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SEPHS1: Its Evolution, Function and Roles in Development and Diseases

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Abstract

Selenophosphate synthetase (SEPHS) was originally discovered in prokaryotes as an enzyme that catalyzes selenophosphate synthesis using inorganic selenium and ATP as substrates. However, in contrast to prokaryotes, two paralogs, SEPHS1 and SEPHS2, occur in many eukaryotes. Prokaryotic SEPHS, also known as SelD, contains either cysteine (Cys) or selenocysteine (Sec) in the catalytic domain. In eukaryotes, only SEPHS2 carries out selenophosphate synthesis and contains Sec at the active site. However, SEPHS1 contains amino acids other than Sec or Cys at the catalytic position. Phylogenetic analysis of SEPHSs reveals that the ancestral SEPHS contains both selenophosphate synthesis and another unknown activity, and that SEPHS1 lost the selenophosphate synthesis activity. The three-dimensional structure of SEPHS1 suggests that its homodimer is unable to form selenophosphate, but retains ATPase activity to produce ADP and inorganic phosphate. The most prominent function of SEPHS1 is that it is implicated in the regulation of cellular redox homeostasis. Deficiency of SEPHS1 leads to the disturbance in the expression of genes involved in redox homeostasis. Different types of reactive oxygen species (ROS) are accumulated in response to SEPHS deficiency depending on cell or tissue types. The accumulation of ROS causes pleiotropic effects such as growth retardation, apoptosis, DNA

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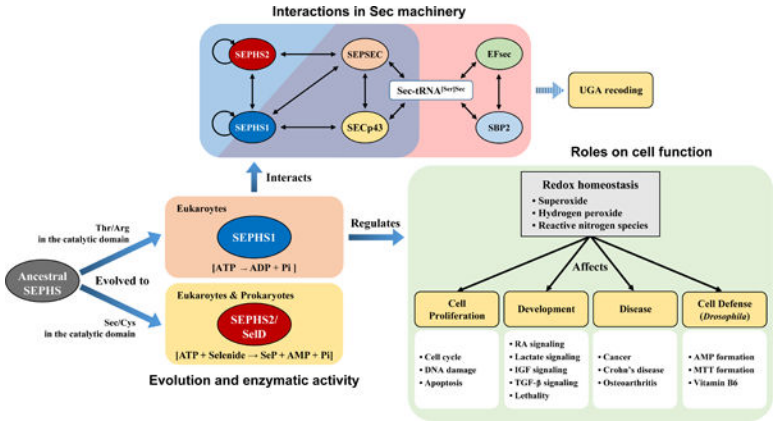
damage, and embryonic lethality. SEPHS1 deficiency in mouse embryos affects retinoic signaling and other related signaling pathways depending on the embryonal stage until the embryo dies at E11.5. Dysregulated SEPHS1 is associated with the pathogenesis of various diseases including cancer, Crohn's disease, and osteoarthritis.

Graphical Abstract



Keywords

Selenium; selenophosphate synthetase; reactive oxygen species; development; osteoarthritis; cell death

Introduction

Selenium is an essential trace element required in the diet of humans and other life forms. An adequate amount of selenium is essential for good health, since it is beneficial in preventing some forms of cancer, inhibiting viral infection and boosting the immune system. An additional important activity of this element is to support the removal of reactive oxygen species (ROS). Selenium also plays key roles in animal development and male reproduction [1, 2 and references therein]. Most of these selenium benefits are mediated by selenoproteins, which contain selenocysteine (Sec) at the active site [3–6].

Selenophosphate synthetase (SEPHS) is an enzyme that catalyzes the synthesis of selenophosphate, which is the selenium donor during selenocysteine (Sec) biosynthesis. Sec is known as the 21st amino acid that is cotranslationally inserted by recoding the UGA stop codon as a Sec codon. The proteins that contain Sec are known as selenoproteins [3, 7–9 and references therein]. Therefore, SEPHS is a key factor for the synthesis of selenoproteins and accordingly for the selenium effects in animals and humans. The overall process of selenoprotein biosynthesis has been described in detail in previous publications [10 and references therein].

The prokaryote *Escherichia coli* is capable of synthesizing three molybdenum cofactor-mediated selenocysteine-dependent formate dehydrogenases. Initially, SEPHS was discovered by analyzing *E. coli* mutants that could not synthesize formate dehydrogenase

[11]. Of the four mutants that could not synthesize either selenoproteins, *selA*, *selB*, *selC* or *selD*, only the mutant with lack of *selD* showed the defect in selenophosphate synthesis. Therefore, prokaryotic SEPHS was named SelD or SPS (selenophosphate synthetase). However, sucrose phosphate synthase in higher eukaryotes is also abbreviated as SPS. Not to be confused with this unrelated protein, selenophosphate synthetase was subsequently renamed SEPHS (selenophosphate synthetase) [10, 12]. The gene name of prokaryotic SEPHS remained as *selD*. In *Drosophila*, because there is no sucrose phosphate synthase, SPS continues to be used as an abbreviation for SEPHS [12]. In *Drosophila melanogaster*, Patufet gene (*ptuf*) was found to be a homolog of the SPS1 gene (*Sps1*), also encoding selenophosphate synthetase [13]. In eukaryotes, the first SelD homolog was cloned from a human cDNA library and identified by comparing the sequence with *E. coli selD* [14]. This eukaryotic SelD homolog was later renamed SEPHS1 because of the subsequent identification of a second SelD homolog. This second SelD homolog was identified in humans and contains Sec at the catalytic domain [15]. It was originally named SPS2, but was later renamed to SEPHS2.

The predicted chemical reaction that SelD catalyzes is as follows:

$$\text{ATP} + \text{selenide} + \text{H}_2\text{O} \rightarrow \text{selenophosphate} + \text{Pi} + \text{AMP}$$

Here, the phosphate group of selenophosphate comes from the γ-phosphate of ATP, and the ADP produced by the first reaction undergoes further hydrolysis to produce AMP and Pi [16, 17]. It was suggested that both eukaryotic SelD homologs (SEPHS1 and 2) exhibit SEPHS activity. However, later only SEPHS2 was found to have SEPHS activity [18, 19]. Interestingly, eukaryotic SEPHS2, which is considered the true SEPHS, contains both an in-frame UGA codon at the catalytic domain and a SECIS element at the 3'-UTR, suggesting that SEPHS2 is a selenoprotein itself [20, 21]. The question remained regarding the biochemical function of SEPHS1. One interesting hypothesis is that SEPHS1 plays a role in Sec recycling [22]. This hypothesis was supported by the observation that *Sephs1* complemented the *selD* mutation when the mutated *E. coli* were supplemented with Sec. On the other hand, this complementation did not occur when inorganic selenium was supplemented. Another interesting hypothesis regarding the function of SEPHS1 is that this protein plays a role in UGA recoding by assisting the transport of Sec-tRNA$^{[Ser]Sec}$ into the nucleus, where Sec-tRNA$^{[Ser]Sec}$ makes a complex with SECIS binding protein 2 (SBP2) and the Sec-tRNA specific elongation factor (EF_{Sec}) [23]. This hypothesis was supported by the observation that SEPHS1 forms a complex with Sec-tRNA$^{[Ser]Sec}$ and localizes to the nucleus. The translocated Sec-tRNA$^{[Ser]Sec}$ forms a new complex with EF_{Sec} and SBP2/mRNA, and this complex in the nucleus protects selenoprotein mRNAs from nonsense-mediated decay [24, 25]. Multimerization of Sec machinery (SEPHS1, SEPHS2, SEPSECS, and SECp43) was also observed both in vitro and in vivo suggesting SEPHS1 participates in the Sec biosynthesis pathway [26]. However, further supporting data are needed to confirm these hypotheses. The most evident and prominent function of SEPHS1 is that this protein plays a key role in the regulation of redox homeostasis. The role of SEPHS1 in the regulation of cellular redox homeostasis has been described in a previous review [10]. Since then, new findings regarding the role of SEPHS1 have been reported. For example,

1) SEPHS1 interacts with SEPHS2 and other molecules to form a heterodimer. Does this complex formation affect the function of SEPHS2 or SEPHS1? We will discuss this issue in relation to their structures; 2) Analyses of SEPHS1 evolution offer insights into its function. It was suggested that SEPHS1 and SEPHS2 evolved by duplication. This issue will be also discussed in more detail; 3) We recently discovered that SEPHS1 regulates cellular redox state in a cell-type and condition-specific manner [10, 27]; 4) Molecular and cellular effects of *Sephs1* deletion on mammalian development have been elucidated [28]; And 5) Targeted removal of *Sephs1* in human and mouse chondrocytes was implicated in the pathogenesis of cartilage tissue which led to the development of osteoarthritis [29].

