

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

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***SEPHS1* is in the selenocysteine biosynthetic pathway. We report nine individuals from eight families with developmental delay, growth and feeding problems, hypotonia, and dysmorphic features, all with heterozygous missense variants in *SEPHS1*. Our findings provide insight into the molecular pathogenesis of this neurodevelopmental disorder.**



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

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Summary

Selenophosphate synthetase (SEPHS) plays an essential role in selenium metabolism. Two mammalian SEPHS paralogues, SEPHS1 and SEPHS2, share high sequence identity and structural homology with SEPHS. Here, we report nine individuals from eight families with developmental delay, growth and feeding problems, hypotonia, and dysmorphic features, all with heterozygous missense variants in SEPHS1. Eight of these individuals had a recurrent variant at amino acid position 371 of SEPHS1 (p.Arg371Trp, p.Arg371Gln, and p.Arg371Gly); seven of these variants were known to be *de novo*. Structural modeling and biochemical assays were used to understand the effect of these variants on SEPHS1 function. We found that a variant at residue Trp352 results in local structural changes of the C-terminal region of SEPHS1 that decrease the overall thermal stability of the enzyme. In contrast, variants of a solvent-exposed residue Arg371 do not impact enzyme stability and folding but could modulate direct protein-protein interactions of SEPHS1 with cellular factors in promoting cell proliferation and development. In neuronal SH-SY5Y cells, we assessed the impact of SEPHS1 variants on cell proliferation and ROS production and investigated the mRNA expression levels of genes encoding stress-related selenoproteins. Our findings provided evidence that the identified SEPHS1 variants enhance cell proliferation by modulating ROS homeostasis. Our study supports the hypothesis that SEPHS1 plays a critical role during human development and provides a basis for further investigation into the molecular mechanisms employed by SEPHS1. Furthermore, our data suggest that variants in SEPHS1 are associated with a neurodevelopmental disorder.

Selenium (Se) is the sole essential trace element to be specified in the genetic code¹ and is incorporated into proteins as the nonstandard amino acid selenocysteine (Sec) which is encoded by the UGA codon.^{2–9} The human genome encodes 25 selenoproteins, many of which are involved in metabolism, maintenance of the cellular redox environment, and oxidative stress management.^{10–13} The Sec

biosynthesis machinery is responsible for generating Sec-tRNA^{Sec} and delivering it to the ribosome for incorporation into the nascent polypeptide chain.^{2–4} The primary Se donor for Sec biosynthesis is provided by selenophosphate synthetase (SEPHS). Using ATP and inorganic Se as substrates, SEPHS cleaves and transfers the γ -phosphate of ATP onto selenide to form mono-selenophosphate (SeP).

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SeP is then delivered to *O*-phosphoseryl selenium transferase (SEPSECS) for the terminal reaction of Sec synthesis and subsequent incorporation into selenoproteins.⁷

Impairment in the Sec pathway has been associated with diverse human diseases including cardiovascular, central nervous, and endocrine system disorders, cancer, and pregnancy complications (miscarriage, premature birth, and preeclampsia).^{1,6–13} Currently, three genes in the Sec biosynthesis pathway have been identified to cause autosomal-recessive disease: *SECISBP2* (MIM: 607693), *SEPSECS* (MIM: 613009), and *TRU-TCA1-1* (MIM: 620198).^{10,12,14–17} Defects in *SECISBP2* are associated with an abnormal thyroid hormone metabolism disorder (MIM: 609698), which is characterized by multiorgan defects including abnormal thyroid hormone metabolism, myopathy, hearing loss, and male infertility.¹⁵ *SEPSECS* pathogenic variants are associated with pontocerebellar hypoplasia type 2D (MIM: 613811) which is characterized by progressive microcephaly, postnatal onset of progressive atrophy of the cerebrum and cerebellum, profound intellectual delay, spasticity, and variable seizures.^{14,18} Pathogenic variants in *TRU-TCA1-1* are associated with autosomal-recessive thyroid hormone metabolism, abnormal 3 (MIM: 165060) which is characterized by euthyroid hyperthyroxinemia, with elevated free T4 and reverse T3 levels and normal TSH (MIM: 188540) and free T3 levels.^{16,17} Affected individuals also show low plasma selenium levels and reduced levels of stress-related selenoproteins.^{16,17}

In the human Sec biosynthesis pathway, there are two *SEPHS* genes encoding *SEPHS1* and *SEPHS2* that both share a high degree of sequence identity and structural homology. However, it has been established that *SEPHS2* catalyzes SeP synthesis.^{19,20} Due to the implications of the Sec biosynthesis pathway to human disease, variants in *SEPHS1* (MIM: 600902) or *SEPHS2* (MIM: 606218) may be linked to a genetic disorder. Interestingly, these two genes have yet to be implicated in human disease in OMIM.

SEPHS1 is an essential protein in mammals and is involved in promoting cell proliferation, differentiation, and survival.^{21–24} *SEPHS1* has been found to modulate the cellular redox environment and promote cell migration, adhesion, and invasion.^{21,24} It is highly expressed in hepatocytes and thyroid glandular cells. The gene is ubiquitously expressed in the brain but has no region specificity.^{21,25} In *Drosophila melanogaster*, *Sps1* is highly expressed during embryonic development and is prevalent in regions of the embryo undergoing rapid cell proliferation and differentiation.²⁶

Given the fact that *SEPHS1* does not directly participate in generating SeP, it is reasonable to propose that *SEPHS1* plays a critical role in cellular processes outside of the Sec biosynthetic pathway. It has been found *SEPHS1* modulates redox homeostasis and is responsible for cell proliferation and defense.^{19,20,25} However, the homeostatic mechanism by which this protein exerts its function is still poorly understood.

Sephs1 knockout mice embryos showed gradual increases in reactive oxygen species (ROS) resulting in growth retardation, structural brain abnormalities, and disrupted cardiac development.^{23,27} Therefore, it is likely that in humans, *SEPHS1* is an essential gene and pathogenic variants may cause a human phenotype resembling other disorders in the Sec biosynthesis pathway.

