REPORT

Mutations Disrupting Selenocysteine Formation Cause Progressive Cerebello-Cerebral Atrophy

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The essential micronutrient selenium is found in proteins as selenocysteine (Sec), the only genetically encoded amino acid whose biosynthesis occurs on its cognate tRNA in humans. In the final step of selenocysteine formation, the essential enzyme SepSecS catalyzes the conversion of Sep-tRNA to Sec-tRNA. We demonstrate that *SepSecS* mutations cause autosomal-recessive progressive cerebellocerebral atrophy (PCCA) in Jews of Iraqi and Moroccan ancestry. Both founder mutations, common in these two populations, disrupt the sole route to the biosynthesis of the 21st amino acid, Sec, and thus to the generation of selenoproteins in humans.

Progressive cerebellocerebral atrophy (PCCA) is a newly described autosomal-recessive phenotype of nondysmorphic profound mental retardation, progressive microcephaly, and severe spasticity.[1] Myoclonic or generalized tonic-clonic seizures are often observed as well. Repeat magnetic resonance imaging of affected individuals showed progressive cerebellar atrophy followed by cerebral atrophy involving both white and gray matter (Figure 1). The PCCA phenotype was identified in nonconsanguineous Jewish Sephardic families; parents of affected individuals were either both of Moroccan ancestry, both of Iraqi ancestry, or of mixed Iraqi-Moroccan ancestry.[1]

DNA samples of all members of two affected nonconsanguineous families of Iraqi ancestry and of two affected families of mixed Moroccan-Iraqi ancestry were available for molecular analysis. Samples were obtained after informed consent was secured under approval of the Soroka Medical Center institutional review board. DNA was isolated from whole blood of affected individuals with the FlexiGene DNA Extraction Kit (QIAGEN) per the manufacturer's instructions. Lymphoblast cell lines were established by Epstein-Barr virus (EBV) transformation via standard methods.

A genome-wide scan of nine individuals of families A and B and two affected individuals of families C and D (Figure 2) was done with the GeneChip Human Mapping 10K Array Xba 142 2.0 (Affymetrix) according to the manufacturer's guidelines. Detailed mapping of the PCCA locus was performed with microsatellite markers selected from public genome databases (Marshfield, Généton, and deCODE genetic maps). No likely disease-associated locus shared by all the affected individuals was identified.

Because the Jewish Iraqi and Moroccan communities are known to be of different ancestral origins, we assumed that the mutations in those two populations, even if in the same gene, could be different. Analysis of the two Iraqi families (Figure 2, families A and B) found that a single ~8.0 cM locus of homozygosity spanning 18 SNPs between rs1396669 and rs2036184 on chromosome 4p15.2 was shared by the two affected individuals (family A individual 4 and family B individual 5). Fine mapping of that locus was done with microsatellite markers. Three novel microsatellite markers, ch4_AC, ch4_AT, and ch4_AC2 (Figure 2), were designed with the *TANDEM REPEATS FINDER* program, the UCSC Human genome database, and the *PRIMER3* program. Haplotypes were manually reconstructed and presented graphically with HaploPainter.[2] As shown in Figure 2, fine mapping narrowed the locus to an approximately 4.0 Mb interval of 7.5 cM that was between markers D4S425 and D4S391 and encompassed 15 genes (see Table S1). Interestingly, at this locus the two affected individuals of mixed Iraqi-Moroccan ancestry (Figure 2: family C individual 4 and family D individual 5) shared one haplotype, inherited from their Iraqi parent, that was identical to that found in the "pure" Iraqi patients. These two individuals shared also an approximately 1.4 Mb haplotype between markers D4S1551 and rs2048506. This haplotype was inherited from their parent of Moroccan origin and harbored 10 of the 15 genes.