Evolutionary significance of SEPHS paralogs

Emerging evidence points to the evolutionary significance of SEPHS functions. SEPHS1 and SEPHS2 have high sequence homology, especially in the conserved functional domains [10]. The most remarkable difference between these two proteins is that all metazoan SEPHS2s contain Sec in the catalytic domain, whereas SEPHS1s have a non-redox amino acid residue at the same position. Bioinformatic studies showed co-occurrence of SEPHS2 with selenoproteins and Sec biosynthesis machinery consistent with the key role of this protein in selenophosphate synthesis. In fact, biochemical experiments provided support that only SEPHS2 has selenophosphate synthesis activity [19]. However, SEPHS1 occurs even in species that have neither SEPHS2 nor other components of the selenoprotein biosynthesis machinery strongly suggesting this protein evolved to have other functions. Figure 1 shows a summarized phylogenetic tree of selected species.

Prokaryotes

Mariotti et al. examined SEPHS/SelD gene sequences from 223 prokaryotic reference genomes and found that 26% of prokaryotic genomes contain *SelD* [31]. It should be noted that prokaryotes use SelD for both selenoprotein and selenouridine biosynthesis. Among SelD-containing prokaryotes, 19%, such as *Geotalea castellanii,* have a Sec codon in the catalytic domain. The remaining 81% of SelD-containing prokaryotes have Cys at the same position. Notably, SelDs in archaea also have either a Sec codon (e.g., *M. fervens* in Fig 1) or Cys codon (*M. petrolearia*, see [10]) in the catalytic domain. As observed in lower eukaryotes, prokaryotes have fused functional domains in their SelD gene, such as the Pyr_redox_2, AIRS, AIRS_C domain (*E. fergusonii*), AIRS, AIRS_C domain (*M. ferverns*), and Cys sulfinate desulfinase (NifS)-like protein domain (see *Geotalea daltonii FRC-32* in Fig. 1) [31–34].

Algae and protists

Although selenoproteins are present in most organisms, terrestrial plants (embryophytes) and most fungi do not contain selenoproteins. However, most algae, except Rhodoplantae, contain selenoproteins and, accordingly, SEPHS2 homologues. In addition to algae, protists were also found to contain selenoproteins and Sec biosynthesis machinery [34]. *A. anophagefferens* and *M. neglectum*, which belong to the protists, have SelD(Cys) homolog; however, only *A. anophagefferens* has selenoproteins (59 selenoproteins have been identified). Therefore, the SelD homolog in *A. anophagefferens* seems to have SEPHS

activity. Interestingly, the SelD(Cys) homolog of *A. anophagefferens* contains a NADH dehydrogenase-like domain at its N-terminal region [31]. In addition, *A. castellanii* also has another functional domain (NifS-like domain) fused in its N-terminal region. Although it is not clear that the ancient SEPHS2 and SelD had redox regulation capacity or that the redox regulation function of this domain is passed to current SEPHS1 during duplication, it seems that the common ancestor of SEPHS paralogs contains a redox-regulatory domain at its N-terminus.

Insects

According to Lobanov *et al.*, who analyzed the selenoproteomes of aquatic and terrestrial arthropods, the red flour beetle, *Tribolium castaneum*, and the silkworm, *Bombyx mori,* lack any selenoprotein genes in their genomes, including *Sephs2,* but have *Sephs1* [35, 36]. These findings suggested that SEPHS1 functions in a pathway unrelated to selenophosphate biosynthesis. Later, a more detailed study on the selenoproteome and Sec biosynthesis machinery in arthropod genomes was carried out by Chapple and Guigo [37]. In addition to *T. castaneum* and *B. mori*, they found three more species, *N. vitripennis*, *A. mellifera* and *D. willistoni* that have only *Sephs1*. All these species belong to the *Endoterygota* of the *Insecta*. It is interesting that only insects show frequent extinction of selenoproteins compared to other metazoan phyla. It is unclear why selenoproteins are dispensable in those species, and why there appear to be fewer selenoproteins in insects than in higher eukaryotes such as vertebrates (Fig. 1). It is also interesting that SEPHS1 is conserved in all arthropods studied. This finding supports the notion that SEPHS1 plays a different role than SEPHS2 in selenium metabolism. Notably, most *Hymenoptera* and some *Paraneoptera* species contain a UGA codon in their SPEHS1 gene, although it is not clear that Sec is inserted by recoding the UGA codon. These species do not contain a SECIS element in their SEPHS1 gene [37]. It is an intriguing possibility that a novel amino acid is inserted at that position.

Ascidians and vertebrates

All eukaryotic SEPHS2s contain Sec at their catalytic domain in the place where Cys is present in prokaryotic SelD. However, eukaryotic SEPHS1s contain other amino acids such as glycine (Gly), threonine (Thr), and arginine (Arg). In the subphylum Tunicata, the Ascidians have SEPHSs with Sec and Gly which correspond to SEPHS2 and SEPHS1, respectively. These organisms provide important insights on the evolution of two forms of SEPHS [30]. The small pelagic tunicate, *Oikopleura dioica,* encodes only the SEPHS2(Sec) gene, which contains a UGA codon and a SECIS element, suggesting only SEPHS2(Sec) is produced. On the other hand, the sea squirt, *Ciona intestinalis*, contains a single gene for SEPHS, but makes both forms of SEPHS, SEPHS1(Gly) and SEPHS2(Sec). Because the SEPHS-encoding gene contains both the UGA codon and a SECIS element, it was annotated as *Sephs2*, but two types of ESTs encoding SEPHS1(Gly) or SEPHS2(Sec) were discovered. The gene contains both N-terminal domains in the first three exons. These data suggest that the two forms are generated by alternative splicing. In case of the sea grape, *Molgula tectiformis,* there are two separate genes for the two proteins. Notably, the SEPHS1(Gly) in *M. tectiformis* contains a SECIS element which is dispensable for the translation of this protein. This SECIS element in SEPHS1(Gly) seems to be an evolutionary relic. The colonial ascidian star tunicate, *Botryllus schlosseri,* has two separate genes for each protein.

Like in higher metazoans, the *Sephs1*(Gly) in *B. schlosseri* does not contain a SECIS element, while the *Sephs2*(Sec) has it. These data suggest that the ancestral SEPHS gene contained domains for both protein forms and the N-terminal domains evolved to support different functions.

