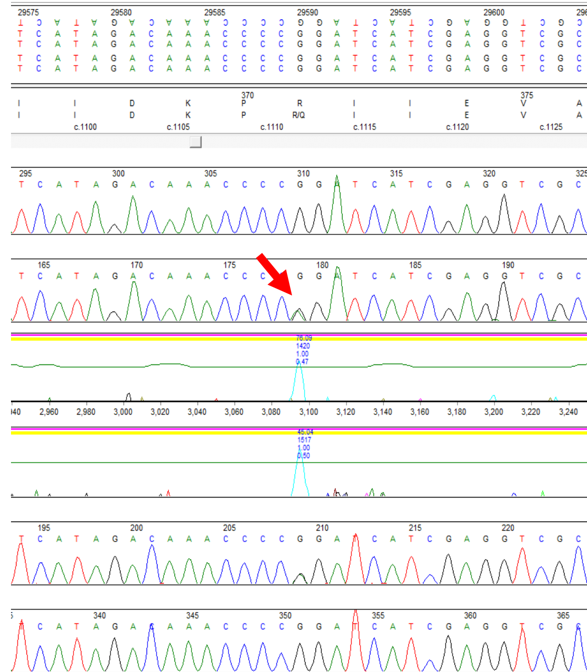
C

Individual 3

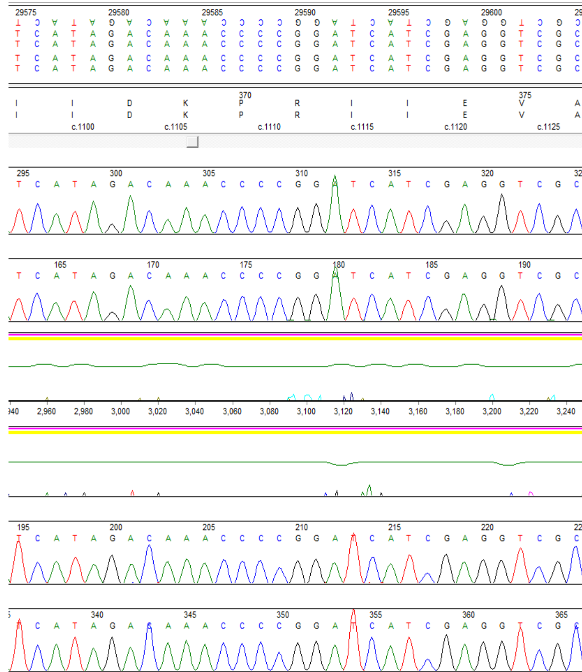


D

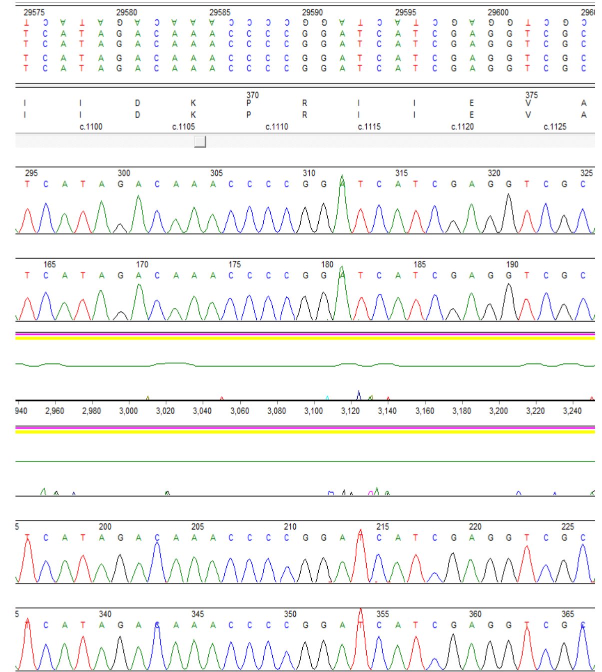
Individual 4



Mother



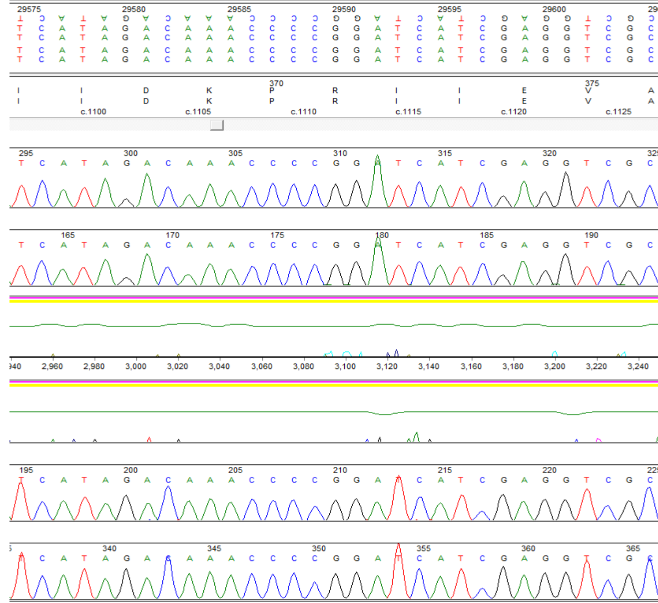
Father



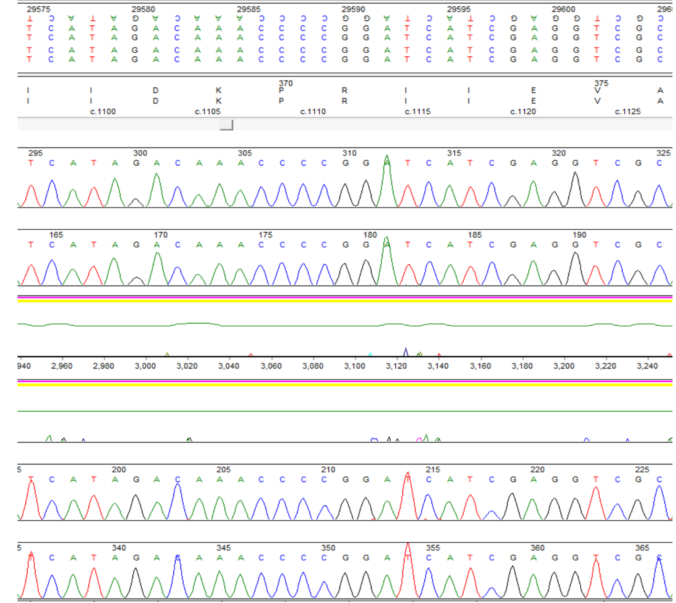
E

Individual 6

Mother



Father



F

Individual 7

Mother

Father

G