Prioritization of genes from within the defined locus was done with our Syndrome to Gene (S2G) software.[3] S2G includes a clinical component that is a revised version of OMIM, and it eliminates negation phrases and integrates overlapping terminology of phenotyes. S2G also contains a molecular component, integrating 16 databases of protein-protein interactions, biochemical pathways, transcription-factor networks, etc. For any syndrome, the software selects a prioritized list of candidate genes on the basis

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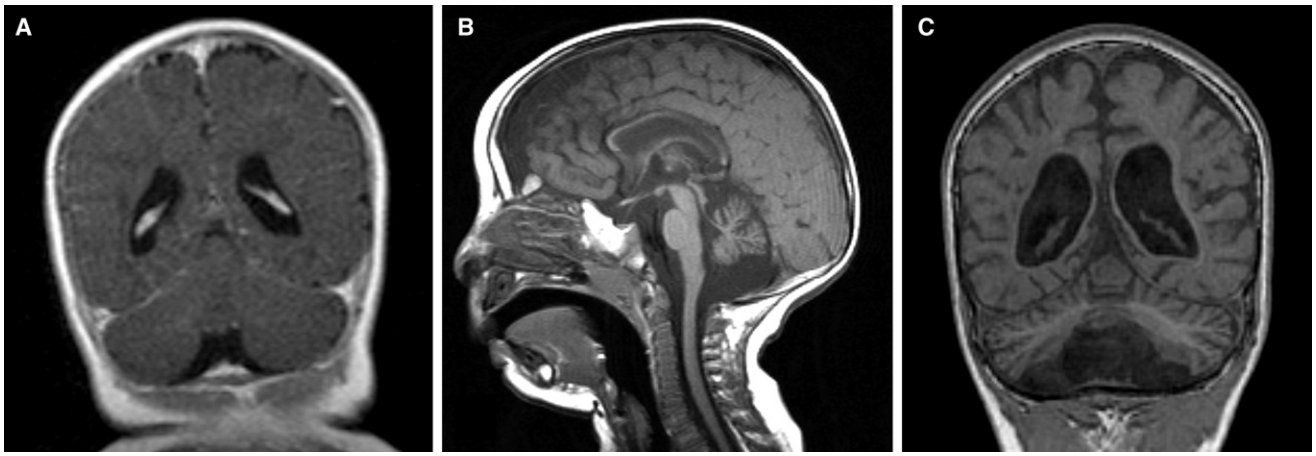

Figure 1. Sequential MRI Studies in PCCA
Images are of individual 4 of family A (Figure 2).
(A) Age 8 months: a T1 coronal image at the level of the occipital lobes demonstrates a normal-looking brain.
(B) Age 18 months: a T1 sagital image showing vermian atrophy with mild cerebral atrophy.
(C) Age 3 years: a coronal image at the level of the occipital lobes demonstrates severe cerebellar and cerebral atrophy.

of their known molecular interactions with genes associated with phenotypically similar syndromes. The clinical presentation of the PCCA patients is most similar to that of ponto-cerebellar hypoplasia type 2 (PCH2A [MIM 277470], PCH2B [MIM 612389], and PCH2C [MIM 612390]), known to be caused by mutations in the following genes involved in tRNA processing: *TSEN54* (MIM 608755), *TSEN2* (MIM 608753), and *TSEN34* (MIM 608754), which encode subunits of the tRNA-splicing endonuclease complex.[4] On that basis, S2G suggested one of the 15 genes in the ~4.0 Mb region as the top candidate: *SepSecS*, encoding *O*-phosphoseryl-tRNA:selenocysteinyl-tRNA synthase, active in catalyzing the conversion of *O*-phosphoseryl-tRNA (Sep-tRNA) to Sec-tRNA during the final step of selenocysteine formation.[5,6]