In this study, nine individuals were identified to have heterozygous missense variants in *SEPHS1* by clinical exome sequencing (ES) (see [supplemental material and methods](#)). They were evaluated by clinical genetic centers in the United States and Canada via clinical exam, laboratory tests, and brain imaging. Clinical data were obtained from the providers via questionnaire. This study was approved by the institutional review board (IRB) (see [supplemental material and methods](#)). All parents or legal guardians provided written informed consent for their children to participate in the study and for publication of clinical information. Seven individuals (individuals 1–4 and 7–9) had ES performed by GeneDx. Individual 5 had ES performed at a pediatric medical center and was ascertained via personal communication. Individual 6 had ES performed through Care4Rare and was ascertained via GeneMatcher.²⁸

Eight individuals (individuals 1–8) had variants at the same amino acid position, p.Arg371 ([Table 1](#)). Individuals 1–3 shared a recurrent variant in *SEPHS1* (GenBank: NM_012247.4: c.1111C>T [p.Arg371Trp]). This variant was *de novo* in individuals 1 and 2, but parental testing for individual 3 was not available ([Figures S1A and S1B](#)). Individuals 4–6 shared a different missense variant at the same residue in *SEPHS1* (GenBank: NM_012247.4: c.1112G>A [p.Arg371Gln]). This variant was *de novo* in individuals 4–6 ([Figures S1D and S1E](#)). Individuals 7 and 8 were similarly affected siblings who shared a third unique missense variant at the p.Arg371 residue in *SEPHS1* (GenBank: NM_012247.4: c.1111C>G [p.Arg371Gly]) ([Figures S1F and S1G](#)). This variant was *de novo* in the siblings, suggesting the possibility of parental germline mosaicism in this family. In addition, individual 7 had a *de novo* *SRCAP* (MIM: 611421) variant, GenBank: NM_006662.2 (c.3833C>A [p.Ser1278*]), that was classified as a variant of uncertain significance (VUS).²⁹ Individual 9 had a *de novo* missense variant at a different residue in *SEPHS1* (GenBank: NM_012247.4: c.1054T>G [p.Trp352Gly]) ([Figure S1H](#)). All variants were located within exon 9 of *SEPHS1* and located in/near the region encoding the AIR synthase-related protein, C-terminal domain of *SEPHS1* ([Figure 1A](#)). None of these variants were reported in Genome Aggregation Database v.4.0.0 (gnomAD). *SEPHS1* contains fewer missense variants than expected (*Z* score = 3.24) and is considered intolerant to loss of function (pLI = 0.98).³⁰ The gene has a relatively %HI score implying that it is likely that variants within this gene operate through a mechanism of haploinsufficiency.³¹ The *in silico* prediction tool Provean predicts each of the identified missense changes to have a deleterious effect on *SEPHS1* ([Table 1](#)).

Table 1. Molecular findings in individuals with heterozygous missense *SEPHS1* variants

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6	Individual 7	Individual 8	Individual 9
<i>SEPHS1</i> variant (NM_012247.4)	c.1111C>T	c.1111C>T	c.1111C>T	c.1112G>A	c.1112G>A	c.1112G>A	c.1111C>G	c.1111C>G	c.1054T>G
Exon	exon 9	exon 9	exon 9	exon 9	exon 9	exon 9	exon 9	exon 9	exon 9
Protein	p.Arg371Trp	p.Arg371Trp	p.Arg371Trp	p.Arg371Gln	p.Arg371Gln	p.Arg371Gln	p.Arg371Gly	p.Arg371Gly	p.Trp352Gly
Inheritance	<i>de novo</i>	<i>de novo</i>	unknown	<i>de novo</i>	<i>de novo</i>	<i>de novo</i>	<i>de novo</i>	<i>de novo</i>	<i>de novo</i>
PROVEAN (cutoff 2.5)	−3.75	−3.75	−3.75	−1.15	−1.15	−1.15	−3.65	−3.65	−10.49
GnomAD frequency	0	0	0	0	0	0	0	0	0
Reported CNV and SNVs	none reported	none reported	CMA: gain of chr13q14.1 (VUS, unknown inheritance) ^a	none reported	none reported	CMA: gain of chr3p25.3 (VUS, maternally inherited) ^a	<i>de novo</i> VUS in <i>SRCAP</i> , NM_006662.2: c.3833C>A (p.Ser1278*) ²⁹	none reported	heteroplasmic variant in <i>MT-TL2</i> ; heterozygous VUS <i>RAPSN</i> ^a
ACMG criteria applied ³³	internal criteria, PM2, PP3	internal criteria, PM2, PP3	internal criteria, PM2, PP3	internal criteria, PS2, PM2, BP4	internal criteria, PS2, PM2, BP4	internal criteria, PS2, PM2, BP4	internal criteria, PM2, PP3	internal criteria, PM2, PP3	PS2, PS3, PM2, PP3
ACMG Class	PATH	PATH	PATH	PATH	PATH	PATH	PATH	PATH	PATH
ClinVar accession	SCV002103293.1	SCV002103293.1	SCV002103293.1	SCV002103295.1	SCV002103295.1	SCV002103295.1	SCV002103294.2	SCV002103294.2	SCV002103296.1

