Forum Review Article

Title

Human disorders affecting the selenocysteine incorporation pathway cause systemic selenoprotein deficiency

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Abbreviated title for the running head

Human disorders resulting in selenoprotein deficiency

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Abstract

Significance

Generalised selenoprotein deficiency has been associated with mutations in *SECISBP2*, *SEPSECS* and *TRU-TCA1-1*, three factors crucial for incorporation of the amino acid selenocysteine (Sec) into at least 25 human selenoproteins. *SECISBP2* and *TRU-TCA1-1* defects are characterised by a multisystem phenotype due to deficiencies of antioxidant and tissue-specific selenoproteins, together with abnormal thyroid hormone levels reflecting impaired hormone metabolism by deiodinase selenoenzymes. *SEPSECS* mutations are associated with a predominantly neurological phenotype with progressive cerebello-cerebral atrophy.

Recent Advances

The recent identification of individuals with defects in genes encoding components of the selenocysteine insertion pathway has delineated complex and multisystem disorders, reflecting lack of selenoproteins in specific tissues, oxidative damage due to lack of oxidoreductase-active selenoproteins and other pathways whose nature is unclear.

Critical Issues:

Abnormal thyroid hormone metabolism in patients can be corrected by T3 treatment. No specific therapies for other phenotypes (muscular dystrophy, male infertility, hearing loss, neurodegeneration) exist as yet, but their severity often requires supportive medical intervention.

Future Directions.

These disorders provide unique insights into the role of selenoproteins in humans. The longterm consequences of reduced cellular antioxidant capacity remain unknown and future surveillance of patients may reveal time-dependent phenotypes (e.g. neoplasia, ageing) or

consequences of deficiency of selenoproteins whose function remains to be elucidated. The role of antioxidant therapies requires evaluation.

Introduction

Selenium is an essential micronutrient which is incorporated as the amino acid selenocysteine (Sec) into human selenoproteins, encoded by 25 separate genes. Most selenoproteins function as oxidoreductases, with the Sec residue involved in catalytic activity. Selenoproteins have diverse functions ranging from maintenance of redox potential, regulating redox sensitive biochemical pathways, protection of genetic material, proteins and membranes from oxidative damage, metabolism of thyroid hormones, regulation of gene expression and control of protein folding (Table1). However, the function of several selenoproteins is unknown (57). Biosynthesis of selenoprotein requires an UGA codon within its mRNA to be recoded as the amino acid Sec, preventing its recognition as a premature stop, possibly targeting the transcript for nonsense mediated decay (NMD) (57, 79). This process is achieved via an unique Sec-insertion machinery, comprising cis-acting SEleniumCysteine Insertion Sequence (SECIS) elements located in the 3'-UTR of selenoprotein mRNAs and the UGA codon, interacting with trans-acting factors (SECIS binding protein 2 (SECISBP2), Sec tRNA specific eukaryotic elongation factor (EEFSEC), Sec-tRNA$^{[Ser]Sec}$) (Figure 1) (4, 24, 33, 58, 85).

In contrast to other amino acids, selenocysteine does not have an aminoacyl-tRNA synthetase, but is synthesised on its own tRNA, encoded by *TRU-TCA1-1* (Figure 1) (5). This tRNA was originally named the human opal suppressor gene and a truncated pseudogene which is not expressed also exists (68). Delivery of to the ribosome and subsequent cotranslational insertion of Sec is mediated by a multiprotein complex (81) which includes EEFSEC and SECISBP2 as well as other factors. The specialised elongation factor EEFSEC (rather than general elongation factors eEf1a and EF-Tu, which delivers all other aa-tRNAs, binds and delivers Sec-tRNA$^{[Ser]Sec}$ to the ribosome acceptor site (85). SECISBP2 interacts with the SECIS element, a stem–loop structure present in the 3'-UTR present of every selenoprotein mRNA

(12, 13, 23). SECIS elements within each selenoprotein mRNA are distinct, but share common structural features, consisting of two helices separated by an internal loop, a GA quartet of non-Watson–Crick base pairs, and an apical loop, resulting in the adoption of a "kink-turn" structure (21, 33, 41, 89, 90). All selenoprotein mRNAs contain a single SECIS element, except for selenoprotein P (SELENOP) which has two, tandemly repeated SECIS elements. This configuration coincides with human SELENOP being the only selenoprotein which contains more than one selenocysteine residue (57). Whilst the SECIS element is required for Sec incorporation, other motifs in some selenoprotein mRNAs (e.g., Sec redefinition element, proximal stem loop element) have been described to contribute to the translation process (15, 22, 47, 48, 62).