Testing samples of affected individuals, we sequenced *SepSecS* as well as the other 14 genes in the above locus. Primer pairs for cDNA and/or each exon including flanking intron sequences of the 15 genes in the putative 4p15.2 locus were designed from cDNA and genomic sequences via Primer 3. RNA was extracted from cultured cells of EBV-transformed lymphoblastoid cell lines with the RNeasy Mini Kit (QIAGEN), and cDNA was reverse transcribed by the Reverse-iT kit (ABgene) according to the manufacturer's protocol. Primer sequences and PCR conditions are available upon request. PCR products were directly sequenced with ABI PRISM 3730 DNA Analyzer according to the protocols of the manufacturer (Applied Biosystems, Foster City, CA). Sequencing of the entire coding region and intron-exon borders of *SepSecS* identified homozygosity for a missense mutation, c.1001A>G (NM_016955.3), in both unrelated affected individuals of Jewish Iraqi ancestry (Figure 3). This mutation leads to substitution of the conserved tyrosine 334 residue with cysteine (p.Tyr334Cys, Figure 4) in the SepSecS protein.

Sequencing the entire coding region and intron-exon borders of all other 14 genes within the 4p15.2 locus identified no further mutations in those individuals (data not shown). In the two unrelated affected individuals of mixed Iraqi-Moroccan origin (Figure 2A, families C and D), sequencing of *SepSecS* identified compound heterozygosity for the c.1001A>G mutation and for another missense mutation (c.715G>A) in the second ("Moroccan") allele (Figure 3). At the protein level, the c.715G>A mutation results in replacement of the conserved alanine 239 residue by threonine (p.Ala239Thr, Figure 4). Interestingly, in two other nonrelated PCCA families in which both parents were of Jewish Moroccan ancestry, there were no *SepSecS* mutations or homozygosity at the *SepSecS* locus (data not shown), suggesting genetic heterogeneity of the phenotype.

We went on to test the frequency of carriers of the *SepSecS* mutations in the normal population: to create a restriction site that would enable easy analysis of the c.1001A>G mutation, we designed PCR primers in which the 5′-to-3′ (forward) primer bordered the mutation site and included one nucleotide change so that the c.1001A>G mutation would create a FokI restriction site. Using this primer set for amplification from genomic DNA gave a 151 bp fragment and generated FokI differential cleavage products of the mutant (121 bp and 30 bp) versus the wild-type (151 bp). For PCR amplification primers, see Table S2.

We analyzed the c.715G>A mutation by restriction analysis of *SepSecS* exon 6 with the restriction enzyme BsrI. The c.715G>A mutation abolishes a recognition site for BsrI and generates differential cleavage products of the wild-type (213 bp and 182 bp) versus mutant (395 bp) alleles amplified from genomic DNA (Table S2). Of 261 unrelated healthy Jewish Moroccan individuals, six were found to be carriers of the "Moroccan" mutation. Of 127 unrelated healthy Jewish Iraqi individuals, three were carriers of