The evolution of the two paralogs, *Sephs1* and *Sephs2*, in vertebrates was described in depth by Mariotti *et al*. [31]. SEPHS1 in vertebrates contains Thr in its catalytic domain, while SEPHS2 of vertebrates has Sec at the same position. In vertebrates, it was suggested that SEPHS1(Thr) originated by duplication of the SEPHS2 gene and conversion of Sec to Thr at the root of *Gnasthostomata*. In metazoans, SEPHS1 underwent convergent subfunctionalization to acquire a new function, after duplication. Interestingly, vertebrates generally have many large selenoproteoms (Fig. 1). It seems that these organisms have strong evolutionary constraints for keeping selenoproteins and acquired selenoproteins with important specialized functions such as iodothyronine deiodinase 2 (DIO2) and selenoprotein P (SELENOP).

Significance of structure and interaction between SEPHSs on their functions

Crystal structures of SelD/SEPHS1 were determined from *Aquifex aeolicus* [38], *E. coli* [39], humans [40] and N-terminal truncated SEPHS2 of *Leishmania major,* which is a member of the Trypanosomatidae family [41]. The overall structures of these SEPHSs are very similar and all protein form dimers. Although it was reported that Cys17 of *E. coli* SelD in the N-terminal Gly-rich loop (catalytic domain) is essential for SEPHS's catalytic activity [42], SEPHSs have sequence variations at this position. SEPHSs which contain Cys or Sec at this position catalyze selenophosphate synthesis [15, 43], whereas those in which Sec/Cys is replaced by other amino acid residues, such as alanine (Ala), Thr, or Gly, cannot catalyze selenophosphate synthesis reactions [14, 44]. SelD of *E. coli* and *A. aeolicus*, human SEPHS1, and Trypanosomatidae SEPHS2 contain Cys, Sec, Thr and Cys in their catalytic domains, respectively.

All three SelD/SEPHS homologs form homodimers with a barrel-like core structure in which each monomer contributes to the formation of one half of the barrel [10]. Each monomer is composed of two layers: the inner layer, which forms the core, comprises the N-terminal half, while the C-terminal half surrounds the N-terminal half, forming the outer layer. There is a narrow channel, through which substrates, such as ATP and selenide, enter to the active site in the N-terminal half of the protein. The catalytic and ligand-binding domains of the N-terminal half and the phosphate binding domain of the C-terminal half are located along this channel.

ATP-binding domain

Co-crystallization of adenosine 5′-(α, β-methylene) triphosphate (AMPCPP) with *A. aeolicus* SelD and human SEPHS1 clearly revealed that dimerization is required for the binding of ATP to the ligand-binding domain (Fig 2A–D, see also references 38 and 40). ATP (here AMPCPP) is located in the ligand-binding pocket and covered almost completely

by protein helix 2, 8 and β-sheet 2 of chain A, and β-sheet 3 and 4 of chain B. The adenine ring establishes hydrogen bonds with T164 of one protein monomer (strand B), while the β- and γ-phosphate are bound to the amino acids (D69, D87, D110, D265) located in the other monomer (strand A) except Q163 which is located in strand B. Phosphate binding to these amino acids is mediated by three Mg^{2+} ions, which interact with the participating amino acids as well as phosphate groups on ATP (Fig 2E–F). Therefore, an ATP-binding pocket is comprised of amino acids from both monomers. Crystal structure of SEPHS2 from *L. major* was determined using the N-terminus-truncated protein and showed that SEPHS2 has a similar 3D-structure to SelD and SEPHS1 [41]. Dimerization of SEPHSs was demonstrated experimentally. Both SEPHS1 and SEPHS2 form a homodimer as well as a heterodimer [45]. Notably, the phosphate groups are oriented towards the catalytic domain, where the γ-phosphate forms a complex with selenide to form selenophosphate (see the pseudo-γ-phosphate, ψγP, in Fig 2A). It is interesting that the ψγP was detached from the AMPCPP used for co-crystallization in both SelD and human SEPHS1. As described in the Introduction, SEPHS1 does not have the ability to synthesize selenophosphate. However, this observation indicates that SEPHS1 has ATP hydrolase activity, although the ADP does not undergo further hydrolysis to AMP and inorganic phosphate. The ATP hydrolysis by SEPHS1 was also observed in biochemical experiments [46].

Catalytic domain

The catalytic domain participates in the formation of selenophosphate using γ-phosphate and possibly selenide. It is located at the N-terminal glycine-rich loop (marked in red in Fig 2G and H). The only difference between SelD/SEPHS2 and SEPHS1 is the presence of a Sec or Cys (Sec/Cys) residue in the catalytic domain. While the SelD/SEPHS2 group has Sec/Cys, SEPHS1 group contains another amino acid residue at the same position. The presence of Sec/Cys is critical for SEPHS activity. Therefore, it can be assumed that the presence of Sec/Cys provides a favorable structure to hold the substrate. In human SEPHS1, there is a Thr at the Sec/Cys position. Comparison of the catalytic domain of SelD from *A. aeolicus* and human SEPHS1 reveals two differences [38]: 1) the side chain of Cys in SelD is oriented inward, while the side chain of Thr in SEPHS1 is oriented outward (compare Fig 2E with F); and 2) the distance between γ-phosphate and Cys in SelD is 8.4 Å, while the distance between γ-phosphate and Thr is 13.1 Å (Fig 2G and H). A similar structure of the catalytic domain in *E. coli* SelD was also observed [39]. Interestingly, it was suggested that Cys17 in the catalytic loop points outward for accepting selenide, and after binding with selenide, Cys17 moves inward to deliver selenide via a selenium-sulfur bond, which then forms selenophosphate with the γ-phosphate of ATP [39, 47]. Therefore, it seems that the position of the side chain of Sec provides a favorable environment for the interaction between γ-phosphate and selenide. This assumption can be shown with co-crystallization of SEPHS-ATP-selenide.

The crystal structures of SelD and SEPHS1 clearly show that both strands in a dimer of SEPHS contribute to holding ATP. The center barrel-like structure in the interface provides easy access of substrates and products. ATP interacts with both monomers using magnesium ions as bridges and this structure explains how the intermediates of ATP hydrolysis, γ-phosphate and ADP, are held until the reaction is completed. It is interesting, but awaiting to

be determined why further hydrolysis of ADP does not proceed in SEPHS1. The structure of the catalytic domain provides some clue as to why SEPHS1 does not support selenocysteine synthesis.

Roles of SEPHS1 in ROS regulation

Reactive oxygen species (ROS) is an umbrella term defined as relatively short-lived partially reduced species of molecular oxygen, with half-lives in aquatic environments in the range of nanoseconds to hours [48 and references therein]. ROS, including various peroxides and free radicals, exhibit different properties such as reactivity and mode of generation. For example, hydrogen peroxide (H_2O_2) which is a non-radical ROS, is generated mainly by plasma membrane NADPH oxidases (NOX) and decomposed by catalase. The superoxide anion radical, $O_2^{\cdot-}$ is a free radical serving as a major source of H_2O_2. The $O_2^{\cdot-}$ is dismutated spontaneously or by superoxide dismutases to H_2O_2 and O_2. The intracellular concentration of H_2O_2 is maintained in the low nanomolar range (approximately 1–100nM), and is associated with physiological redox signaling. The overall cellular concentration of the $O_2^{\cdot-}$ is maintained at about 10^{-3} nM, much lower than that of H_2O_2 [49]. The physiological targets of oxidants serve as molecular redox switches in signal transduction acting at various levels of cell regulation affecting protein function; thus leading to changes in signaling outputs, enzyme activity, gene transcription and membrane and genome integrity [50].

Due to the short lifetimes and typically low concentrations of ROS in aquatic systems, analyses of ROS have proven to be challenging [49]. Issues such as bioavailability of the probe, tissue penetrance of the signal and signal-to-noise ratio need to be considered in applications of ROS probes. These issues make *in vivo* detection of ROS relatively more difficult than *in vitro* detection [51]. Methods for ROS detection can be broadly classified as either direct or indirect. The direct observation is only possible on the sub-millisecond timescale, with the relatively stable H_2O_2 being an exception. Indirect methods involve the reaction of a particular ROS with a probe molecule to yield a more stable, long-lived analyte [52].