The EEFSEC - Sec-tRNA$^{[Ser]Sec}$ - SECISBP2 complex, bound to SECIS in the selenoprotein mRNA, is thought to be in close proximity to the ribosomal complex, thereby preventing recognition of UGA as a translational stop and poised to mediate Sec incorporation into the polypeptide when the UGA codon is presented. Ribosomal protein L30 (RPL30), selenocysteine 1 associated protein 1 (TRNAU1AP), eukaryotic translation initiation factor 4A3 (EIF4A3) and nucleolin are other factors which regulate the Sec insertion process (Figure 1) (49, 57, 79). However, three factors, Sec-tRNA$^{[Ser]Sec}$, EEFSEC and SECISBP2, have been shown to be essential and sufficient for Sec incorporation *in vitro* (42, 59, 63, 82).

The biological importance of selenoproteins is highlighted by the fact that Trsp (mouse tRNA$^{[Ser]Sec}$) and Secisbp2 null mice are both embryonically lethal (19, 78). Mutations in individual human selenoproteins and their consequences have recently been reviewed in detail elsewhere (36): selenoprotein N (myopathy); glutathione peroxidase 4 (respiratory failure and skeletal defects); thioredoxin reductase 2 (associated with familial glucocorticoid deficiency and dilated cardiomyopathy); thioredoxin reductase 1 (generalized epilepsy); two reports

describe patients with a complex, hereditary spastic paraplegia and mutations in SELENOI, which catalyzes the transfer of phosphoethanolamine from CDP-ethanolamine to diacylglycerol to produce phosphatidylethanolamine (2, 46). Here, we describe mutations in genes (*SEPSECS*, *SECISBP2* and *TRU-TCA1-1*) encoding three components of the selenocysteine insertion pathway, affecting general incorporation of selenocysteine into selenoproteins and their clinical consequences.

SECISBP2

SECISBP2 is an obligate limiting factor for selenoprotein synthesis, as first shown by absence of selenoprotein synthesis in SECISBP2-depleted cell lysates, with restoration of production by repletion with SECISBP2 (23, 24). Human SECISBP2 is a large (854 amino acids, 120-kDa) protein, with the first 400, amino (N-) terminal, residues being dispensable for its function *in vitro* (25, 26) (Figure 2). In contrast, the C-terminal region (amino acids 399-784) is both necessary and sufficient for SECIS-binding and Sec incorporation *in vitro* and contains several functional domains. The Sec incorporation domain (SID), located centrally in SECISBP2, is not essential for SECIS binding but required for Sec incorporation. The RNA binding domain (RBD) contains an L7Ae-type RNA interaction motif identified in a large family of ribosomal proteins (e.g., RPL30, SUP1, eRF-1 and 15.5-kD/Snu13p) (4, 5, 16, 25, 26), which interacts with the "kink-turn" structure adopted by the SECIS-element. The RBD mediates interaction with the SECIS element (34, 90) and 28S ribosomal RNA (25, 49, 56, 59). A domain N-terminal to the L7Ae module, referred to as either the bipartite, SID or K-rich region, is involved in specific recognition of SECIS elements and other regulatory RNA motifs, thereby also controlling selenoprotein expression levels (17, 30, 83). SECISBP2 also contains several other functional motifs shown in Figure 2 (69).

Alternative splicing events in the 5'-region of human *SECISBP2*, with use of alternative initiation of translation from downstream ATG start codons in exons 2, 3a, 3b, 5 and 7, generates five different protein isoforms, each containing varying N-terminal protein sequence (Figure 2) (24, 70). These alternate splicing events alter content of the dispensable N-terminal region, but not the essential C-terminal domain, within protein isoforms. Nevertheless, it is possible that the alternately spliced isoforms do play a role in regulation of SECISBP2-dependent Sec incorporation and selenoprotein expression in *vivo*. During protein synthesis dynamic interaction of SECISBP2 with the ribosome and SECIS element is essential for recruitment of the EEFsec/Sec-tRNA$^{[Ser]sec}$ complex to the UGA codon, enabling incorporation of Sec into the polypeptide.