Individual 8

H

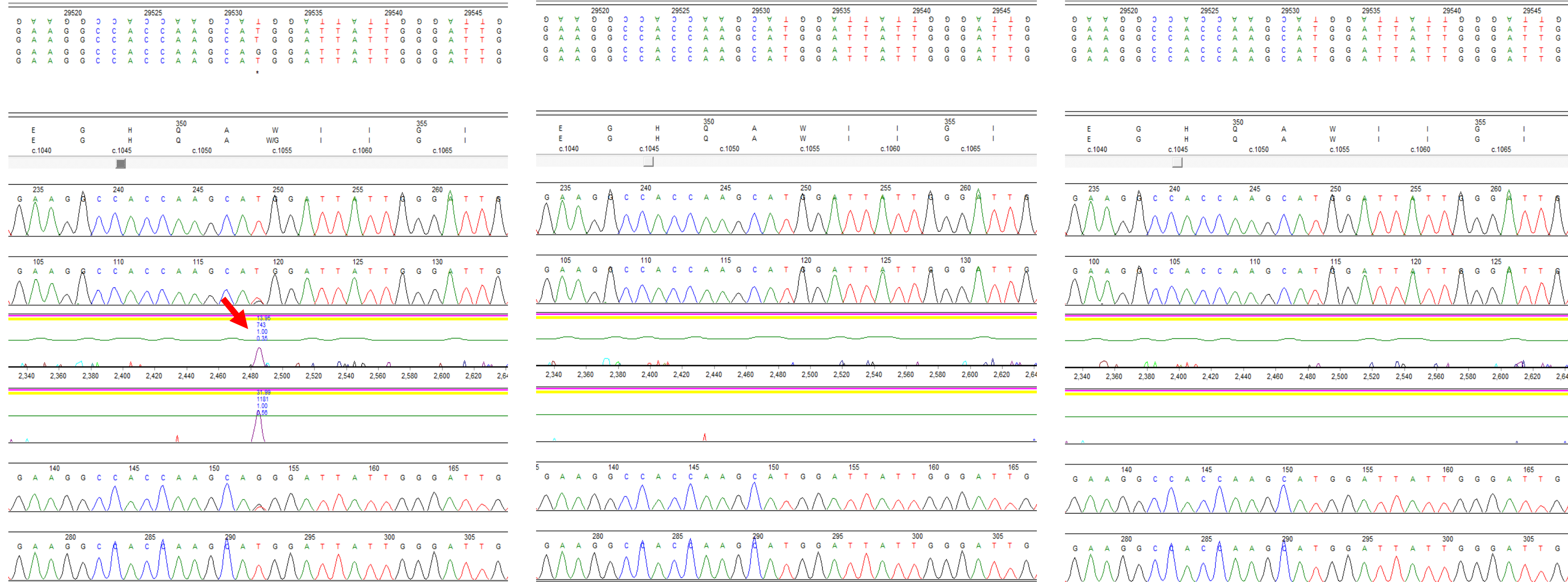


Figure S1. Sequence traces of all available individuals with variants in *SEPHS1* and their available parents (A-H). (A) Individual 1; *SEPHS1*, NM_012247.4: c.1111C>T, Arg371Trp (B) Individual 2; *SEPHS1*, NM_012247.4: c.1111C>T, Arg371Trp (C) Individual 3; *SEPHS1*, NM_012247.4: c.1111C>T, Arg371Trp (D) Individual 4; *SEPHS1*, NM_012247.4: c.1112G>A, p.Arg371Gln (E) Individual 6; *SEPHS1*, NM_012247.4: c.1112G>A, p.Arg371Gln (F) Individual 7; *SEPHS1*, NM_012247.4: c.1111C>G, p.Arg371Gly (G) Individual 8; *SEPHS1*, NM_012247.4: c.1111C>G, p.Arg371Gly (H) *SEPHS1*, NM_012247.4: c.1054T>G, p.Trp352Gly