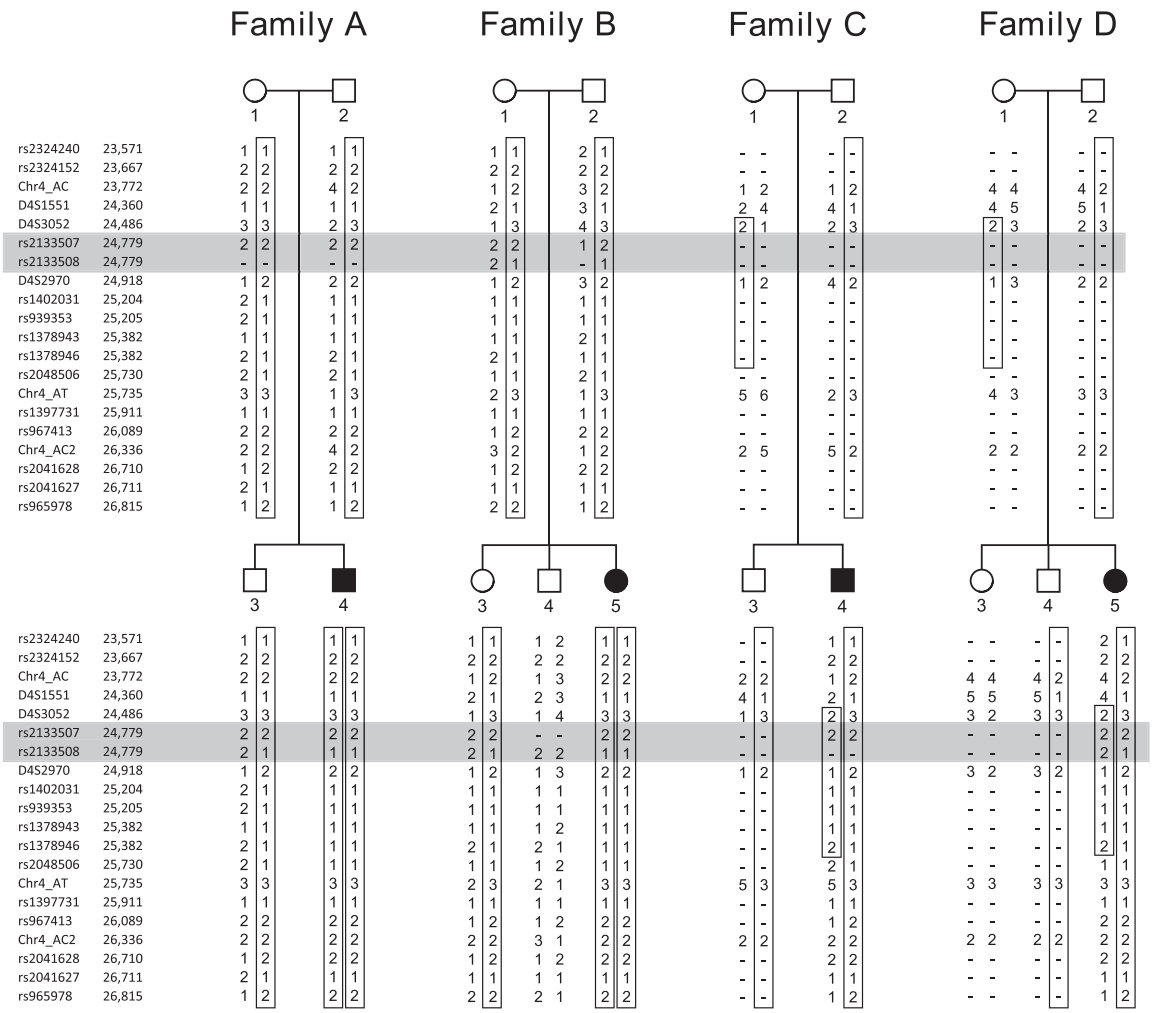

Figure 2. Genetic Mapping of the Disease-Associated Locus
Haplotypes of all four families on chromosome 4p15.2 are shown. The disease-associated haplotype is boxed. Additional microsatellite markers were used for fine mapping the candidate region identified by the genome-wide scan with the 10K SNP arrays. Families A and B are of Iraqi origin. Families C and D are of Iraqi and Moroccan origin. The minimum disease-associated haplotype lies between markers D4S425 and D4S391 for the "Iraqi" haplotype and between markers D4S1551 and rs2048506 for the "Moroccan" haplotype. Gray shading indicates the SepSecS position within the haplotype.

the "Iraqi" mutation. The "Moroccan" mutation was not found in any of the 127 Iraqi controls, and the "Iraqi" mutation was not found in any of the 261 Moroccan controls. None of the mutations was found in 192 healthy Ashkenazi Jewish controls, and no homozygous mutants were found in any of the normal controls.