Homozygous or compound heterozygous mutations in *SECISBP2* have been described in 13 individuals from 11 families (Table 2). Disruption of SECISBP2 function prevents appropriate Sec incorporation into selenoproteins during their biosynthesis resulting in a multisystem disorder due to deficiency of diverse selenoproteins (Table 3). The biochemical signature which identifies SECISBP2-deficient patients consists of low circulating selenium (reflecting low plasma SELENOP and GPX3) and abnormal thyroid hormone levels due to diminished activity of deiodinases (Table 3) (31, 75). Most cases present in childhood due to growth retardation with raised circulating FT4, normal to low FT3 and raised reverse T3 levels, reflecting deficiency of all three deiodinase enzymes.
Muscle weakness, due to progressive rigid spine muscular dystrophy, affecting axial and proximal limb muscles with raised creatine kinase (CK) levels and fatty infiltration on imaging, is similar to that seen in patients with mutations in selenoprotein N. In one patient (proband E), male azoospermic infertility was described, reflecting loss of testis selenoproteins (mitochondrial GPX4, thioredoxin reductase, selenoprotein V) required for spermatogenesis (75).

Significantly decreased expression levels of antioxidant selenoenzymes are associated with increased levels of cellular reactive oxygen species (ROS). Clinical consequences of raised cellular ROS include skin photosensitivity, progressive sensorineural hearing loss and possibly increased total body adipose tissue mass paradoxically associated with enhanced systemic insulin sensitivity (75).

Reduced red blood cell and total lymphocyte counts, with impaired mononuclear cell cytokine secretion and T-cell proliferation (similar to findings in T cell-specific Trsp null mice (80)), were recorded in one case, proband E (75). Although other haematological and immune cell phenotypes have not been formally evaluated, immunodeficiency is not a reported feature in other *SECISBP2* mutation cases nor seen in mouse models of selenoprotein deficiency. Additional age-dependent phenotypes such as neurodegeneration, premature ageing or neoplasia may emerge but have not been described hitherto.

Oral selenium supplementation in some *SECISBP2* patients raised total serum Se levels, but without clinical effect (9, 20, 28) or altering synthesis (circulating GPX's, SELENOP) or action (thyroid hormone metabolism) of selenoproteins (77). Treatment of probands C and F with T3 alone (T4 was not effective in one case) or in combination with growth hormone (proband G) resulted in an improvement in growth, development and bone maturation. Treatment of proband G with a combination of alpha tocopherol (vitamin E) and T3 resulted in the most promising response, with decreased serum levels of lipid peroxidation products, altered FT4 and FT3 concentrations and increased circulating white blood cells and neutrophils, all of which reversed after treatment withdrawal (74). These observations suggest that treatment with antioxidants, to counteract effects of elevated cellular ROS, are the best available therapeutic option for this disorder.

Most *SECISBP2* mutations identified to date cause premature stop codons, resulting in absence of full length SECISBP2 protein. However, elegant minigene experiments have shown that, for premature stops located in the N-terminal part of the protein, initiation of translation from alternative, downstream ATG codons in exons 5 (Met233) and 7 (Met300), permits low-level synthesis of shorter SECISBP2 isoforms (28, 75). Some premature stop mutations are situated downstream of Met300 and may completely eliminate synthesis of functional protein. However, stop mutations (e.g. R770X, Q782X), distal to the RBD might generate C-terminally truncated proteins whose RNA binding and nuclear localisation functions remain, partially, intact (Figure 2). In a patient with defective mRNA splicing due to an intronic mutation IVS8ds+29G>A (31) it has been shown that levels of normally spliced transcript are only reduced by 50% and if a similar mechanism operates with other splice site mutations, this suggests some preservation of normally spliced SECISBP2 mRNA in such cases.

Three missense *SECISBP2* mutations (R540Q, C691R and E679D) have been described: The R540Q mutation localises to the K-rich region within the RBD (Figure 2), with the R540Q mutant exhibiting reduced binding to SECIS elements in GPX1 and DIO2 mRNAs, correlating with diminished GPX1 and DIO2 enzyme activity in patient-derived primary cells and mouse model. Detailed analyses suggests that R540Q mutant SECISBP2 fails to bind only a subset of SECIS-elements, consistent with the K-rich region mediating recognition of specific (type I and type II) SECIS elements (14) and a mouse model revealed a possible tissue specific pattern of SECISBP2 protein stability correlating with varying loss or preservation of expression of different selenoproteins (14, 31, 96).