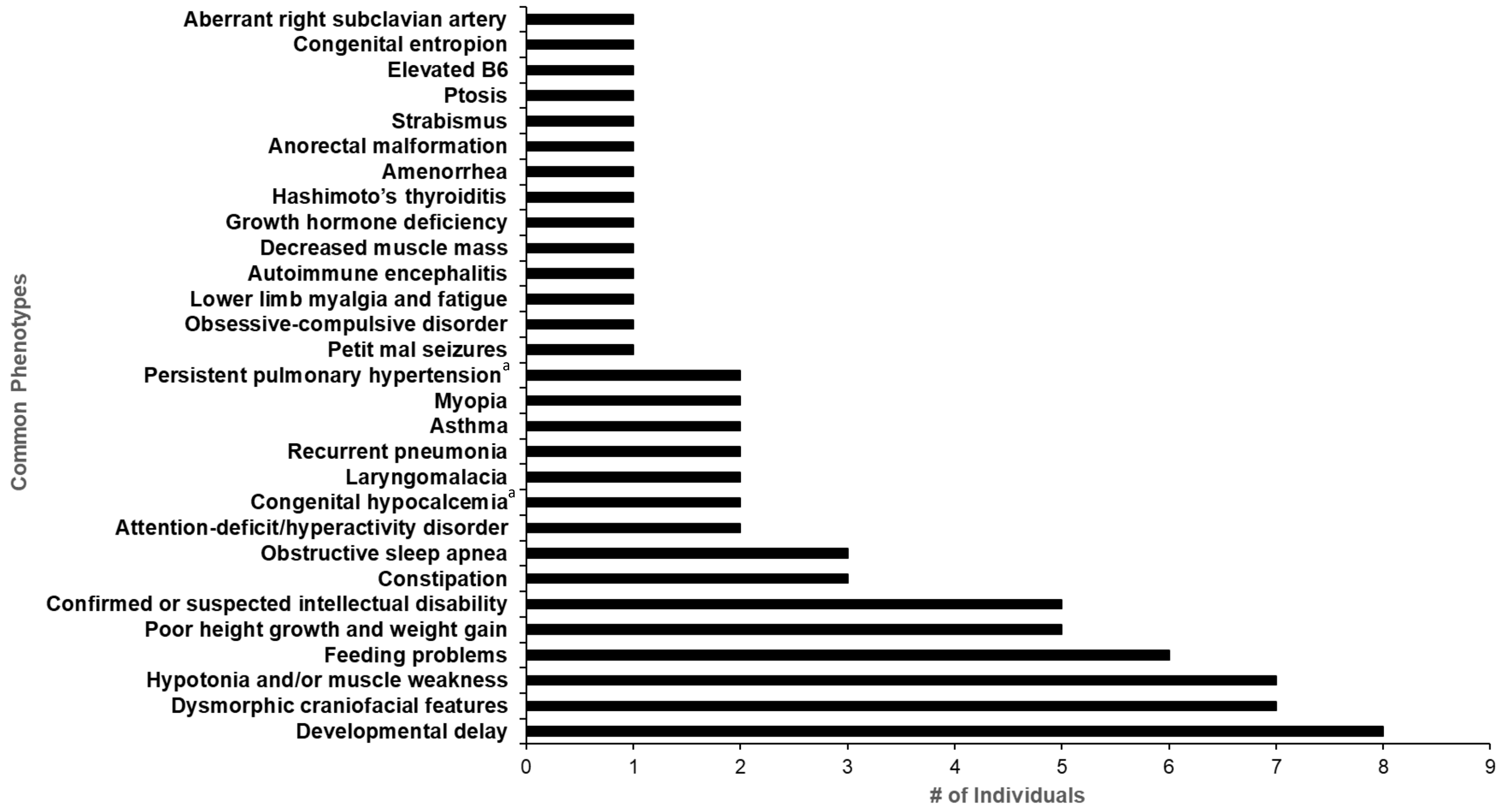


Figure S2. Phenotypes present in individuals with *SEPHS1* variants

[a]These individuals are siblings.