The two mutations abrogate SepSecS activity in vivo in an anaerobic assay that has been readily used in the past for the characterization of archaeal,[5,7] human,[5,6] and trypanosomal[8] SepSecS proteins (Figures 5 and 6). In this system, both p.Ala239Thr and p.Tyr334Cys SepSecS mutants could not complement an *Escherichia coli* strain that lacks its endogenous selenocysteine synthase (SelA) activity (Figures 5 and 6). Further details are as follows: the human SepSecS mutants (p.Lys284Thr, p.Ala239Thr, and p.Tyr334Cys) were generated with the QuikChange site-directed mutagenesis kit (Stratagene) and cloned into the pET15b vector with an N-terminal His tag. Construction of the *E. coli* Δ*selA* deletion strain (DE3) and cloning of the *M. jannaschii PSTK* into the pACYC-Duet vector were described previously.[5] The human wild-type and mutant *SepSecS* were transformed into the *E. coli* Δ*selA* strain with the *M. jannaschii PSTK*. Aerobic overnight cultures were streaked on LB-agar plates supplemented with 0.01 mM isopropyl β-D-1-thiogalactopyranoside, 1 μM Na_2MoO_4, and 1 μM Na_2SeO_3 as described previously.[7] The cells were grown anaerobically for 20 hr at 37°C. The plates were then overlaid with agar containing 1 mg/ml benzyl viologen, 0.25 M sodium formate, and 25 mM KH_2PO_4 (pH 7.0). The appearance of a blue or purple color is the indication of active formate dehydrogenase H. It should be noted that the addition of high selenite levels (up to 0.1 mM) to the medium did not alter the result (data not shown). In vitro assays could not be performed because the SepSecS mutant proteins proved to be insoluble after expression.

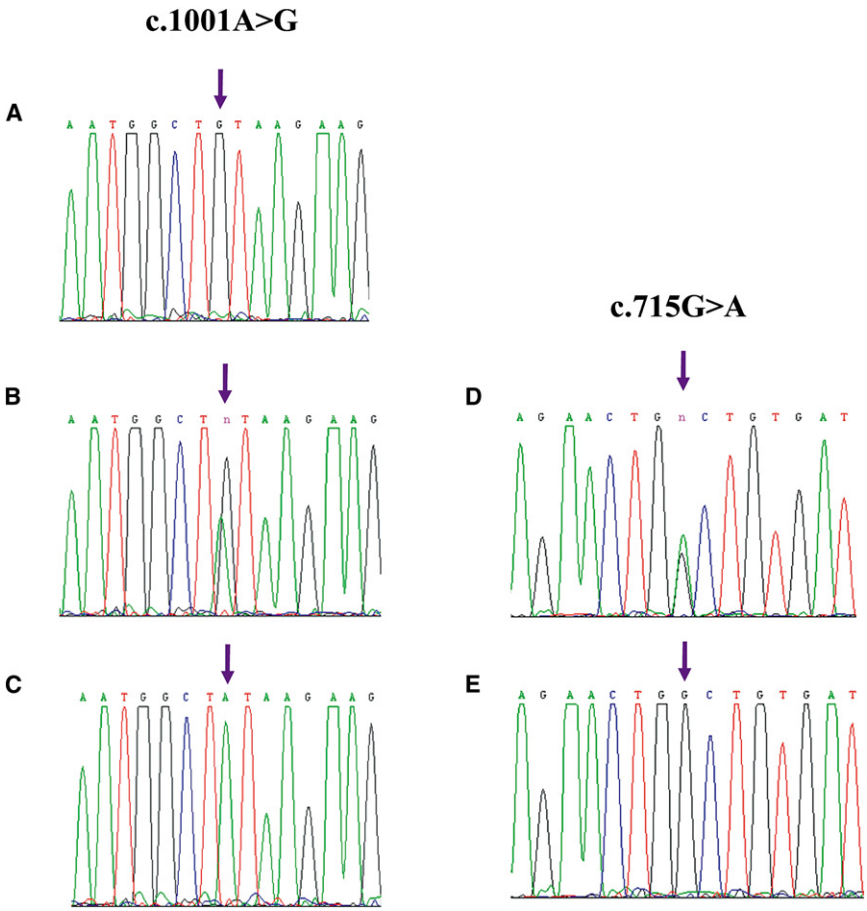

Figure 3. *SepSecS* Mutations in PCCA Families

(A–C) The c.1001A>G *SepSecS* mutation in exon 8. Sequence analysis is shown for an affected individual (A), a carrier (B), and an unaffected individual (C) in families A and B.