Humans have 25 genes encoding selenoproteins [53], and among these many are oxidoreductases, as 15 selenoproteins exhibit a thioredoxin fold, and there are three additional thioredoxin reductases and one methionine sulfoxide reductase [54 and references therein]. Here, we briefly review selenoproteins participating in redox regulation. Glutathione peroxidases (GPXs) are the enzymes that catalyze the reduction of H_2O_2 and lipid hydroperoxides with glutathione. There are eight GPXs in mammals, five of which are selenoproteins (GPX1–4, GPX6), and three are Cys-containing paralogs (GPX5, GPX7, and GPX8). Most GPXs reduce small organic hydroperoxides, but are unable to reduce complex hydroperoxides. Notably, GPX4 has a broad substrate specificity representing the only GPX enzyme capable of reducing large and complex lipid hydroperoxides and cholesterols [55]. Thioredoxin reductases (TXNRDs) are selenocysteine-containing flavoenzymes, which reduce thioredoxins. TXNRD1 is primarily localized in the cytosol and nucleus and TXNRD2 is localized in the mitochondria. TXNRD3 is predominantly expressed in testis [56], and is particularly abundant in elongating spermatids at the site of mitochondrial sheath formation [57]. Regarding male reproduction, TXNRD3 was recently discovered to be

essential for sperm maturation [58, 59]. SELENOP is a secreted glycoprotein that contains most of the selenium measurable in plasma. *SELENOP* knockout mice showed enhanced ROS production [60]. SELENOW is a selenoprotein localized mainly in the cytosol. It plays an important role in cell protection against oxidative stress, and in the regulation of redox-related calcium homeostasis. Overexpression of SELENOW reduced intracellular peroxide concentrations [61]. One critical role of selenium is the reduction of methionine sulfoxide in proteins. This role is fulfilled by MsrB1, a methionine-R-sulfoxide reductase located in the cytosol and nucleus. This protein is also involved in regulation of actin function through reversible oxidation of two critical methionine residues. When these methionine residues are oxidated to methionine-R-sulfoxide by MICALS, F-actin is depolymerized. However, the reduction of methionine-R-sulfoxide by MsrB1 promotes actin polymerization. There are also several other thioredoxin fold selenoproteins of unknown function: SELENOT, SELENOV, SELENOM, SELENOF and SELENOH.

During the last few decades, the role and significance of SEPHS1's function in redox homeostasis has been studied with direct and indirect observations of ROS accumulation in SEPHS1-deficient cells. The first study demonstrating that SEPHS1 deficiency caused ROS accumulation was performed in *Drosophila* embryos [62]. *Sephs1* knockout in *Drosophila* by P-element insertion, named $SelD^{ptuf}$, led to the increase of ROS levels in the imaginal disc at the larval stage. Moreover, $SelD^{ptuf}$/WT heterozygous flies (SEPHS1 haplo-insufficient flies) presented a significant decrease on lifespan when treated with oxidizing agents [63]. However, under normal conditions, there was no difference in the lifespan between normal and SEPHS1 haplo-insufficient flies. These observations suggest that SEPHS1 plays an important role in regulating cellular redox balance and that the accumulation of ROS upon SEPHS1 deficiency or insufficiency results in the inhibition of growth.

The studies performed in mice have further elucidated how SEPHS1 deficiency affects the cellular system to control redox homeostasis (Fig 3). Liver-specific SEPHS1 knockout mice showed an upregulation of glutathione S-transferase alpha 1(*Gsta1)*, glutathione S-transferase alpha 2(*Gsta2)*, glutathione S-transferase mu 1 (*Gstm1)*, glutathione S-transferase mu 2 (*Gstm2)*, and glutathione S-transferase mu 3 (*Gstm3)*, and downregulation of glutathione peroxidase 1 (*Gpx1)*, selenoprotein W (*SelenoW)*, glutaredoxin 1 (*Glrx1)*, glutathione S-transferase omega 1 (*Gsto1)*, and glutathione S-transferase alpha 4 (*Gsta4)* [64]. Most of the genes are glutathione (GSH)-related and the alteration of their expression suggests that ROS is accumulated and the ratio of GSH/GSSG is altered in the cell. However, no evidence was found that the targeted removal of *Sephs1* by knockout causes ROS accumulation in the liver. This seems to result from the fact that liver has many oxidoreductases. A transcriptome analysis performed with *Sephs1*-knockout mouse embryos also showed a similar expression pattern of genes regulating oxidoreduction as found in other cell lines [28]. Gene Set Enrichment Analysis (GSEA) using gene expression data obtained from RNA-seq of SEPHS1-deficient mouse embryos predicted a 'ROS pathway', where, in the leading-edge subsets, some typical ROS-regulating genes such as *Txnrd1, Txnrd2*, and *NAD(P)H quinone dehydrogenase 1(Nqo1)* were upregulated or others such as *Glrx1, peroxiredoxin 2 (Prdx2)*, and peroxiredoxin 6 (*Prdx6*) were downregulated. This alteration of gene expression also suggests that ROS are accumulated in the cell.

In addition to SEPHS1-deficient embryos showing ROS accumulation *in vivo*, SEPHS1-downregulated embryonic cell lines derived from *Drosophila*, mouse, and human also showed ROS accumulation or significant changes in the expression of ROS-related genes. SEPHS1 knockdown in *Drosophila* embryonic SL2 cells resulted in the accumulation of ROS and inhibited the biosynthesis of pyridoxal phosphate, the active form of vitamin B6 [65, 66]. The treatment of SL2 cells with 4-DPN, a vitamin B6 inhibitor, led to ROS accumulation [67], suggesting that the inhibition of vitamin B6 synthesis by SEPHS1 deficiency is responsible for the ROS accumulation. Bioinformatic analysis of transcriptomes in SEPHS1-deficient human embryonic stem cells (hESC) predicted that ROS would be accumulated [68]. However, experimental evidence supporting the bioinformatic prediction is still lacking.

SEPHS1 knockdown in mouse embryonic carcinoma F9 cells resulted in the accumulation of H_2O_2 [64]. In *Sephs1*-knockout F9 cells, the expression of redox-homeostasis-related genes encoding proteins such as GLRX1 and various GSTs was dysregulated. *Glrx1, Gsto1* and *SelenoW* expression is significantly reduced by targeted downregulation of *Sephs1* [64]. The accumulation of H_2O_2 was reversed by over-expression of *Glrx1* suggesting that the accumulation of H_2O_2 in SEPHS1-deficient F9 cells was mainly due to the lack of intracellular GLRX1 activity. On the other hand, *Sephs1* null mutation using CRISPR-Cas9 system in 2H11 cells, a mouse endothelial cancer cell line, resulted in the accumulation of superoxide. Staining SEPHS1-deficient cells with dihyrdoethidium showed that the superoxide levels were significantly increased (approximately 2.2-fold) in SEPHS1-deficient cells [27]. The major sources of superoxide accumulation in SEPHS1-deficient endothelial cells were the increase of xanthine oxidase expression and NADPH oxidase 4 (NOX4), and the decreased expression of superoxide dismutases [27]. It should be noted that chondrocyte-specific *Sephs1*-knockout mouse also showed the accumulation of superoxide in the chondrocytes of the cartilage [29].

Taking the above results together, it seems that different types of ROS are accumulated upon SEPHS1 deficiency depending on cell types, presumably through regulating the expression of different sets of genes. Furthermore, these observations suggest that this protein has a critically important role in cellular redox homeostasis.