CMA, chromosomal microarray

^aNo HGVS notation available

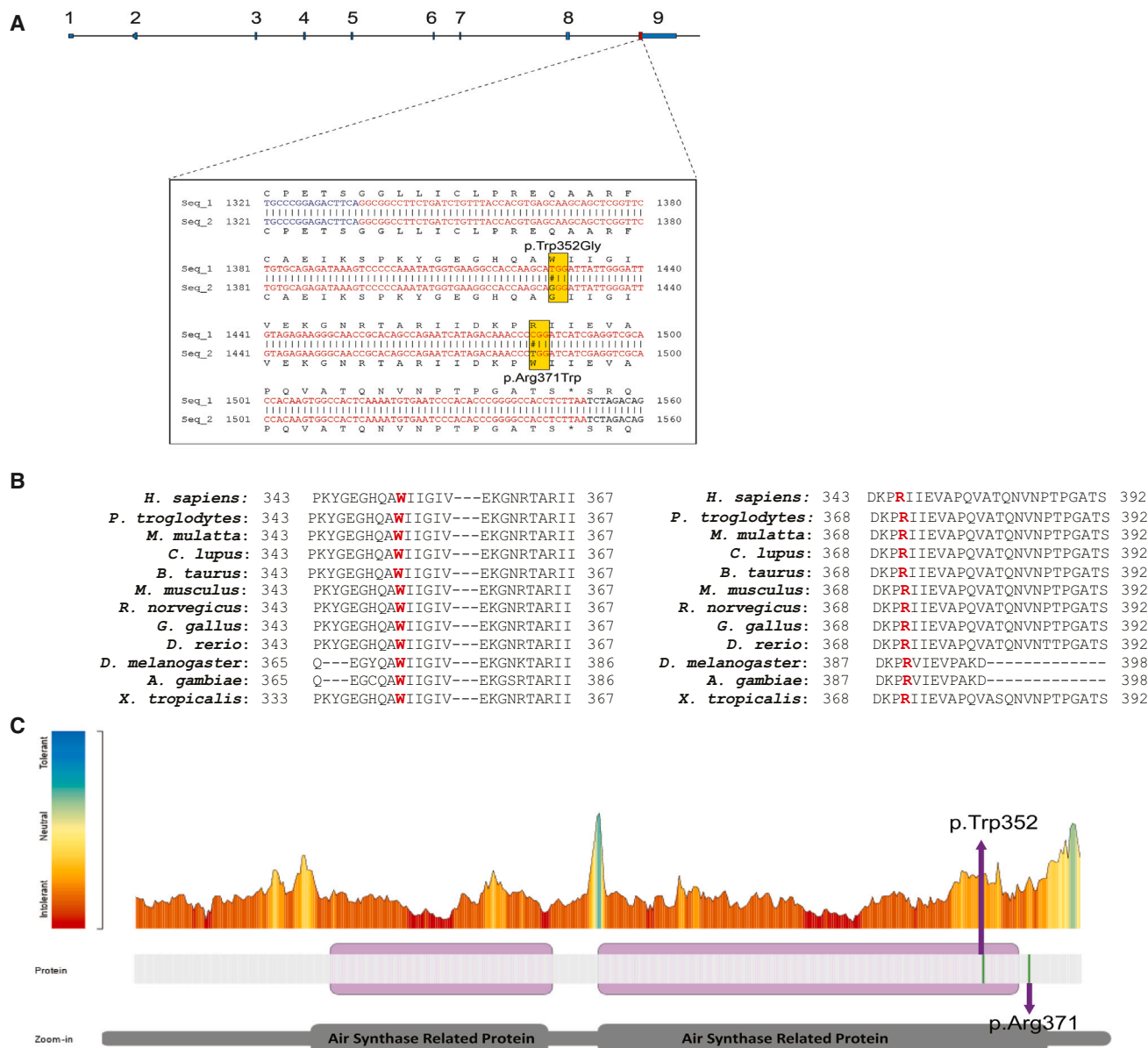


Figure 1. Identification of missense *SEPHS1* variants in nine individuals

(A) Schematic of *SEPHS1* organization. The blue boxes reflect exons. The yellow box depicts *SEPHS1* variant position.
 (B) Evolutionary conservation of missense variants observed in *SEPHS1*. Protein alignment of *SEPHS1* orthologs was performed to determine protein sequence conservation of the region of interests. Residues impacted by variants within our cohort are highlighted in red. The Trp352 and Arg371 amino acids are highly conserved from human to frog.
 (C) Tolerance Landscape of *SEPHS1*. The tolerance landscape of *SEPHS1* was generated using Metadome (<https://stuart.radboudumc.nl/metadome/>). The color in the plot is an indication for the tolerance (red, intolerant; blue, tolerant). Graphical representation of the linear protein structure of *SEPHS1* with two functional Air Synthase Related Protein domains is below the plot. Missense variants observed in this study are labeled, p.Arg371 and p.Trp352G. The missense variants in *SEPHS1* are located in regions that are intolerable for variation (red).

The amino acids at the two variant sites identified in the nine affected individuals (Trp352 and Arg371) are highly conserved throughout multiple species (Figure 1B) indicating that the tryptophan (Trp) and arginine (Arg) play an essential role in protein function. To identify the tolerance landscape of these missense variants, we utilized Metadome regional tolerance plot for genetic variation based on the ratio of observed missense and synonymous (dN/dS) variants that are included in gnomAD.³² Both

altered positions in *SEPHS1* are in regions that are intolerant to missense variants (Figure 1C).

The clinical features of the nine individuals are summarized in Tables 2 and S1 and Figure S2. The cohort is comprised of 5 females and 4 males. The ages of the cohort ranged from two days to 16 years. Individual 7 expired at two days of age due to complications of persistent pulmonary hypertension of the newborn (PPHN); therefore, most of the phenotypic comparison was made among the other

Table 2. Clinical characteristics of individuals with *SEPHS1* variants

Demographics	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6	Individual 7	Individual 8	Individual 9
Age at last evaluation	2.5 years	23 months	8 years	11 years	16 years	7.6 years	2 days	3.5 years	4 years
Neurocognitive features									
Developmental delay	+	+	+	+	+	+	NR	+	+
Intellectual disability	NR	-	+	NR	+	possible ^a	NR	+	possible ^a
Neurobehavioral diagnoses	NR	NR	NR	NR	+	+	NR	NR	-
Hypotonia and/or muscle weakness	+	+	+	-	+	+	+	NR	+
Clinically significant brain malformation on MRI	NR	NR	-	-	-	NR	NR	-	-
Other neurologic findings	-	-	+	-	+	+	NR	NR	-
Growth-related features									
History of poor height growth	+	+	-	+	+	-	-	-	+
History of poor weight gain	+	+	-	+	+	-	NR	-	+
Feeding difficulties	+	+	+	-	+	+	NR	NR	+
Additional features									
Dysmorphic craniofacial features ^b	+	+	+	+	+	+	NR	NR	+
Endocrine	-	+	NR	+	+	NR	+	+	-
Respiratory	+	-	+	+	+	-	+	+	-
Ophthalmologic	+	-	-	NR	+	+	NR	NR	+
Cardiac	-	-	-	-	+	NR	+	+	-
Gastrointestinal	+	-	+	+	-	-	NR	NR	NR