The C691R SECISBP2 mutation, also located in its RBD, is expected to affect RNA binding. Homology modelling, based on the crystal structure of the spliceosomal 15.5 kDa protein (87), suggests that mutation of cysteine to a bulky and charged arginine residue (75) may destabilise

its hydrophobic core, disrupting local protein structure (Figure 2 B and C). In vitro assays, showing enhanced proteasomal degradation of the C691R mutant SECISBP2 protein, confirmed this (75). A mouse model suggests that the C691R mutant SECISBP2 is unable to bind RNA and non-functional (96). The E679D SECISBP2 mutation is predicted to be deleterious (PolyPhen-2 algorithm score of 0.998 and also located in the RBD and may therefore affect its RNA binding function, but this has not been investigated in detail (37) (Figure 2).

Knowledge that knockout of Secisbp2 in mice is embryonic lethal (78), with no evidence for an alternative Sec-incorporation mechanism in humans, suggests that there is some residual SECISBP2 activity in all patients. All patients described to date are expected to harbour at least one allele which directs synthesis of SECISBP2 at either reduced levels or which is only partially functional, in combination with either a mutant or shorter form of the protein synthesised from Met300 (Table 2). A limited number of patients, mostly compound heterozygous for different *SECISBP2* mutation combinations, with limited knowledge of phenotypes in heterozygous relatives, have been described, making it difficult to assess the effect of a specific *SECISBP2* mutation or its correlation with severity of phenotype. However, since SECISBP2 is rate limiting for Sec incorporation, a significant reduction in functional SECISBP2 protein levels will result in diminished but not complete loss of selenoprotein synthesis. A further variable is that differences in architecture of SECIS elements within different selenoprotein mRNAs may dictate the extent to which reduced SECISBP2 protein limits their biosynthesis and expression levels *in vivo*, as suggested by elegant experiments testing SECISBP2 with luciferase reporter genes containing different SECIS elements (60, 84). Future studies, undertaking ribosomal RNA (96) or selenoprotein expression profiling in secisbp2 mutant mouse models and *in vitro* reconstitution experiments with different mutant SECISBP2 proteins and SECIS element containing luciferase reporter genes (84), may help us better understand the effect of specific

SECISBP2 mutations. In turn, greater understanding of how different mutations affect selenoprotein expression, may enable better prediction of clinical outcome or targeting of therapy in patients.

TRU-TCA1-1

Selenocysteine is synthesised on its own tRNA (Figure 1), $tRNA^{[Ser]Sec}$ (encoded by *TRU-TCA1-1*) which has several unique features, being longer (90 nucleotides versus usual 78), with an atypical long acceptor- and D-stem with few modified bases (57) that distinguishes it from other tRNAs (Figure 3A). Its promoter region, containing both tRNA and U snRNA gene regulatory elements (18, 39, 44) forming a new class of RNA polymerase III transcribed genes, also differs from other tRNA genes (Figure 3B) and consists of four regulatory elements: an activator element, containing an SPH motif, but is octamer independent; the proximal sequence element; an extended TATA-motif, essential for efficient transcription and an internal B box (64, 65).

Two major isoforms of $Sec\text{-}tRNA^{[Ser]Sec}$ have been identified, containing either 5-methoxycarbonyl-methyluridine (mcm^5U) or its methylated form 5-methoxycarbonylmethyl-2'-O-methyluridine (mcm^5Um) at position 34 (Figure 3C). Uridine 34 is located in the anticodon loop and its methylation may contribute to stabilization of the codon-anticodon interaction (19, 27, 45, 79). Methylation at uridine 34 is executed by a methylase whose identity is not known and the relative abundance of the two $Sec\text{-}tRNA^{[Ser]Sec}$ isoforms is known to be influenced by systemic Se-status, increasing selenium levels resulting in more mcm^5Um isoform (19, 27, 45). Each $Sec\text{-}tRNA^{[Ser]Sec}$ subtype has a different role, with synthesis of cellular housekeeping selenoproteins (e.g., TXNRD1, TXNRD3, GPX4) being dependent on the mcm^5U isoform, whereas expression of cellular, stress-related selenoproteins (e.g., GPX1, GPX3, SELENOW), requires the mcm^5Um isoform (19, 79). Methylation of mcm^5U, the final step in

posttranscriptional maturation of Sec-tRNA$^{[Ser]Sec}$, is dependent on correct aminoacylation (53, 54), intact secondary/tertiary structure (53) and other, prior, base modifications (Figure 3A) of the transfer RNA (27).