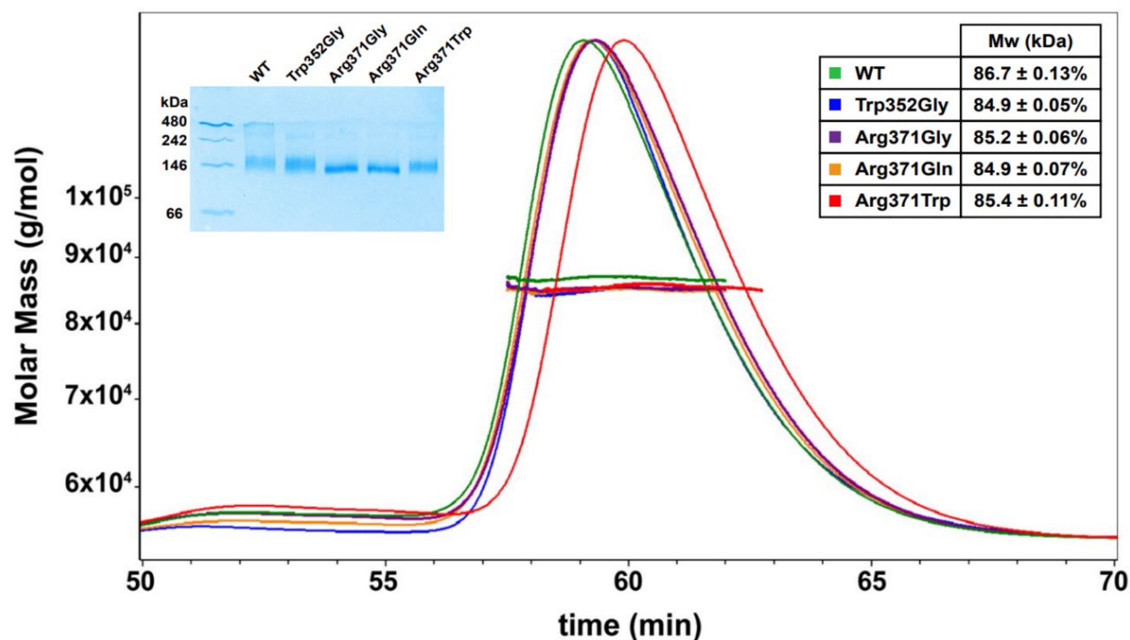


Figure S3. Native-PAGE and SEC-MALS of SEPHS1 samples. **Figure S3. Native-PAGE and SEC-MALS of SEPHS1 samples.** Accurate molecular weight and size was determined for WT, Trp352Gly, Arg371Gly, Arg371Gln, and Arg371Trp. Light scattering curves are plotted against time and molecular weight is plotted across each peak. All samples had an average polydispersity of 1.0 ± 0.001%.

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6	Individual 9
Eyes		Deep set palpebral fissures with mild asymmetry	Deep set eyes, dark and thick lashes, horizontal palpebral fissures		Heavy eyebrows with a lateral flare	Long lashes, ptosis	Mildly arched eyebrows with long lashes; entropion; bilateral ptosis requiring surgery
Ears	Cupped, asymmetric, low set, hypoplastic with bilateral preauricular pits		Small, cupped	Mildly dysplastic, thick helix, Stahl's Ear	Ears are small with poor development of the superior helix	Low set, cupped, fleshy helices	
Nose	Broad nasal root			Slightly prominent nose	Prominent nasal bridge, nasal turbinate reduction	Overhanging columella	Mildly bulbous nasal tip
Mouth	Ankyloglossia	Ankyloglossia	Small widely spaced teeth	Mild bifid uvula	Ankyloglossia; thin vermillion	Abnormal philtrum, thin lips	Ankyloglossia

Table S1. Description of facial features of the seven individuals

Target	Forward (5' - 3')	Reverse (5' - 3')
GPX1	CAG TCG GTG TAT GCC TTC TC	GCT CGT TCA TCT GGG TGT AG
MSRB1	CAC ACT GTC GGC TGA CTT AG	GAG GGT GTA ACG TCA TTC AGA G
SELENOW	TCC AGG TGG GAG GTT AGT	CCT CAA GCG CCA CAA TAA AC
SELENOT	CGT GCC CAG CAA GAG ATT A	CCG CAT GTA CTC CTC AAA CA
GSTO1	TCA TCT GGC CCT GGT TTG	GCT GAG ACT GTG GGA TCT TC
PRDX1	CCC ACG GAG ATC ATT GCT T	GTC CCA GTC CTC CTT GTT TC
GSTA4	CCC GAA GCA TTC TCC ACT AC	CCC TCC ACG TAC ATG TCA ATC
SOD3	AGG TCT CAC CTT CGC CTT TG	TCA GAC CTA CTG AGT GGG GG
PolB	ACC GAC ATG CTC ACA GAA C	AGC TTC AGC TCC ACT CTT TAT T

Table S2. Real-Time Quantitative Reverse Transcription PCR primer sequences for mRNA expression analysis

Materials and Methods

IRB

This study was conducted under GeneDx's research protocol ''Research to Expand the Understanding of Genetic Variants: Clinical and Genetic Correlations", approved by the Western Institutional Review Board (IRB) (protocol 20171030). All research subjects provided written consent to participate through either GeneDx's research protocol or as required by their clinical institution.