Function of SEPHS1 during cell growth and development

Direct evidence demonstrating that SEPHS1 is essential for cell growth and proliferation was obtained by targeted removal of *Sephs1* or the corresponding mRNA in *Drosophila*, mouse and human systems. In *Drosophila*, studies using embryonic SL2 cells revealed that cell proliferation was retarded after targeted downregulation of *Sephs1* [62]. The major phenotypic change in response to SEPHS1 deficiency was megamitochondria formation, which was mediated by the elevation of intracellular glutamine levels [69]. SEPHS1 knockdown activated the expression of a novel glutamate transporter (dmGLUT) and glutamine synthetase 1 (GS1), which resulted in the increase of intracellular glutamine levels that then led to megamitochondria formation. Another interesting finding is that SEPHS1 knockdown inhibits vitamin B6 synthesis [69]. The decreased levels of PLP, which is the active form of vitamin B6, by downregulation of SEPHS1 inhibited cell growth

and led to the formation of megamitochondria [66]. Therefore, it seems that SEPHS1 knockdown in *Drosophila* SL2 cells leads to the lack of vitamin B6, ROS accumulation, increase of glutamine levels, and finally megamitochondria formation and inhibition of cell proliferation. The biological significance of megamitochondria formation, often seen as a cellular response to metabolic disturbances, has yet to be determined.

The effect of SEPHS1 deficiency on the development of *Drosophila* was studied with a $SelD^{ptuf}$ mutant, and the mutant showed aberrant disc morphologies such as a reduction in disc mass and disorganization of disc cells. In addition to the aberrant morphologies, the *Sephs1*-knockout larva also showed inhibition of cell proliferation and apoptosis in imaginal disc cells [13, 62]. Mutant flies transformed with the full-length SelD/SEPHS1 cDNA showed complete inhibition of lethality and rescued the disc phenotype indicating that SEPHS1 is essential for *Drosophila* development.

SEPHS1 knockdown in embryonic mouse F9 cells resulted in the growth retardation and inhibition of other hallmarks of cancer, such as invasiveness [64]. These phenotypic changes were recovered by the overexpression of *Glrx1,* suggesting that the phenotypic changes were caused by accumulation of ROS such as H_2O_2. It was also demonstrated the effect of SEPHS1 loss in 2H11 mouse endothelial cancer cells by the targeted removal of *Sephs1* [27]. The superoxide accumulated in 2H11 cells mediated by *Sephs1* knockout led to the following phenotypic changes: 1) the proliferation rate of SEPHS1-deficient 2H11 cells was reduced compared to normal cells; 2) the decrease of cell proliferation was accompanied by cell cycle arrest in the G2/M phase; and 3) DNA damage was induced presumably by oxidative stress, because γH2AX foci were formed following *Sephs1* knockout. In addition, angiogenic ability, which is the major function of endothelial cells, was lost in *Sephs1* knockout mice. Furthermore, production of nitric oxide (NO), which is known to be reduced by oxidative stress, was downregulated as a result of SEPHS1 knockout. Therefore, it can be assumed that SEPHS1 plays an essential role in angiogenesis by regulating the NO and ROS levels in endothelial cells.

Endothelial cell-specific *Sephs1* knockout in a mouse model using the *TieII-Cre* system resulted in vast hemorrhage and embryonic lethality at E13.5 and E18.5, respectively [70]. Hemorrhage in endothelial cell-specific knockout embryos occurred near the burst site of blood vessels. Notably, all the blood vessels in knockout mice were thin and an endothelial cell layer was not found, suggesting SEPHS1 deficiency is lethal to endothelial cells in the embryos and the dead cells were reabsorbed. A systemic SEPHS1 knockout mouse model using the *EIIa-Cre* system showed embryonic lethality at the early stage via oxidative stress-induced apoptosis [28]. SEPHS1-deficient embryos revealed structural abnormalities in the dermal layer at E6.5, abnormal initiation of organ development at E7.5, retarded organ development of the central nervous and cardio-vascular systems at E8.5, and axial turning and chorio-allantoic fusion at E9.5 (Fig. 4). Pathway analysis with RNA-seq data predicted that systemic knockout of *Sephs1* affected retinoic acid (RA) signaling throughout gastrulation, and Wnt, Prl, and IGF signaling were additionally affected at E6.5, E7.5, and E8.5, respectively (Fig. 4). These signaling pathways are closely correlated with the phenotypic changes observed at each embryonic day. Oxidative stress-induced DNA damage was verified by immunostaining the embryos with antibodies against 8-oxo-guanine and

γH2AX at E10.5. Severe reduction of cell proliferation and apoptosis were also observed in the SEPHS1-deficient embryos at E10.5, followed by embryonic death at E11.5. Unlike with other tissue-specific models, there was no apparent physical phenotype in liver-specific SEPHS1 knockout mice using *Alb-Cre* until 8~10 weeks after birth [64]. This may be due to the tissue-specific nature of the liver. Liver has been known to contain much more antioxidant proteins than other organs, and the abundance of antioxidant proteins leads to maintaining redox homeostasis [71].

Compared to the progress made with elucidating the function of SEPHS1 in *Drosophila* and mouse, only a few studies were performed using human cells/tissues. There are five splice variants of human SEPHS1, and these alternative splice variants showed unique characteristics in terms of their intermolecular interactions, subcellular localizations, specific tissue expression and cell cycle suggesting that each splice variant has a specific function for cell growth [45]. The average amount of each splice variant was different. Approximately 90% of SEPHS1 population consists of major type (MT). ΔE2 and ΔE8 comprise 2% of the total SEPHS1 mRNA, while +E9 and +E9a make up less than 1%. Notably, the relative amounts of +E9 and +E9a are extremely small compared to that of MT [45]. It is not clear whether these two low abundant variants are generated by splicing error or by normal pathway. Assou *et al.* compared the gene expression profiles of mature metaphase II oocytes and hESC with that of human somatic cells, and found that SEPHS1 is a marker of oocytes and hESCs [72]. SEPHS1 deficiency in hESC induced by shRNA resulted in growth retardation. Furthermore, the number of hESC colonies was significantly reduced in shSEPHS1-treated cells, and the ROS-related gene expression patterns were changed to increase cellular ROS levels [67]. Interestingly, SEPHS1 downregulation in human umbilical vein cells (HUVECs) led to cell death rather than the inhibition of cell proliferation [70]. It is not clear, how and why normal cells are more sensitive to SEPHS1-mediated increases in ROS than transformed cells.

The accumulating data strongly suggest that SEPHS1 plays an essential role during development and proliferation via regulating cellular redox homeostasis. The absence of SEPHS1 leads to growth retardation in cancer cells and to cell death in normal cells. Lack of SEPHS1 also leads to embryonic lethality at an early stage of development in both insects and mammals. However, the underlying mechanisms of SEPHS1's primary function of regulating cellular redox homeostasis are still not fully understood. In addition, the questions whether and how SEPHS1 participates in selenium metabolism need to be solved.

SEPHS1 and its implication in diseases

The role of SEPHS1 in the regulation of ROS, cell growth, and organismal development was discussed in the previous sections. Extending from these findings, emerging evidence suggests a role for SEPHS1 in the regulation of pathogenesis and progression of human diseases. Embryonic lethality of *Sephs1*$^{-/-}$ mice and severe organismal defects shown in the tissue-specific *Sephs1*-deficient embryos suggest a physiological significance of SEPHS1 and its potential implications in various disease pathogeneses.