A single patient, homozygous for a single nucleotide change (C65G) in *TRU-TCA1-1* has been identified (Figure 3A) (76). The proband exhibits a similar phenotype to that seen in *SECISBP2* mutation patients. However, comparison of cellular selenoprotein expression profiles in the two disorders has revealed differences, with expression of relative housekeeping selenoproteins (e.g., TXNRDs, GPX4) being more preserved than in *SECISBP2* cases. In contrast, expression of stress-related selenoproteins (e.g., GPX1, GPX3) was similarly reduced in both disorders. In primary cells from the *TRU-TCA1-1* mutation patient, lower total tRNA$^{[Ser]Sec}$ expression with disproportionately greater diminution in Sec-tRNA$^{[Ser]Sec}$ mcm^{5}Um levels was observed, with decreased i^{6}A modification at position 37 suggesting that its posttranscriptional maturation is impaired. The mutation had no effect on tRNA$^{[Ser]Sec}$ aminoacylation with serine or Sec synthesis or its interaction with SEPSECS. Low levels of tRNA$^{[Ser]Sec}$ in the proband were insufficient to direct normal synthesis of stress-related selenoproteins, but were not rate limiting for adequate synthesis of some housekeeping selenoproteins and similar, differential preservation of selenoprotein synthesis has been observed in murine tRNAsec mutant models (19, 57).

The human SEPSECS - tRNA$^{[Ser]Sec}$ structure shows that C65 is situated in the acceptor arm, next to C64 in TΨC–arm (Figure 3D). C64 interacts with Lys38 and Glu37 in helix 1 of the non-catalytic part of the SEPSECS moiety (Figure 3E), raising the possibility that the C65G mutation could affect the stability of the Sec-tRNA$^{[Ser]Sec}$ - SEPSECS complex and the selenoprotein synthesis.

In summary, these observations indicate that reduction in Sec-tRNA$^{[Ser]Sec}$ levels, with particular deficiency of the Sec-tRNA$^{[Ser]Sec}$ mcm^{5}Um subtype, contributes to the selective pattern of selenoprotein deficiency seen in the proband. The precise mechanism mediating reduction in mutant Sec-tRNA$^{[Ser]Sec}$ presence remains unclear, with defective posttranscriptional modification of mutant Sec-tRNA$^{[Ser]Sec}$ or instability of the mutant Sec-tRNA$^{[Ser]Sec}$ -SEPSECS complex being possibilities.

SEPSECS

Human SEPSECS was initially identified as an autoantigen (soluble liver antigen/liver pancreas, SLA/LP) in autoimmune hepatitis (52). Subsequent studies in which mammalian cell extracts were treated with autoimmune hepatitis patients' serum, showed that SEPSECS co-precipitated with Sec-tRNA$^{[Ser]Sec}$, as part of a ribonucleoprotein complex (40). This led to the identification of SEPSECS as the enzyme which catalyses conversion of O-phosphoserine-tRNA$^{[Ser]Sec}$ to Sec-tRNA$^{[Ser]Sec}$, using selenophosphate as donor substrate (81, 93) (Figure 1).

Crystal structures of the archaeal and murine Sepsecs apo-enzymes as well as human wild type and mutant SEPSECS complexed with Sec-tRNA$^{[Ser]Sec}$ have been solved, suggesting that SEPSECS is a distinct member of the fold type I family of pyridoxal phosphate-dependent enzyme family (7, 38, 71, 73). The human structure shows a complex containing a SEPSECS tetramer binding two Sec-tRNA$^{[Ser]Sec}$ molecules through their long acceptor-TΨC arms, with the non-catalytic SEPSECS dimer mediating RNA-protein interactions which stabilize the complex and the CCA end of Ser-tRNA$^{[Ser]Sec}$ residing in the active site of the catalytic SEPSECS dimer (Figure 3D, 4B). The conversion of Ser-tRNA$^{[Ser]Sec}$ to Sec-tRNA$^{[Ser]Sec}$ by SEPSECS is pyridoxal-5-phosphate (PLP) cofactor dependent, with the proposed mechanism involving a

conformational change in SEPSECS upon Ser-tRNA$^{[Ser]Sec}$ binding, enabling the phosphoserine of Ser-tRNA$^{[Ser]Sec}$ to be oriented correctly for conversion to occur (71).