(D and E) The c.715G>A mutation in exon 6. Sequence analysis is shown for a carrier (D) and unaffected individuals (E) in families C and D. Affected individuals in families C and D were compound heterozygous for the two mutations.

The arrows indicate the positions of the mutations.

The human SepSecS protein catalyzes the final step in the biosynthesis of the 21st genetically encoded amino acid, selenocysteine.[5,6] Sec is the major form of the essential micronutrient selenium in the human body. Although chemically similar to cysteine, Sec is ionized at physiological pH and is thus more reactive. Hence, in Sec-containing enzymes (or selenoenzymes), Sec is an essential component of the active site.[9] The codon for Sec is UGA, which serves as a stop codon in nonselenoprotein mRNAs. The presence of a conserved stem-loop structure in the 3′-untranslated region of mammalian selenoprotein mRNAs directs recoding of UGA from stop to Sec in the translating ribosome.[10] The human selenoproteome comprises 25 members, whose biological functions have been implicated in diverse human diseases ranging from cardiovascular and endocrine disorders to abnormalities in immune responses and cancer.[11] Five human glutathione peroxidases (GPxs) and three thioredoxin reductases (TrxRs) are selenoenzymes that orchestrate the human antioxidant defense mechanisms.[9] They protect the cell from reactive oxygen species such as hydrogen peroxide and lipid hydroperoxides and are responsible for recycling nutritional antioxidants, such as vitamin C, vitamin E, and coenzyme Q, as well. Sec is also the catalytic residue in three iodothyronine deiodinases, the enzymes that potentiate circulating thyroxine (T4) by converting it to its active cellular form, triiodothyronine (T3), in peripheral tissues.[9] Mutation of the active-site Sec residue to Cys is detrimental for the catalytic activity of selenoenzymes.[12,13]

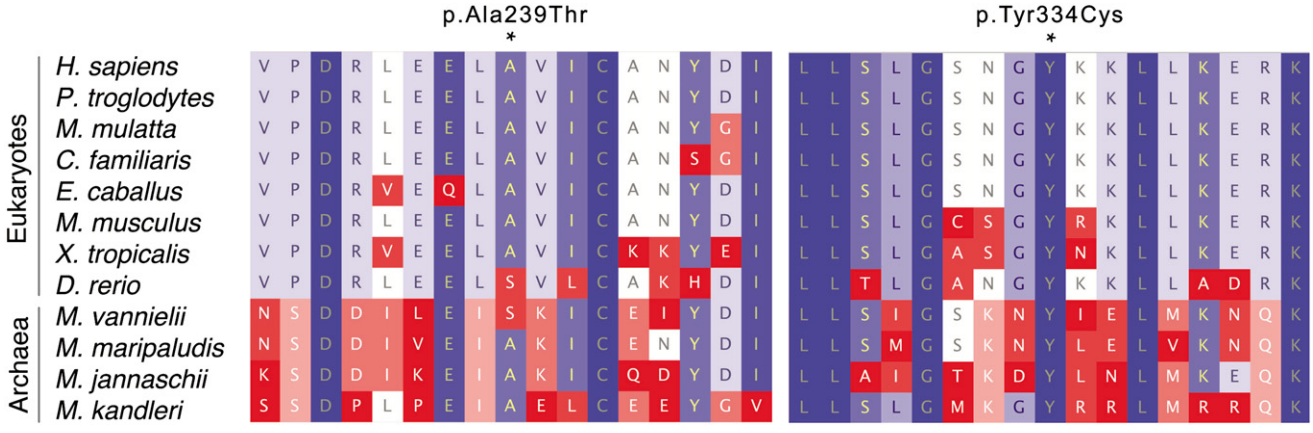

Figure 4. Alignment of the Mutated Sequence Regions in Some Eukaryotic and Archaeal SepSecS Proteins