The disease-related function of SEPHS1 has been actively explored, especially in the context of cancer development. Choi *et al.* sought out the difference in human rectal carcinoma as compared with adjacent normal rectal tissue using a hybridization method [73]. SEPHS1 was overexpressed in 10 of 12 rectal carcinoma cases, on average 4.6-fold in their mRNA levels. In an investigation of potential relationships between the selenoproteome and five leading cancer types by analyzing GSCALite database [74], an increased expression of SEPHS1 and TXNRD1 in liver hepatocellular carcinoma (HCC) was associated with poor prognosis. In this case, the increased expression of SEPHS1 might reduce oxidative damage and increase cancer cell proliferation in HCC, thus promoting cancer. In an independent study, upregulation of SEPHS1 was similarly noted in HCC and in another liver cancer subtype, cholangiocarcinoma [75]. SEPHS1 positively regulated smooth muscle actin/mothers against decapentaplegic 2/3/4 (SMAD2/3/4) expression in HCC cells, and stimulated HCC migration and invasion [76]. SMAD2/3/4 are known to participate in TGF-β signaling to promote cancer metastasis, developmental process, and immune response. SEPHS1 knockdown resulted in the decrease of migration and invasion in hepatocellular carcinoma cells (HCCs) [76]. SEPHS1 levels were increased in HCC compared with adjacent normal liver tissues. SEPHS1 knockdown in HCC led to the decrease of SMAD2/3/4 as well as mesenchymal marker genes such as snail, slug, and N-cadherin. The suppression of migration and invasion abilities by SEPHS1 knockdown was reversed by TGF-β treatment. In addition, the knockdown of SMAD3 in SEPHS1 overexpressed HCC inhibited the increase of cell invasion ability. These results suggest that SEPHS1 participates in cancer cell malignancy by regulating TGF-β downstream genes and ROS production. However, it is not clear which of ROS accumulation or TGF-β target gene expression comes first by SEPHS1 deficiency. The correlation between SEPHS1 expression and prognosis (overall survival and disease-free survival) in HCC patients was further corroborated in a five-gene prognostic model in colorectal cancer based on the Cancer Genome Atlas (TCGA), Genotype-Tissue Expression (GTEX), and Gene Expression Omnibus (GEO) databases [77]. By conducting weighted gene co-expression network analysis (WGCNA) and Cox regression analysis of cohorts derived from three databases, Yang *et al.* identified SEPHS1 among five hub genes defining a risk score calculated based on their expression levels. Among these five hub genes, only SEPHS1 was localized to the cell membrane, suggesting a possible druggability of SEPHS1 for colorectal cancer treatment. The role of SEPHS1 in carcinogenesis and its diagnostic or prognostic significance still remain to be further investigated. Nonetheless, overall effects of SEPHS1 to promote cell survival appears to undesirably support tumor growth and cancer progression.

Two single nucleotide polymorphisms (SNPs) in SEPHS1, as well as serum selenium levels were associated with the development of Crohn's disease, one of the inflammatory bowel diseases [78]. In this study, three SNP sites, two (rs17529609 and rs7901303) on *SEPHS1* and one (rs1553153) on *SEPSECS,* were identified among the 29 SNPs tested. rs17529609 is located approximately 2,600 bases away from 3′ end of SEPHS1 gene. rs7901303 is located in intronic region between exon 5 and exon 6 of SEPHS1 gene. rs1553153 is located in intronic region between exon 5 and exon 6 of SEPSECS gene. Neither exact function nor association with any other human pathology was reported for those sites. Therefore,

the underlying molecular mechanisms that account for the association of SEPHS1 and SEPSECS with Crohn's disease remain to be explored.

SEPHS1 has been implicated in the maintenance of redox homeostasis in chondrocytes and its deficiency plays a causal role in the progression of osteoarthritis. Osteoarthritis is the most common form of arthritis and is best characterized by loss of cartilage extracellular matrix, and an enrichment with collagen fibril and proteoglycans [79]. The risk factors associated with osteoarthritis, such as age and mechanical stress, collectively lead to the accumulation of oxidative stress in chondrocytes. Oxidative stresses cause chondrocyte senescence and onset of their inflammatory phenotypes, which in turn lead to osteoarthritic cartilage degeneration [80]. SEPHS1 was identified as a key factor modulating redox in chondrocytes; its loss was critically linked to the accumulation of ROS and emergence of chondrocyte senescence [29]. Transcriptome analysis of genes comprising the selenium metabolic pathways revealed specific downregulation of SEPHS1 in human and mouse osteoarthritic cartilage. The downregulation of SEPHS1 was aligned with concomitant suppression of stress-responsive selenoproteins. Indeed, SEPHS1 knockout in chondrocytes reduced the expression of stress-responsive selenoproteins, elevated ROS levels, and promoted cellular senescence. Chondrocyte-specific *Sephs1* knockout in adult mice accelerated both aging-associated and post-traumatic osteoarthritis development. Collectively, this study demonstrates a key role of SEPHS1 in maintaining cartilage homeostasis.

Conclusions

SEPHS1 was originally discovered as a homolog of prokaryotic SelD and assumed to have selenophosphate synthesis activity. However, since another eukaryotic SelD homolog, SEPHS2, was identified, it was found that only SEPHS2, among these two paralogs, contains the ability to catalyze selenophosphate synthesis. Nevertheless, SEPHS1 is indispensable for cell survival, embryogenesis and cell defense. An increasing body of evidence suggests that SEPHS1 participates in maintaining cellular redox homeostasis by regulating the expression of genes related to redox homeostasis. It, therefore, seems that SEPHS1 obtained new function of regulating intracellular ROS levels, instead of losing selenophosphate synthesis activity from its ancestor.

Comparative genomic analyses provide some insights on the evolution of SEPHS1 and possibly its functional relationship with ROS. Both prokaryotes and lower eukaryotes (protists) have only one type of SEPHS homolog that contains a Cys or Sec codon in the catalytic domain, which is characteristic of SEPHS2 [10, 31]. In Tunicates, the N-terminal domains of SEHPS1 and SEPHS2 are located in the same gene in different exons that give rise to two isoforms by alternative splicing, suggesting the common ancestor of SEPHS contains both SEPHS1 and SEPHS2 activities (Fig. 1 and [31]). Whether these two paralogs were generated by direct duplication of the gene or by retrotransposon-like insertion is not clear. Crystal structures of SelD and SEPHS1 clearly show that dimerization is required for activity of ATP binding and hydrolysis [38–40]. ATP is hydrolyzed by the SEPHS1 dimer, although the role of γ-phosphate has not been determined. The fact that SEPHS1

and SEPHS2 can form heterodimers and that their 3D structures are similar suggests that SEPHS1 may affect SEPHS2 activity or *vice versa*.

The effect of SEPHS1 deficiency is pleiotropic. Depending on cell and tissue types where SEPHS1 is targeted, different types of ROS are produced and accumulated, different signaling pathways are affected, and there are different effects on cell proliferation, cell survival, and phenotypic changes are dependent on cell and tissue type where *Sephs1* is downregulated [10, 27, 54]. Notably, systemic knockout of *Sephs1* in mouse embryos affects retinoic acid signaling throughout gastrulation; however, Wnt, prolactin (Prl), and insulin-like growth factor (IGF) signaling are affected sequentially according to the developmental stages [28]. An essential role of SEPHS1 in the cell function implicates this protein in disease pathogenesis. In fact, SEPHS1 deficiency or overexpression may be used as a biomarker of various diseases including cancer. For example, it was found that SEPHS1 dysregulation is strongly associated with the progression of osteoarthritis [29].