NR, data not reported
^aNo formal assessment of intellectual disability
^bDysmorphic facial features are described in [Table S1](#)

individuals. Despite the small sample size, there was a remarkably strong clinical overlap ([Table 2](#); [Figure S2](#)). The most consistent feature observed in our cohort was developmental delay (8/8; 100%), with five of those individuals known or suspected to have intellectual disability. Two of the affected individuals also had neurobehavioral diagnoses (attention-deficit/hyperactivity disorder in two; obsessive-compulsive disorder in one). Hypotonia and/or muscle weakness was reported in all but one affected individual. Additional neurologic/neuromuscular findings present in single affected individuals include petit mal seizures, lower limb myalgia and fatigue, autoimmune encephalitis, and decreased muscle mass. None of the affected individuals who underwent brain MRI (n = 5) had a major malformation. Growth was also widely impacted. Five affected individuals had a history of both

poor height growth and weight gain, four of whom had history of feeding difficulties. Two individuals of typical size also had a history of feeding problems; tube feedings were required in three affected individuals. Multiple facial dysmorphisms were observed in seven affected individuals ([Table S1](#)). Issues affecting other body systems were more variable within the cohort. Endocrine abnormalities reported in a single affected individual include Hashimoto's thyroiditis (individual 5) and elevated B6 (individual 2). Two affected individuals had growth hormone deficiency (individuals 4 and 9) and the siblings had congenital hypocalcemia (individuals 7 and 8). Respiratory issues seen in multiple affected individuals include obstructive sleep apnea (n = 3), laryngomalacia (n = 2), recurrent pneumonia (n = 2), and asthma (n = 2). Gastrointestinal issues included constipation (n = 3) and anorectal malformation

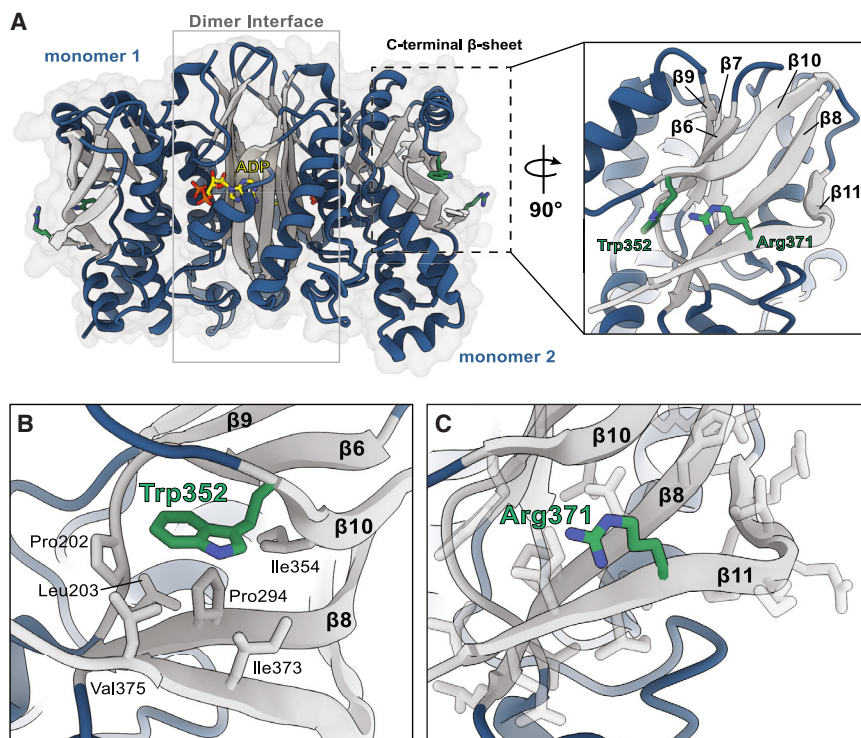


Figure 2. Structural modeling of SEPHS1
 (A) Overall structural organization of SEPHS1 (PDB: 3FD6). Interactions between the N-terminal regions of monomers 1 and 2 form a β -barrel-like structure to stabilize the homodimer and active sites. ADP is shown in yellow. Residues Trp352 and Arg371 are situated in the C-terminal β -sheet and are highlighted in green.
 (B) Trp352 resides in a hydrophobic pocket stabilized by interactions with neighboring hydrophobic residues.
 (C) Arg371 is located on the solvent-exposed face of β 11 and does not appear to form any significant interactions with surrounding residues. All modeling was performed using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

($n = 1$). Two affected individuals had myopia, and ophthalmologic issues reported in single affected individuals included strabismus, astigmatism, ptosis, and congenital entropion. Cardiac/vascular anomalies were seen in three affected individuals (aberrant right subclavian artery in one affected individual; persistent pulmonary hypertension of the newborn in the siblings), with three affected individuals having normal echocardiograms. Overall, it is likely that *de novo* variants in *SEPHS1* that disrupt SEPHS1 function result in a syndromic neurodevelopmental disorder. Suggestions for clinical care based on the medical and developmental histories of our cohort can be found in the [supplemental note](#).

To further demonstrate whether these variants in *SEPHS1* disrupt SEPHS1 function, we investigated the effect of the variants on protein function by using both biochemical assays and structural modeling. When we mapped the location of the altered residues, we observed that both residues Trp352 and Arg371 are situated within a six-stranded β -sheet in the C-terminal domain (Figure 2A). The architecture of this β -sheet is notated as $\beta 7 \uparrow \beta 9 \downarrow \beta 6 \uparrow \beta 10 \downarrow \beta 8 \uparrow \beta 11 \uparrow$ with Trp352 located on the N-terminal tail of β 10 and Arg371 found in the middle of β 11 (Figure 2A). The side chain of Trp352 makes extensive contacts with Pro202, Leu203, Pro294, Ile354, Ile373, and Val375, which contribute to the formation of a hydrophobic pocket that stabilizes three antiparallel β -strands β 8, β 10, and β 11 (Figure 2B). By contrast, the side chain of Arg371 is solvent-exposed and does not appear to participate in any interactions stabilizing the enzyme structure (Figure 2C).