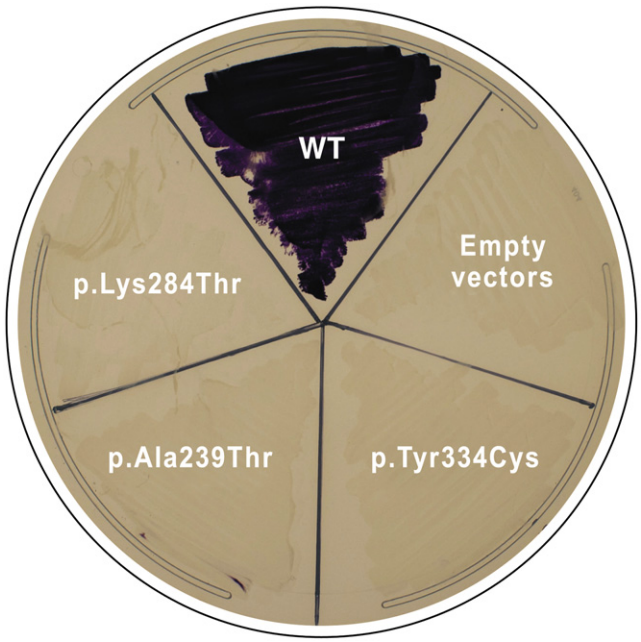

Figure 5. Functional Studies of the SepSecS Mutations
In vivo assays of human SepSecS mutants. Formation of Sec-tRNA^{Sec} in vivo is assayed by the ability of the wild-type human SepSecS and the mutant variants p.Lys284Thr, p,Ala239Thr, and p.Tyr334Cys to restore the benzyl-viologen-reducing activity of the selenoprotein FDH_H in the *E. coli* Δ*selA* deletion strain. The *Methanocaldococcus jannaschii* PSTK is cotransformed to generate the Sep-tRNA^{Sec} intermediate. K284T is a previously described inactive SepSecS mutant.

The pathway to Sec biosynthesis in humans has recently been deciphered,[5] and the structure of the human SepSecS in complex with the tRNA molecule that delivers Sec to the translating ribosome, tRNA^{Sec}, has provided key insights into the reaction mechanism.[6] Biosynthetic pathways starting from glycolysis or Krebs-cycle metabolic intermediates establish free cellular pools for all twenty standard amino acids. Sec is the sole exception to this paradigm; it is only made attached to tRNA^{Sec} in three enzymatic steps (Figure 6). Seryl-tRNA synthetase (SerRS) acylates serine to tRNA^{Sec}, and the resulting Ser-tRNA^{Sec} is converted by *O*-phosphoseryl-tRNA kinase to Sep-tRNA^{Sec}. In the presence of the cofactor pyridoxal phosphate and the selenium donor selenophosphate, SepSecS then converts Sep-tRNA^{Sec} to Sec-tRNA^{Sec}.

On the basis of our knowledge of SepSecS structure and function,[6,14] we expected the two founder mutations seen in Jews of Iraqi and Moroccan ancestry to disrupt folding and, thus, the catalytic function of the human SepSecS protein. In particular, introduction of Thr in place of Ala239 introduces steric clashes that disrupt interactions between two helices in the enzyme's core. Moreover, a Cys residue instead of Tyr in position 334 would disturb the architecture of the active site and the position of the catalytically essential pyridoxal phosphate. Indeed, these two mutations abrogate SepSecS activity in vivo and disrupt the sole route to Sec biosynthesis in humans and thus, the generation of selenoenzymes.