Several issues remain to be elucidated regarding SEPHS1's function. What is its molecular/biochemical function? What is the significance of protein dimerization and ATP hydrolysis in this function? Does SEPHS1 affect SEPHS2 activity? How does SEPHS1 regulate the expression of genes participating in redox homeostasis? What is the relationship to retinoic acid metabolism? How many diseases are related to SEPHS1 dysfunction and what are the mechanisms of pathogenesis? Answering these questions will provide important insights regarding cellular redox regulation and cell proliferation, and potential future molecular targets for therapy.

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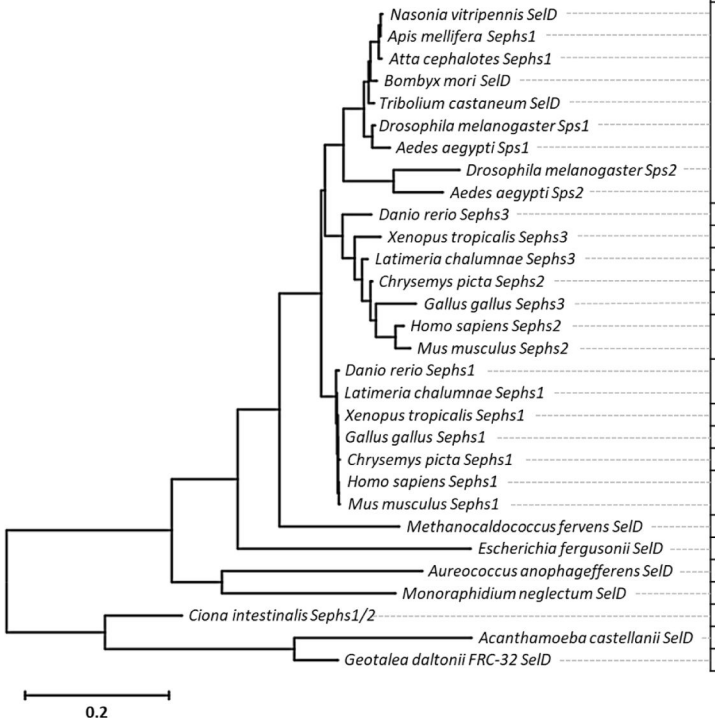
Highlights

- Sec/Cys residue in the catalytic domain is the key functional determinant in selenophosphate synthetase function.
- SEPHS1 deficiency disrupts cellular redox homeostasis and causes cell type-specific accumulation of different types of ROS.
- The accumulation of ROS due to SEPHS1 deficiency causes cell- and tissue-specific pleiotropic effects such as growth retardation, apoptosis, and embryonic lethality.
- SEPHS1 is an essential enzyme and its null-mutation in mouse embryos leads to the activation of retinoic acid signaling.
- Dysregulation of SEPHS1 is associated with the development of cancers and degenerative diseases.



Group	Sec /Cys	Sec machinery	Selenoprotein number	Categories*
Insect	UGA(Sec)	No	0	SEPHS1
	UGA(Sec)	Incomplete	0	SEPHS1
	UGA(Sec)	No	0	SEPHS1
	AGA(Arg)	No	0	SEPHS1
	CGU(Arg)	No	0	SEPHS1
	CGC(Arg)	Complete	3	SEPHS1
	CGC(Arg)	Complete	3	SEPHS1
	UGA(Sec)	Complete	3	SEPHS2
	UGA(Sec)	Complete	3	SEPHS2
Fish	UGA(Sec)	Complete	39	SEPHS2
Amphibia	UGA(Sec)	Complete	23	SEPHS2
Fish	UGA(Sec)	Complete	25	SEPHS2
Reptile	UGA(Sec)	Complete	25	SEPHS2
Bird	UGA(Sec)	Complete	25	SEPHS2
Mammal	UGA(Sec)	Complete	25	SEPHS2
	UGA(Sec)	Complete	24	SEPHS2
Fish	ACA(Thr)	Complete	39	SEPHS1
	ACA(Thr)	Complete	25	SEPHS1
Amphibia	ACU(Thr)	Complete	23	SEPHS1
Bird	ACA(Thr)	Complete	25	SEPHS1
Reptile	ACA(Thr)	Complete	25	SEPHS1
Mammal	ACA(Thr)	Complete	25	SEPHS1
	ACA(Thr)	Complete	24	SEPHS1
Archaea	UGA(Sec)[1)]	Complete	8	SelD
Bacteria	UGC(Cys)[2)]	Complete	5	SelD
Protist	UGC(Cys)[3)]	Complete	59	SelD
Chlorophyte	UGC(Cys)[1)]	No	0	Unclear
Tunicata	UGA(Sec)	Complete	19	SEPHS1/2[#]
Amoebae	UGC(Cys)[4)]	Incomplete	0	SEPHS2
Bacteria	UGA(Sec)[4)]	Complete	8	SelD

Fig 1. Phylogenetic profile of SEPHSs.

A phylogenetic tree with the highest log likelihood (−29560.22) was inferred using the maximum likelihood method and Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA11 [30]. The common names, codon sequence of Sec/Cys residue, information regarding Sec utilization machinery, the number of selenoproteins, proposed functional forms are listed in the table. Some prokaryotes and protists contain extra functional domains at the N-terminus, and are marked as superscripts. The superscripted numbers denote N-terminal fused domain(s); 1) Pyr_redox_2, 2) Pyr_redox_2, AIRS, AIRS_C, 3) NADH-dehydrogenase like domain, and 4) NifS-like domain. [#]Both SEPHS(Sec) and SEPHS(Gly) occur by alternative splicing. *Predicted functional homologues of prokaryotic selenophosphate synthetase, eukaryotic SEPHS2 and the eukaryotic SEPHS1 homologues are represented as SelD, SEPHS2 and SEPHS1, respectively.

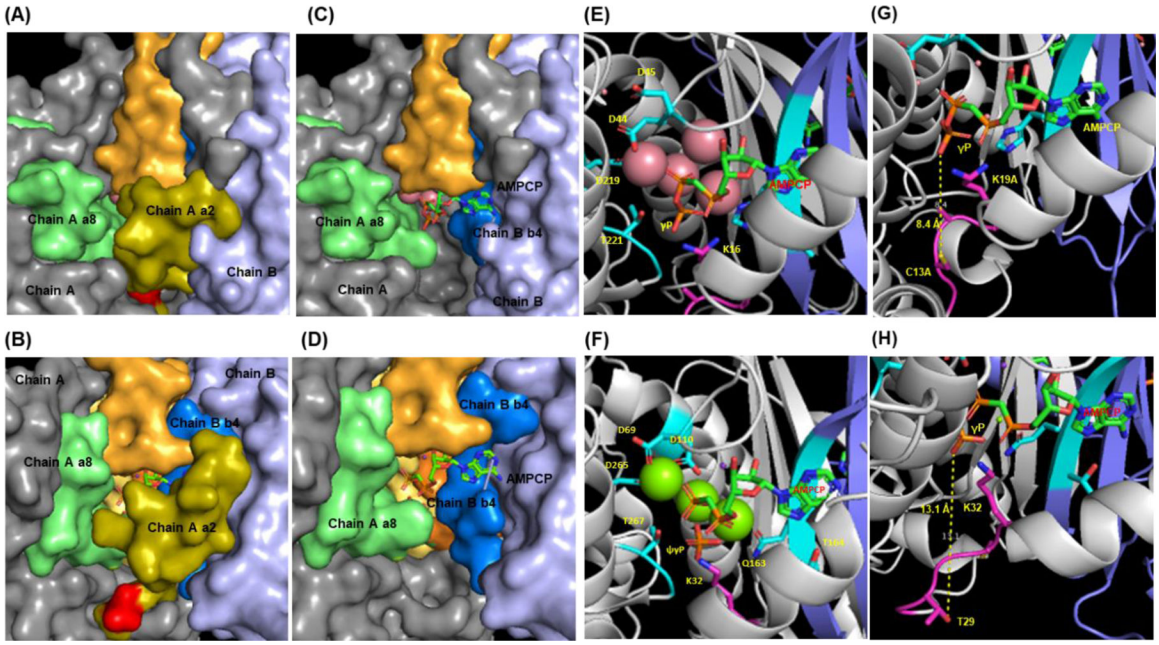


Fig 2. Structural comparison between *A. aeolicus* SelD (A,C,E,G) and *H. sapiens* SEPHS1 (B,D,F,H).