Homozygous and compound heterozygous mutations in *SEPSECS* (Table 4, Figure 4, 5) are associated with profound intellectual disability, global developmental delay, spasticity, epilepsy and hypotonia with progressive microcephaly due to cortical and cerebellar atrophy on magnetic resonance imaging (1). Additional phenotypes described in other patients include axonal neuropathy, optic atrophy and early onset epileptic encephalopathy with burst suppression (6, 67, 72). The timing of presentation in patients with *SEPSECS* mutations is variable, ranging from severe prenatal onset to delayed postnatal presentation and a mild, late onset phenotype in three patients (50, 86). This disorder is classified as autosomal recessive pontocerebellar hypoplasia type 2D (PCH2D, OMIM # 613811), also known as progressive cerebellocerebral atrophy (PCCA) (1, 11). Mice, homozygous for a *SEPSECS* mutation (Y334C) present with Sedaghatian

-type spondylometaphyseal dysplasia and die shortly after birth, in contrast to humans with the same mutation. This divergence in phenotype may be due to species differences, dietary environment or the genetic background of the mouse line used (35).

The effect of *SEPSECS* mutations on selenoprotein expression has only been studied in 4 patients (Family E, G, F; 6). In brain tissue levels of TXNRD1, TXNRD2, GPX1 and GPX4 proteins are reduced, correlating with increased cellular oxidative stress. However, selenoprotein deficiency is not generalised with normal TXNRD levels in patient's fibroblast and muscle cells suggesting that residual SEPSECS activity preserves selenoprotein synthesis in some tissues or existence of alternative pathway(s). Consistent with findings in human tissues Sepsecs mutant mice exhibit decreased Gpx4 expression in neurons but not hepatocytes (35).

Although the selenium content of brain is not high (95) it is kept very stable (10, 66) exemplified by the fact that in systemic selenium deficiency circulating selenoprotein P delivers this trace element preferentially to this organ at the expense of other tissues (66). This may explain why a reduction in SEPSECS activity could have a greater effect on brain development and function compared to other tissues.

It is remarkable that an overt central nervous system phenotype is not reported in patients with *SECISBP2* and *TRU-TCA1-1* defects, but with most individuals with these mutations being children the possibility of a late onset neurological phenotype cannot be discounted. However, a neurodegenerative phenotype has been described in brain-specific *Gpx4*, *Trsp* and *Secisbp2* KO mouse models suggesting that Se deficiency and/or reduced redox capacity has a larger impact on the mouse brain compared to the human (78, 91, 92).

Biochemical hallmarks of selenoprotein deficiency in *SECISBP2* and *TRU-TCA1-1* mutation cases include low circulating selenium and abnormal thyroid hormone levels, reflecting deficiency of circulating selenoproteins (SEPP, GPx3) or all three deiodinase enzymes respectively (31, 75). In *SEPSECS* mutation patients low serum selenium is not reported to be part of the phenotype and thyroid status has only been partially investigated in four patients, documenting either normal thyroid hormone (Family H; 72) or normal T4 but elevated TSH levels (Family E, G, F; 6). However more detailed investigation, with measurement of T3 or reverse T3 levels, which would be abnormal with decreased deiodinase enzyme activity, has not been undertaken in *SEPSECS* mutation cases (6). Myopathic features with raised CK levels, abnormal mitochondria, cytoplasmic bodies and increased lipid accumulation in muscle have been documented in one *SEPSECS* mutation case (Family H; 72), with broad-based gait and postural instability suggesting muscle weakness in another patient (Family O, 86). Similar findings have been noted in adult *SECISBP2* mutation patients, reflecting deficiency of

selenoprotein N and altered redox capacity in skeletal muscle (75). Overall, these observations suggests that some *SEPSECS* mutation patients can exhibit phenotypes associated with more global deficiency of selenoproteins. It is also possible that severity of neurological problems in patients has precluded detailed investigation and ascertainment of non-neurological phenotypes.

The availability of the crystal structure of human SEPSECS - $tRNA^{[Ser]Sec}$ (71, 73) (Figure 4 and 5) together with an SEPSECS activity assay using an *E. coli* strain lacking endogenous Sec-synthase (SelA) activity (94), has enabled detailed studies of some *SEPSECS* (A239T, Y334C, T325S and Y429X) mutations (1, 6, 73). These pathogenic variants were found to be less soluble than WT protein *in vitro*, with loss of functional activity. A239T mutant SEPSECS failed to form stable tetramers, possible as result of a steric clash destabilizing two helices (H8-H9) within the enzyme's core. Y334C and T325S SEPSECS mutants are predicted to fold like wild type SEPSECS in the crystal structure and retain binding to $tRNA^{[Ser]Sec}$ (73) but affect its catalytic pocket, reducing enzyme activity (1, 6, 73). Mutation of Tyr334 to Histidine is also recorded (6) and *in silico* analysis predicts that this variant is likely to have a similar deleterious effect as the Y334C mutation (Figure 5). The premature stop SEPSECS mutant (Y429X) is insoluble and inactive (6, 73) and three other premature stop mutants (Table 4) can be expected to have a similar effect.