Given that affected residues are far removed from the dimer interface and active sites, we sought to determine how pathogenic *SEPHS1* variants may affect overall protein stability and architecture, local conformational stability, and enzyme function. Using site-directed mutagenesis, we recapitulated the *de novo* variants (p.Trp352Gly, p.Arg371Gly, p.Arg371Gln, and p.Arg371Trp) and wild-type (WT) SEPHS1 enzymes in *E. coli*. All proteins were purified with a combination of affinity and size-exclusion chromatography. The native PAGE analysis confirmed all variants maintained the native dimer form, with the expected molecular weight of ~ 85 kDa (Figure 3A). We then employed size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) to accurately determine the molecular weight (MW) and polydispersity of all SEPHS1 samples. The average MW of all enzymes was calculated to be $85.41\% \pm 0.87\%$ kDa with a polydispersity constant of $1.0\% \pm 0.001\%$ (Figure S3), confirming that all recombinant SEPHS1 enzymes exist as dimers in solution.

Though not directly participating in the Sec biosynthetic pathway, SEPHS1 retains the ATPase activity. We investigated the possibility that pathogenic variants impact the active-site conformation and enzymatic activity. Using a luciferase-based ATPase assay, we monitored the relative change in ATP concentration over time. Our data show that WT, p.Trp352Gly, p.Arg371Gly, and p.Arg371Gln all consumed $\sim 50\%$ – 60% of the available ATP over the course of the assay (Figure 3D). The p.Arg371Trp exhibited a moderate reduction in activity consuming only $\sim 30\%$ of the total ATP, suggestive of hypomorphic properties. Ultimately, we found all SEPHS1 variants are capable of hydrolyzing ATP *in vitro*, suggesting the dimer interface and active sites are not detrimentally impacted by the variants for this function.

To investigate whether the overall protein stability was altered by the variants, we used the Nanotemper

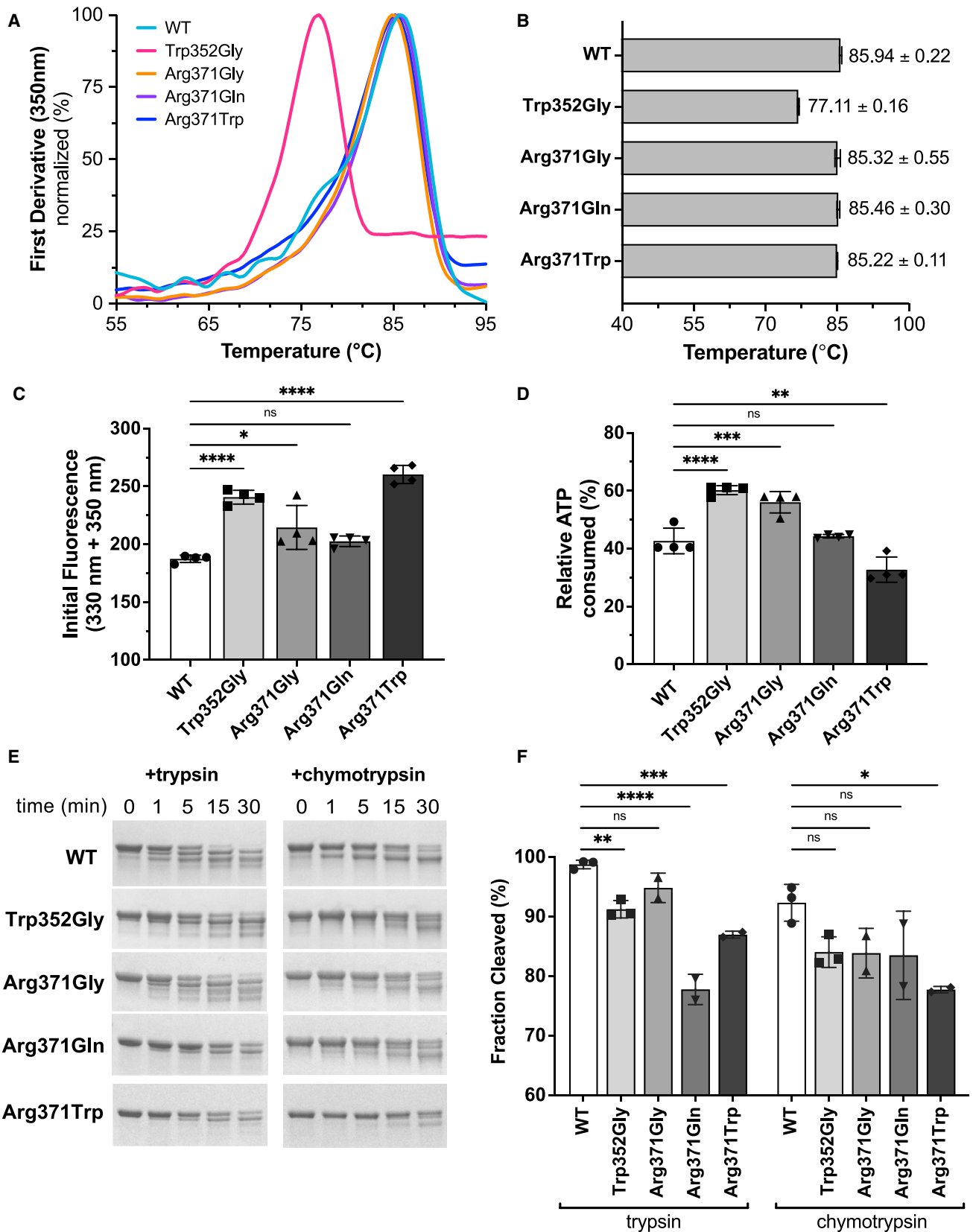


Figure 3. Analysis of SEPHS1 stability and enzymatic activity

(A) Protein thermal stability was measured using the Tycho NT.6 instrument. Intrinsic protein fluorescence was monitored as a thermal ramp was applied to each sample. The resulting curves are plotted as the first derivative and used to calculate the inflection temperature (T_i) for each sample.