(A) Surface image of ligand binding pocket of SelD. **(C)** α-helix 8 of chain A was removed to show inside of the pocket from **(A)**. α-helix 2 and 8 in chain A, and β-sheet 4 in chain B form a ligand binding pocket. C16 in the catalytic domain is highlighted in red. **(B)** Surface structure of human SEPHS1. **(D)** α-helix 8 of chain A was removed to show inside of the pocket from **(B)**. Like SelD, α-helix 2 and 8 in chain A and β-sheet 4 in chain B form a ligand binding pocket. T29 in the catalytic domain is highlighted in red. Ribbon model of ligand binding pocket of SelD (**E** and **G**) and SEPHS1 (**F** and **H**). ψγP represents pseudo-gamma phosphate. Pink spheres indicate magnesium ions. Green spheres represent cobalt ions. The catalytic domain of SelD and SEPHS1 in (**A**) and (**B**) are shown in red. The yellow dotted lines shown in (**G**) and (**H**) indicate the distance between C16 (SelD) /T29 (SEPHS1) and γ phosphate. Redrawn from refs 38 and 40 using Pymol software.

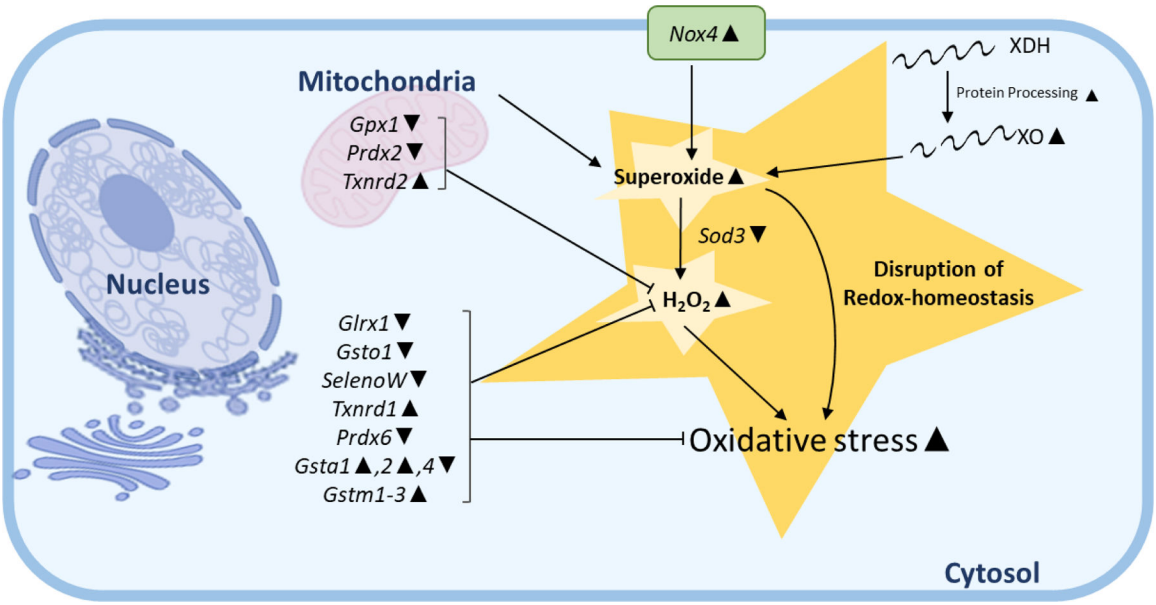


Fig 3. ROS-related gene set expression affected by SEPHS1 deficiency.

The upregulated and downregulated genes in SEPHS1-deficient cells are represented as ▲ and ▼, respectively. The levels of xanthine oxidase (XO), which is the processed form of xanthine dehydrogenase (XDH), are increased by SEPHS1 deficiency. Abbreviations: *Glrx1*, glutharedoxin1; *Gsto1*, glutathione S-transferase omega1; *Gsta1*, glutathione S-transferase alpha1; *Gstm1–3*, glutathione S-transferase mu1–3; *Gpx1*, glutathione peroxidase1; *Nox4*, NADPH-oxidase 4; *SelenoW*, selenoprotein W; *Sod3*, superoxide dismutase 3; *Prdx2*, peroxiredoxin2; *Txrnd1*, thioredoxin reductase1; *Txrnd2*, thioredoxin reductase2.

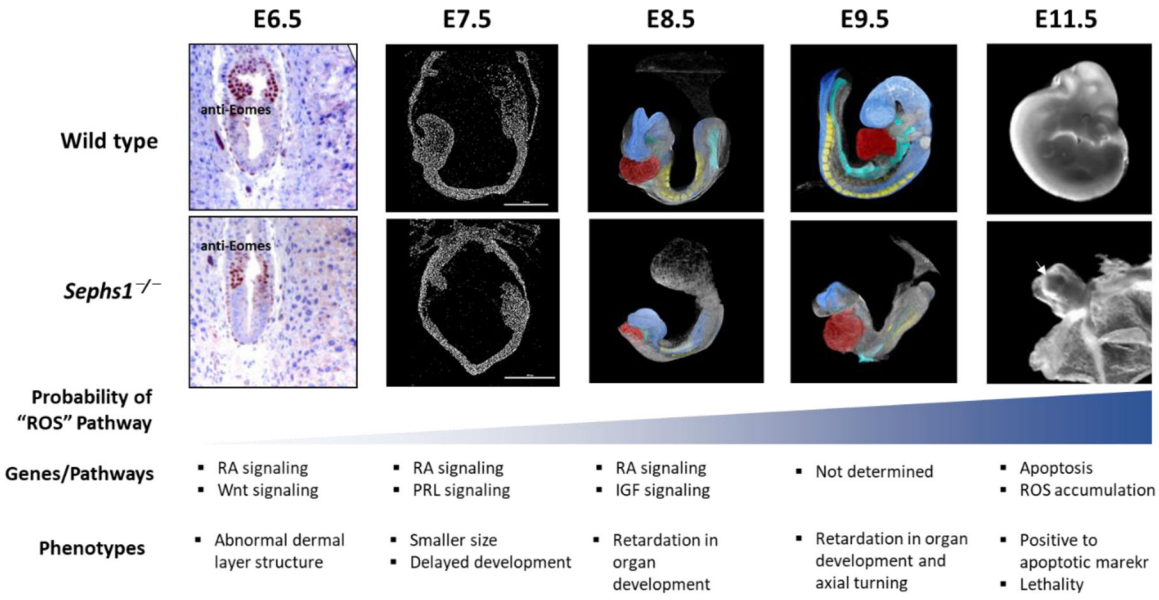


Fig 4. Effects of SEPHS1 deficiency on the development of mouse embryos.

Photos representing typical changes at each embryonic day were adopted from Bang *et al.* [28]. Images of E6.5 show that EOMESs were expressed at different positions. Images from E7.5 to E9.5 are whole embryo x-ray micrographs, images of E11.5 are stereo micrographs. White arrow in the lower panel of E11.5 indicates the dead embryo undergoing resorption. Abbreviations: RA, retinoic acid; PRL, prolactin; IGF, insulin-like growth factor.