In contrast with the lethal phenotype of tRNA^{Sec}-null mutant mice,[15] affected humans with *SepSecS* mutations survive. This is concordant with many examples of different severity of phenotypes in mice and humans, and it is well in line with the human phenotype of PCH2, stemming from a defect in another tRNA. The progressive brain atrophy caused by the p.Ala239Thr and p.Tyr334Cys SepSecS mutations in affected individuals is in line with the known crucial role of selenium and selenoproteins in brain development and function. Compared to that of other organs, the selenium content of the brain is not particularly high.[16] Nevertheless, under dietary selenium deficiency, circulating selenoprotein P (SelP) ensures the preferential transport of selenium reserves to the brain at the expense of other organs.[17] It is thus crucial that selenium brain content remain stable.[17,18] Indeed, knockout of SelP[19] or its brain receptor ApoER2[20] leads to cerebral selenium deficiency and neurodegeneration phenotypes, such as ataxia and seizures, in mice.[21,22] Moreover, neuron-specific deletion of tRNA^{Sec} ablated expression of all 24 selenoproteins present in the mouse brain and resulted in a neurodevelopmental and degenerative phenotype of astrogliosis in the cerebral cortex, cerebellum, and hippocampus.[15]

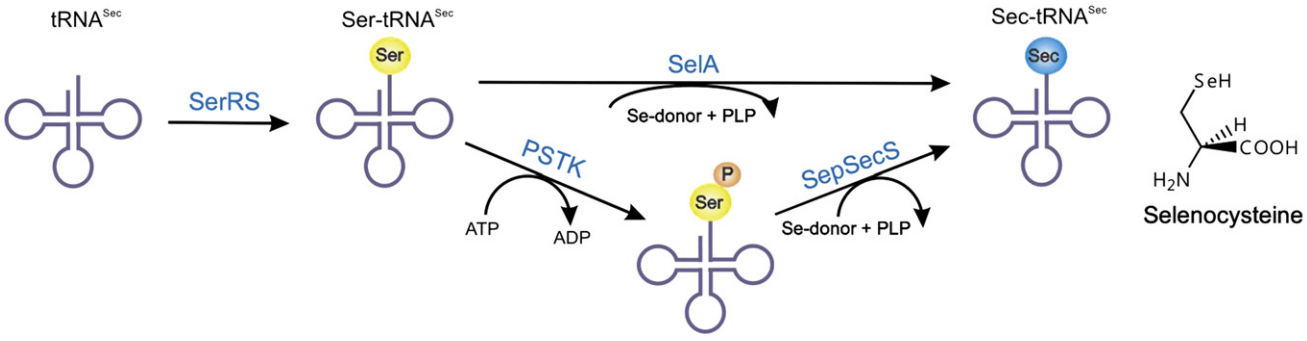

Figure 6. Structure of Selenocysteine and Its Route of Biosynthesis
Seryl-tRNA synthetase attaches serine to tRNA^{Sec}.
(Top) Bacteria use selenocysteine synthase (SelA) to convert Ser-tRNA^{Sec} directly to Sec-tRNA^{Sec}.
(Bottom) Eukaryotes and archaea employ a two-step conversion; a kinase (PSTK) provides the phosphorylated intermediate Sep-tRNA^{Sec}, the required substrate of SepSecS (see text).

Healthy expression of selenoproteins is, thus, crucial for neuronal function.[23] The cerebral and cerebellar neurodegeneration in mouse models of defective neuronal selenoprotein expression strengthens the notion that the PCCA phenotype caused by *SepSecS* mutations is the result of disrupted selenoprotein biosynthesis in the brain of affected individuals. It remains to be seen which selenoproteins are most crucial in the disease mechanism. Attempts at delaying the clinical progression of the PCCA phenotype with dietary selenium supplementation can be made, although our in vitro experiments suggest that this approach might be futile. Moreover, because selenocysteine is generated on its cognate tRNA, selenocysteine supplementation might also not be effective. Other, more complex strategies, circumventing the *SepSecS* defect, are being sought.

Supplemental Data

Supplemental Data include two tables and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
Primer3, (v. 0.4.0) Pick primers from a DNA sequence, http://frodo.wi.mit.edu/primer3/
Syndrome to Gene (S2G), (http://fohs.bgu.ac.il/s2g)
UCSC Genome Browser website, http://genome.ucsc.edu/

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