(B) Calculated T_i values.

(legend continued on next page)

instrument Tycho NT.6 to monitor intrinsic fluorescence changes of each enzyme sample during heat denaturation. When comparing the inflection temperatures (T_i) of the variants to WT *SEPHS1*, we observed a substantial decrease ($\sim 10^\circ\text{C}$) in p.Trp352Gly while there was very little change with any of the Arg371 variants (Figure 3B). As this method is based on the intrinsic fluorescence of Trp residues, we expected the initial fluorescence of the p.Trp352Gly variant would decrease as a Trp residue was removed. Unexpectedly, the initial fluorescence for p.Trp352Gly was significantly higher than WT *SEPHS1* (Figure 3C). This suggests that the variant causes other Trp residues, which are normally buried in the WT protein, to be exposed in the altered enzyme. Furthermore, p.Arg371Gly exhibited a small increase in initial fluorescence, suggesting slight changes in the local environment surrounding residue 371 but not enough to reduce thermal stability of the entire protein.

These results led us to speculate that pathogenic variants may elicit local structural changes in the C-terminal region of *SEPHS1*. To probe this, we used limited proteolysis with trypsin and chymotrypsin proteases (Figure 3E). In comparison to WT, *SEPHS1* variants exhibited similar cleavage patterns for both proteases but exhibited varying degrees of proteolytic resistance. p.Trp352Gly, p.Arg371Gln, and p.Arg371Trp variants exhibited the highest resistance to proteolytic cleavage. Taken together, our results suggest that Trp352 stabilizes the C-terminal domain of *SEPHS1* and, when replaced with Gly, induces local structural changes across the 6-stranded β -sheet. While exhibiting small changes in initial fluorescence and resistance to proteolysis, most of the Arg371 variants did not have a significant impact on protein stability. Thus, we suggest Arg371 variant does not greatly compromise the structural integrity of the C-terminal β -sheet and instead may play an integral role in modulating protein-protein interactions in the cell.

To investigate the physiological functions of the *SEPHS1* variants, we introduced FLAG-tagged *SEPHS1* variants into human neuroblastoma SH-SY5Y cells through lentiviral-mediated transduction. We initiated our assessment by examining the impact of *SEPHS1* variants on cell proliferation using the alamarBlue viability assay (Figure 4A). The results indicated that the growth rate of p.Arg371Gln was comparable to that of the control and WT, while p.Trp352Gly, p.Arg371Gly, and p.Arg371Trp exhibited substantially increased cell proliferation, with p.Arg371Trp demonstrating the most pronounced effect among them.

Furthermore, immunoprecipitation using the M2 anti-FLAG antibody revealed that the dimerization capability of FLAG-tagged *SEPHS1* variants with endogenous *SEPHS1* remained unaltered (Figure 4B). These findings align with our earlier observations from bacterial recombinant expression, indicating that the identified variants would not influence *SEPHS1* functions by affecting *SEPHS1* dimerization.

Given *SEPHS1*'s pivotal role in regulating stress-related selenoproteins and maintaining redox homeostasis, we further investigated whether the identified variants influence cell proliferation through ROS production (Figure 4C). In the absence of oxidative stress stimulation, there were no significant differences in intracellular ROS production observed between the variants and WT (Figures 4C and 4D, top). Intriguingly, when the oxidant tBHP was employed in the cells to induce oxidative stress, p.Trp352Gly, p.Arg371Gly, and p.Arg371Trp exhibited a significant reduction in ROS production compared to WT. Among the variants, p.Arg371Trp displayed the most substantial reduction, while p.Arg371Gln had a marginal effect on tBHP-induced ROS production (Figures 4C and 4D, bottom).

The observed impact of *SEPHS1* variants on ROS accumulation prompted us to investigate the mRNA expression levels of genes encoding stress-related selenoproteins, specifically glutathione peroxidase 1 (*GPX1* [MIM: 138320]), glutathione peroxidase 4 (*GPX4* [MIM: 138322]), thioredoxin reductase 1 (*TXNRD1* [MIM: 601112]), selenoprotein T (*SELENOT* [MIM: 607912]), selenoprotein W (*SELENOW* [MIM: 603235]), and methionine sulfoxide reductase B1 (*MSRB1* [MIM: 606216]). Additionally, we examined the expression of genes encoding ROS-scavenging enzymes, including peroxiredoxin 1 (*PRDX1* [MIM: 176763]), glutathione S-transferase alpha 4 (*GSTA4* [MIM: 605450]), glutathione S-transferase omega 1 (*GSTO1* [MIM: 605482]), and superoxide dismutase 3 (*SOD3* [MIM: 185490]). Quantitative real-time PCR analysis revealed that the mRNA expression of *GPX1*, *MSRB1*, *SELENOW*, *SOD3*, *GPX4*, *MSRB1*, and *TXNRD1* was increased in the p.Trp352Gly, p.Arg371Gly, and p.Arg371Trp variants compared to the WT, whereas the expression of *GSTA4*, *GSTO1*, *PRDX1*, and *SELENOT* remained unaffected. Once again, the variant p.Arg371Gln exhibited little to no effect (Figure 4E).

In this study, we used ES to identify variants affecting the C-terminal region of *SEPHS1* in nine individuals with an overlapping phenotype. The variants are classified as pathogenic based on the ACMG recommendations

(C) Initial fluorescence values measured for each sample using the Tycho NT.6. All T_i and initial fluorescence values are reported with \pm SD from 4 independent runs.

(D) *SEPHS1*-mediated ATP hydrolysis monitored using the CellTiter-Glo Assay 2.0 assay kit. Relative ATP consumption was measured after 18 h of incubation at $+37^\circ\text{C}$. Assays were performed in triplicates and reported with \pm SD.