I*n silico* analysis predicts that the A59V SEPSECS mutation results in steric hindrance destabilising helix 2 and helix 3, possible affecting its catalytic function and/or dimerization (Figure 5). The N119S SEPSECS mutation is a conservative amino acid substitution with a probable small effect, weakening an H-bond network in the non-catalytic dimer possibly affecting RNA interaction. Likewise, mutation of Arg156 to Glutamine in SEPSECS is a conservative change, predicted to perturb local structure minimally and possibly reducing activity of the catalytic site (Figure 5). The G441R SEPSECS mutation, situated close to the

catalytic site, changes the side chain of this amino acid from small and neutral to large and polar, but as Gly441 is situated within a loop, its possible effect on catalytic activity might be limited. Absence of two SEPSECS mutations (M1V, D489V) from crystal structures precludes *in silico* analyses: M1V affects the first methionine in SEPSECS and unless an alternative start codon (e.g., position 61) is used, no protein will be generated; the D489V mutation changes the size and charge of this amino acid and can therefore be expected to have a major impact on protein function/stability.

Three SEPSECS mutation patients (patient I (R26Pfs*42 - N119S); J (N119S - R156Q); O (G441R) presented with late onset PCH2D, with progressive but milder degree of CNS atrophy (50, 86). I*n silico* analyses suggest that these mutations have a less deleterious effect on SEPSECS function (Figure 5), but it is also conceivable that environmental factors or patients' genetic background may have modulated their phenotype. Future studies need to investigate the relationship between mutations, their effect on SEPSECS protein function and general expression of selenoproteins in different tissues and patient phenotypes in more detail.

Conclusions

SECISBP2, Sec-tRNA$^{[Ser]Sec}$ and SEPSECS are essential components of the selenoprotein biosynthesis pathway. Unsurprisingly, in patients, harbouring mutations in any of these genes, expression of most members of the selenoproteome is affected, sometimes in a tissue specific manner, resulting in a complex, multisystem phenotype. The combination of the nature of the gene defect, genetic/ethnic background of individuals and environmental factors (e.g., selenium and/or iodine status), might also contribute to inter-individual differences in phenotypes. Further complexity is due to the fact that most patients harbour compound heterozygous mutations with monoallelic mutations in individuals having no reported

phenotype. This makes it difficult to assess the effect of a particular mutation on the Sec-incorporation pathway. Although a substantial body of knowledge regarding the individual functions of SECISBP2, Sec-tRNA$^{[Ser]Sec}$ and SEPSECS exists we do not have a comprehensive understanding of the Sec-insertion pathway. Nevertheless most observations in patients with gene defects accord with our current knowledge of this pathway. However, it is interesting that mutations in *SECISBP2* and *TRU-TCA1-1* present with similar phenotypes (growth retardation and myopathy together with abnormal thyroid function), whereas the dominant phenotype in *SEPSECS* mutation cases is progressive cerebellocerebral atrophy.

In all patients some phenotypes (e.g., photosensitivity, age-dependent hearing loss, neurodegeneration) are clearly progressive, perhaps reflecting absence of antioxidant selenoenzymes resulting in cumulative oxidative damage to DNA, proteins and membrane lipids and dysfunction of redox-dependent signalling pathways. Some phenotypes can clearly be linked to deficiency of specific selenoproteins (e.g., abnormal thyroid function and DIO1,2,3; low plasma Se and SELENOP, GPX3; azoospermia and SELENOV, GPX4, TXRND3; myopathy and SELENON).