Exome Sequencing

For Individuals 1-4 and 7-9, we used genomic DNA from the proband, parents and affected sibling (when available). The exonic regions and flanking splice junctions of the genome were captured using the IDT xGen Exome Research Panel v1.0 (Integrated DNA Technologies, Coralville, IA). Massively parallel (NextGen) sequencing was done on an Illumina system with 100bp or greater paired-end reads. Reads were aligned to human genome build GRCh37/UCSC hg19, and analyzed for sequence variants using a custom-developed analysis tool. Reported variants were confirmed, if necessary, by an appropriate orthogonal method in the proband and, if submitted, in selected relatives. Additional sequencing technology and variant interpretation protocol has been previously described[1]. The general assertion criteria for variant classification are publicly available on the GeneDx ClinVar submission page (http://www.ncbi.nlm.nih.gov/clinvar/submitters/26957/)

For Individual 5, exome sequencing was performed as previously described[2]. Briefly, Whole exome sequencing (WES) is performed using genomic DNA from the submitted sample, prepared using the Kapa or TruSeq library prep. Samples are enriched using IDT xGen exome research panel v 1.0 and sequenced to a minimum of 7 Gb of 2x125 paired end reads for a mean of 80x average coverage or greater on the Illumina NOVA Seq. Bidirectional sequence is assembled, aligned to reference gene sequences based on human genome build GRCh37/UCSC hg19, and analyzed using custom-developed software.

Individual 6 used genomic DNA from the proband and unaffected parents. Exonic DNA was selected using the xGen Exome Research Panel with custom-designed capture probes (Integrated DNA Technologies, Coralville, IA). Sequencing was performed using the Illumina sequencing system with 2 x 150bp chemistry. Read alignment, variant calling, annotation, and analysis of sequencing data were done as outlined for previous FORGE and Care4Rare Canada projects [3].

Plasmid Construction

The *SEPHS1* was cloned from plasmid pANT7-SEPHS1, a kind gift from Dr. Christian Baron from the University of Montreal. The *SEPHS1* coding sequence was inserted into the pET15b expression vector (Novagen 69661) to generate pET15b-SPS1. Site-

directed mutagenesis was performed on pET15b-SPS1 to generate expression plasmids for each mutant (Trp352Gly, Arg371Gly, Arg371Gln, Arg371Trp).

Protein Expression and Purification

Recombinant SEPHS1 possessing an N-terminal 6xHis tag was produced in *E. coli* strain OverExpress C41(DE3) (Sigma-Aldrich). Cells were grown at +37ºC shaking until reaching an OD_{600} ~0.6-0.8. To induce protein expression, isopropyl B-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5mM. All proteins were produced for 18 hours at +18ºC shaking at 180 rpm. Cells were then pelleted and resuspended in lysis buffer (50mM HEPES pH 7.5, 300mM NaCl, 1mM DTT, 5% glycerol) containing an EDTA-free protease inhibitor tablet (Roche). Cells were lysed via sonication on ice and the cell lysate was clarified at 27,000*g* for 45min at +4ºC. Clarified lysate was loaded directly onto a His-Trap FF Crude 5 mL column (Cytiva) and washed with lysis buffer containing 20mM imidazole. Recombinant SEPHS1 was eluted from the column with lysis buffer containing 350mM imidazole. Samples were further purified by size-exclusion chromatography (SEC) using a Superose 6 Increase 10/300 column GL (Cytiva). Fractions containing SEPHS1 were pooled and concentrated in storage buffer (20 mM HEPES pH 7.5, 200mM NaCl, 0.5mM TCEP, 5% Glycerol). Final samples were flash-frozen in liquid nitrogen and stored at -80ºC.

Native PAGE

For each SEPHS1 protein, 2µg of recombinant protein was loaded onto a 4% stacking and 10% resolving non-denaturing polyacrylamide gel. Gels were run in the NativePage Cathode Buffer (Invitrogen). Gels were stained with Coomassie R-250, destained, and imaged with the Gel Doc XR+ system (Bio-Rad).

ATPase Assay

Reactions were set up as follows: 15 µM SEPHS1, 50mM HEPES-KOH pH 7.5, 50mM KCl, 5mM MgCl2, 1mM DTT, 1mM ATP. Reactions were incubated at 37°C for 18 hours. ATP availability was measured at 0, 3, 6, and 18 hours. Reactions were stopped by heat denaturation, centrifuged at 16,000*g*, and either used immediately in the assay or flash frozen and stored at -80°C. ATP was quantified using the CellTiterGlo 2.0 assay kit (Promega). Samples were diluted 1000-fold and 100µL of diluted sample was added to 100µL of CellTiter-Glo Reagent in opaque white 96-well plates. The plate was incubated for 10 minutes at room temperature and measured in the FLUOstar Omega plate reader (BMG Labtech). ATP standards were prepared from 1pM - 10 µM to generate a standard curve. Total ATP concentration was interpolated from the generated standard curve.