(E) Trypsin or chymotrypsin was added to *SEPHS1* samples and allowed to digest the protein for 30 min. Time points were taken at 0, 1, 5, 15, and 30 min. Digested products were analyzed on 4%–20% TGX gels. Representative gels are shown from 2 to 3 independent cleavage reactions.

(F) Densitometric quantification performed with ImageJ of total fraction cleaved after 30 min. Statistical significance for all panels was determined by one-way ANOVA with the Bonferroni correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

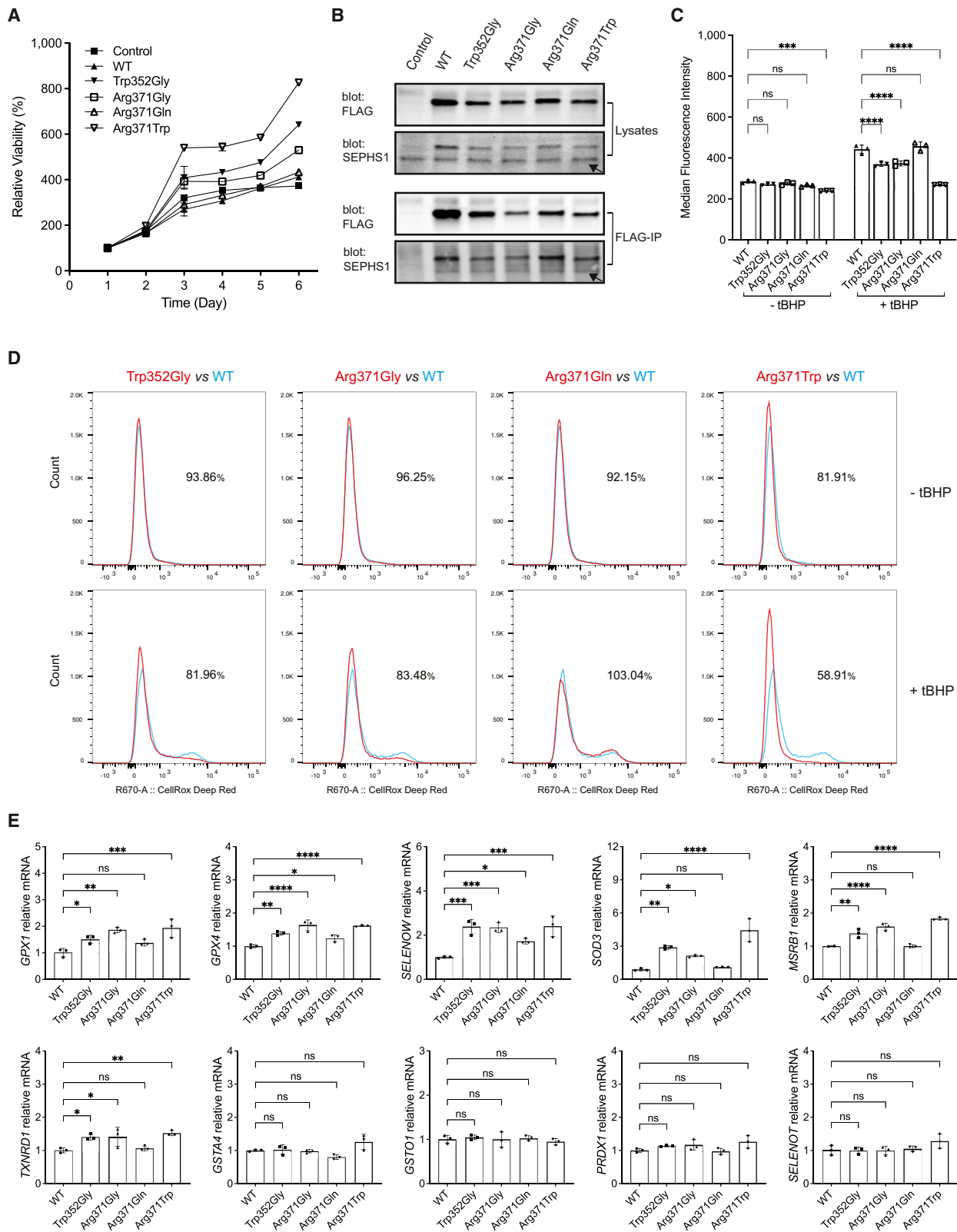


Figure 4. Analysis of SEPHS1 cellular functions in SH-SY5Y cells

(A) Cell proliferation analysis of SH-SY5Y cells overexpressing *SEPHS1* variants at the indicated days. The % live cell values were normalized to the day 1 cells (considered as 100% viable).

(B) Immunoblotting for immunoprecipitation of FLAG-tagged *SEPHS1* variants. Arrows indicate the endogenous *SEPHS1*.

(C) Fluorescence-activated cell sorting (FACS) analysis of CellROX Deep Red fluorescence in SH-SY5Y cells with or without treatment with ROS inducer tBHP, showing the quantitative bar graphs and statistical analysis of the median fluorescence intensity (MFI). Error bars derived from three independent measurements.

(legend continued on next page)

for interpreting and reporting sequence variants (Table 1).³³

Functional characterization revealed that variant p.Trp352Gly significantly reduced the overall thermal stability of the protein, due to the disruption of a network of hydrophobic interactions. When compared to WT SEPHS1, we determined that p.Trp352Gly was more resistant to proteolytic cleavage and found that the initial fluorescence of p.Trp352Gly was drastically enhanced due to the exposure of Trp residues that are usually buried in the core of the WT protein. Replacement of Trp352 with a Gly residue destabilizes a C-terminal hydrophobic pocket containing residues spanning the entire β -sheet, leading to local structural changes which may modulate protein-protein interactions in the cell. With eight out of nine individuals having Arg371 variants, it was intriguing to us that they behaved differently than p.Trp352Gly and resembled WT SEPHS1. Arg371 variants did not significantly affect protein stability or proteolytic cleavage pattern. Thus, we propose that Arg371 does not contribute to stabilization of the C-terminal region but rather likely participates in recognition and/or regulation of SEPHS1 binding partners. Our findings in neuronal SH-SY5Y cells provide evidence that the identified SEPHS1 variants could enhance cell proliferation by modulating ROS homeostasis. However, the specific impact of the p.Arg371Gln variant on cellular function remains unclear. Further investigation is needed to elucidate the precise mechanism by which SEPHS1 variants influence the expression of genes encoding stress-related selenoproteins and ROS-scavenging enzymes.

Based on the cellular data, the variants in SEPHS1 most likely act through a gain-of-function mechanism. Proper function of selenoproteins is shown to be crucial for neuronal function.^{6,9,11,13,18,34} It is conceivable that defective SEPHS1 could affect development of limbic system structure, including the hypothalamus, thalamus, basal ganglia, cingulate gyrus, hippocampus, and amygdala.^{19,20} In addition, individuals with impaired function of SEPHS1 may have neurodevelopmental deficits due to possible loss in the total number of pluripotent stem cells that survive during fetal development by an overactivation of the RAS/MAPK signaling pathway in these cells.²³ Additionally, a deficiency of growth hormone-releasing hormone and thyroid-stimulating hormone secondary to diminished hypothalamic input to the anterior pituitary gland may exacerbate the neurodevelopmental and growth delays seen in our cohort.^{35–37}

SEPHS1 has high evolutionary conservation across species, which allows for the study of the gene in various animal models.³⁷ Based on findings from the *in vitro*, mouse, and fly models, SEPHS1 plays an essential role during cell

development and proliferation and regulates cellular redox homeostasis and defense. *Seps1*-targeted knockouts in the mice and the knockout of *Sps1* mRNA in *Drosophila* show neurodevelopment being affected as well as embryonic lethality.^{23,26} Heterozygous *Sps1* variants in *Drosophila* resulted in eye phenotypes, which could coincide with the variable eye phenotypes seen in our cohort.

In mouse and human cells, knockdown of SEPHS1 suppresses cell proliferation. *Seps1* knockout mice had embryos that were underdeveloped by day E8.5 and virtually resorbed by day E14.5.²⁷ Knockdown of *Seps1* mRNA in a *Drosophila* SL2 immortalized cell line led to the inhibition of cell proliferation.²⁶ In this model, megamitochondria were formed through the inhibition of pyridoxal phosphate which is the active form of vitamin B6 and necessary for SepSecS function, among other roles. Interestingly, one individual had elevated B6 levels. Taken together, our study helps support the role of SEPHS1 in human growth and development. In our study, individuals with SEPHS1 pathogenic variants exhibit hypotonia, muscle weakness, poor weight gain, growth delays, and short stature. The phenotypes point to the role of SEPHS1 in regulating growth via modulation of ROS. In the Ras/MAPK signaling pathway, ROS are messengers for growth and gain-of-function variants in this pathway are associated with a group of disorders termed RASopathies.³⁸

To gain a deeper understanding of the potential link between SEPHS1 variants and cell proliferation via alterations in ROS homeostasis, it is imperative to conduct future experiments either in cell culture systems or animal models. These experiments will provide a more comprehensive and certain insight into the underlying mechanisms at play. By examining specific ROS homeostasis mechanisms and the signaling pathways affected by SEPHS1 variants, we can identify the precise molecular interactions responsible for the observed cellular changes. Additionally, a comparative analysis between cells with wild-type SEPHS1 and those with SEPHS1 variants will offer valuable insights into the differences in ROS regulation and cell proliferation rates, further confirming the role of these variants. Such investigations will not only advance our understanding of this neurodevelopmental disorder but also provide a foundation for the development of targeted therapeutic strategies aimed at restoring cellular homeostasis and improving patient outcomes.

Overall, individuals with pathogenic variants in SEPHS1 appear to have a neurodevelopmental and growth disorder, with variable effects on other body systems. We provide some considerations for immediate and long-term care of these individuals (see supplemental note). In conclusion, missense variants in exon 9 of SEPHS1

(D) FACS histograms showing ROS production as described in (C). The numerical values accompanying each histogram signify the percentage of MFI for SEPHS1 variants relative to the wild-type (WT).

(E) Quantitative real-time PCR for genes encoding stress-related selenoproteins and ROS-scavenging enzymes in SH-SY5Y cells harboring SEPHS1 variants. mRNA levels were normalized to *PolB*. Data are represented as mean \pm SEM, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 and ns, not statistically significant.

underlie a neurodevelopmental syndrome associated with developmental delay, growth problems, feeding difficulties, hypotonia, and dysmorphic features. Our findings provide insight into the molecular pathogenesis of this disorder.

Data and code availability

The sequence variants in *SEPHS1* (NCBI ID 22929, transcript GenBank: NM_012247.4) reported in the paper have been deposited in ClinVar database (SCV002103293.1, SCV002103295.1, SCV002103294.2, SCV002103296.1) (<https://www.ncbi.nlm.nih.gov/clinvar/>) (Table 1). Exome sequencing data were generated in diagnostic setting and can not be shared due to privacy concerns.

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Supplemental information

Supplemental information can be found online at <https://doi.org/10.1016/j.ajhg.2024.02.016>.

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Declaration of interests

S.V.M., E.T., H.G., T.B.P., A.T., D.A.C., M.M.M., I.M.W., K.G.M., and J.J. are employees of GeneDx., LLC. This article was prepared while M.S. was employed at the University of Illinois at Chicago. The opinions expressed in this article are the author's own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.

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Web resources

Clustal Omega, <https://www.ebi.ac.uk/Tools/msa/clustalo/>
DGV, <http://dgv.tcag.ca/dgv/app/home>.
GeneMatcher, <https://genematcher.org/>
gnomAD, <https://gnomad.broadinstitute.org/>
MetaDome, <https://stuart.radboudumc.nl/metadome/>
OMIM, <https://www.omim.org/>
The Human Protein Atlas, <https://www.proteinatlas.org/>
PROVEAN, <http://provean.jcvi.org/>

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