Thermal Shift Assays

Initial fluorescence values and thermal unfolding profiles of recombinant SEPHS1 samples were measured using the Tycho NT.6 (Nanotemper). Total initial fluorescence was calculated using the combined sample fluorescence at 260nm and 280nm. Thermal unfolding profiles were generated by monitoring the change in fluorescence at 260nm and 280nm while samples were gradually heated up to 95°C. Normalized fluorescence (260nm) was plotted against temperature to generate the final curves. Melting temperature was determined using the inflection point of each profile. Data analysis was performed in GraphPad Prism version 9.2.0 (GraphPad Software, San Diego, California USA, www.graphpad.com). Statistical significance between samples was determined using one-way ANOVA followed by Tukey's multiple comparisons test.

Limited Proteolysis

Proteolysis was carried out in SEPHS1 storage buffer using recombinant SEPHS1 samples at a final concentration of 0.5mg/ml. Trypsin or Chymotrypsin proteases were added to the reactions at a final concentration of 10 ug/ml. Reactions were incubated for 30 minutes at 30°C for trypsin and 37°C for chymotrypsin. 2µg of total digested protein was analyzed with SDS-PAGE using 4-20% TGX precast gels (Bio-Rad). Gels were imaged with the Imager 2000 (Bio-Rad).

Size-Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS)

SEC was performed in SEPHS1 storage buffer without glycerol where 250µL of recombination SEPHS1 at 1mg/ml was injected into the HPLC system (Shimadzu) and run over a Superdex s200 Increase 10/300 GL column (Cytiva). Directly following SEC, molecular weight was determined using the DAWN multi-angle light scattering (MALS) detector in combination with the OptiLab dRI detector (Wyatt Technology). Baseline detection, signal alignment, peak picking, and molecular weight determination was performed using Astra 8.0 (Wyatt Technology).

Cell Culture

Human neuroblastoma SH-SY5Y cells were obtained from American Type Culture Collection (ATCC, CRL-2266) and grown in DMEM containing 10% FBS, 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37ºC.

Lentivirus-mediated Gene Overexpression

The 3XFLAG tagged *SEPHS1* cDNA of wild type and mutants were PCR amplified and cloned into the XbaI/EcoRI sites of lentivirus expression vector pLV-tetO-CMV-SV40-PuroLoxP through Gibson assembly, as previously described [4]. To produce infectious lentivirus, each construct was transfected into 293FT packaging cells together with the

lentiviral packaging plasmids using lipofectamine 2000 (Invitrogen). The empty vector was used as a negative control. The 293FT cells were cultured for 48 h and the virus-containing medium were collected, centrifuge-clarified and stored in aliquots at -80°C. lentiviral transduction of SH-SY5Y cells was performed in the presence of 4 μg/ml polybrene at MOI of 1.0. The medium was changed 24 h later, and the cells were cultured for an additional 48 h prior to further selection with 2 μg/ml puromycin for 3 days. Stable transfectants from a mixed population were used in the experiments.

Co-immunoprecipitation and Western Blot Analysis
Trypsinized SH-SY5Y cells were pelleted, washed with ice-cold phosphate-buffered saline (PBS), and then lysed in cell lysis buffer (Cell Signaling Technology, Cat #: 9803; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40) containing 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and additional protease Inhibitor cocktail (Sigma-Aldrich, P8340). Cell lysates were centrifuged at 16,000x g at 4°C for 10 min and protein concentration was determined using the BCA assay (Thermo Scientific).

For co-immunoprecipitation, the supernatants containing equal amounts of proteins were added to 1:1 Protein A and G Dyna beads (Thermo Fisher Scientific) mixture and incubated with anti-FLAG M2 antibody (Sigma Aldrich, F1804) by rotation overnight at 4°C. The bead-antibody-protein complex was washed thrice with lysis buffer. After the final washing, the beads were resuspended and boiled in Laemmli buffer before western blot analysis was performed.

For western blots, proteins were resolved on 4-20% Criterion TGX precast SDS-PAGE gels (Bio-Rad) and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were blocked for 1 h at room temperature with Odyssey blocking buffer (LI-COR) and incubated overnight at 4°C with primary antibodies. After incubation with IRDye 680/800-conjugated secondary antibodies, the membranes were scanned using an Odyssey infrared imaging system (LI-COR). The following primary antibodies were used: anti-SEPHS1 (Abcam, ab96542), anti-FLAG M2 (Sigma Aldrich, F1804) and anti-αTubulin (Genscript, A01622).

Cell Viability Assay
The alamar Blue viability assay was performed to assess the effect of *SEPHS1* mutants on cell viability. SH-SY5Y cells were seeded (1×10^4 cells/well) in Corning Costar 96-well, cell culture-treated, flat-bottom microplates (Day 0) and allowed to recover overnight at 37°C with 5% CO_2. For growth curve detection, 10 μL of alamarBlue HS cell viability reagent (Invitrogen, A50100) was added to the wells containing 100 μL of complete growth media and the attached cells. Cell plates were incubated at 37°C for exactly 2 hours prior to reading the fluorescence on days 1 - 7. Fluorescence was measured with excitation and emission values of 560 nm and 590 nm, respectively, using microplate reader Tecan infinite M200. Readings relative to Day 1 (100%) were used to plot against time.

Intracellular ROS Detection
Intracellular ROS levels were determined by using CellROX Deep Red Flow Cytometry Assay Kit (Thermo Scientific). Briefly, SH-SY5Y cells were treated with PBS or the common inducer of ROS production tert-butyl hydroperoxide (TBHP, 200 μM) for 30 minutes before labeling with the CellROX Deep Red reagent. The SYTOX Blue Dead Cell stain was further used to differentiate live cells from dead cells. Stained samples were analyzed by flow cytometry using Becton Dickinson LSRFortessa cytometer and FlowJo software (v10.9.0).

RNA Extraction and Quantitative Real-time PCR (qRT-PCR)
Total RNA was isolated using TRI reagent (Sigma-Aldrich, T9424). For gene expression analyses, 2.5 μg of RNA was reverse transcribed using iScript gDNA Synthesis Kit (Bio-Rad) in a 20 μl reaction mixture. The cDNA product was amplified using Sso Advanced SYBR Green Supermix (Bio-Rad) with gene-specific primers (Table S2). The PCR plates were denatured for 3 min at 95 °C and then subjected to 40 cycles of 10 s at 95 °C and 30 s at 60 °C in a CFX96 real-time PCR detection system (Bio-Rad). PCR products were subjected to melting curve analysis to assure that a single amplification product was produced. Quantification of relative changes in mRNA level was determined by the ΔΔCt method (normalized to DNA polymerase beta, POLB) and expressed as the fold change between experimental and control samples.

Statistical Analysis
Data analysis was performed in GraphPad Prism version 9.5.1 (GraphPad Software, San Diego, California USA, www.graphpad.com). For multiple group experiments, ANOVA was used with Dunnett's multiple comparisons test for group-wise comparison. P values > 0.05 were not statistically significant (ns).

References:

1. Retterer, K., Juusola, J., Cho, M.T., Vitazka, P., Millan, F., Gibellini, F., Vertino-Bell, A., Smaoui, N., Neidich, J., Monaghan, K.G., et al. (2016). Clinical application of whole-exome sequencing across clinical indications. Genet Med *18*, 696-704. 10.1038/gim.2015.148.
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4. Yang, J., Tian, B., Sun, H., Garofalo, R.P., and Brasier, A.R. (2017). Epigenetic silencing of IRF1 dysregulates type III interferon responses to respiratory virus infection in epithelial to mesenchymal transition. Nat Microbiol *2*, 17086. 10.1038/nmicrobiol.2017.86.