However, the precise role of many selenoproteins in human biological processes is unknown, making identification of causal links between altered expression of specific selenoproteins and human disease a particular challenge. Furthermore, the role of individual selenoproteins needs to be analysed in the context of a complex cellular biochemical environment, where antagonistic, additive and synergistic effects can occur. Recent advances including analysing selenoprotein expression in mouse models, RNA ribosome profiling (96) *in vitro* technologies like CRISPR-Cas9-VLP (88), dissection of SECIS and other functional RNA elements (22, 62) using luciferase-based reporter assays (84), modelling using crystal structures (e.g., SEPSECS - Sec-tRNA$^{[Ser]Sec}$, EEFSEC) (29), can help us better understand the complex Sec-insertion

pathway in general and more specifically the effect of mutations in genes within this pathway and their consequences on selenoprotein expression. This knowledge will provide the essential basis for understanding the pathogenesis of human disease due to generalised or specific selenoprotein deficiencies and may enable identification of therapies targeted at specific processes (e.g. oxidative stress) in which selenoproteins play a key role.

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Disclosure Statement

No competing financial interests exist.

List of Abbreviations

CRD	Cysteine rich domain
C-terminal	carboxy-terminal
FT3	free triiodothyronine
FT4	Free thyroxine
NES	nuclear export signals
NLS	nuclear localisation signal
NMD	nonsense mediated decay
N-terminal	amino-terminal

PCCA	progressive cerebellocerebral atrophy
PCH	Pontocerebellar Hypoplasia
PCH2D	pontocerebellar hypoplasia type 2D
PLP	pyridoxal-5-phosphate
RBD	RNA binding domain
ROS	reactive oxygen species
Sec	selenocysteine
SECIS	SEleniumCysteine Insertion Sequence
SECISBP2	SECIS binding protein 2
SID	Sec incorporation domain
SID	Sec incorporation domain
SLA/LP	soluble liver antigen/liver pancreas
SPH motif	proximal sequence element
T3	triiodothyronine
T4	thyroxine
TSH	thyroid stimulating hormone

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Figure legends Erik Schoenmakers

Figure 1 Pathway of selenocysteine synthesis and its incorporation into selenoproteins.

Sec is synthesised on its own tRNA ($tRNA^{[Ser]Sec}$), which undergoes maturation through sequential modifications, with initial attachment of serine by seryl-tRNA synthetase (SARS) resulting in $Ser\text{-}tRNA^{[Ser]Sec}$. Subsequent phosphorylation of this serine residue by phosphoseryl-tRNA kinase (PSTK) generates $O\text{-}Phosphoseryl\text{-}tRNA^{[Ser]Sec}$. Finally, O-phosphoserine tRNA:Sec tRNA synthase (SEPSECS) catalyses the acceptance of a selenophosphate, generated from selenide and ATP by selenophosphate synthetase 2 (SEPHS2), resulting in $Sec\text{-}tRNA^{[Ser]Sec}$. An intermediate complex which includes $Sec\text{-}tRNA^{[Ser]Sec}$, tRNA selenocysteine 1 associated protein 1 (TRNAU1AP) and EEFSEC, is formed subsequently. This complex is guided by interaction with SECISBP2 to the SECIS-element of selenoprotein mRNA, ready for incorporation into the nascent polypeptide. Other factors (ribosomal protein L30, eukaryotic initiation factor eIF4a3, nucleolin, …) also have regulatory roles and influence the Sec insertion process. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

Figure 2. A. The organisation of the SECISBP2 gene (top), with naturally-occuring aminoterminal splice variants, each containing distal exons 8 to 17 shown below; the functional domains of SECISBP2 protein with the location of human mutations superimposed is shown (bottom). Arrowheads denote the location of ATG codons, which could function as alternative sites for initiation of translation. Functional domains in SECISBP2 protein: N-terminal domain (1-399); minimal functional protein (shaded grey, 399-784); Sec incorporation domain (SID, 399-517); minimal RNA-binding domain (RBD,

517-784); Lysine-rich domain involved in RNA specificity and ribosome binding (517-544); L7Ae homology module (620-745); nuclear localisation signal (NLS, 380-390); redox-sensitive cysteine-rich domain (CRD, 584-854) and two nuclear export signals (NES1: 634-657; NES2: 756-770) (69).

B. and C. Model of the L7ae RNA-binding domain of SECISBP2. The position of wild type residues (E679, C691) (panel B) and corresponding mutations (E679D, C691R) at these locations (panel C) is shown. The model was generated using the phyre2 (Protein Homology/analogy Recognition Engine 2) web portal, which predicts and analyses protein structures based on homology/analogy to solved protein crystal structures (51) The figures were generated with MacPyMOL Molecular Graphics System, Schrödinger, LLC.

Figure 3 A. The primary structure of human Sec-tRNA$^{[Ser]Sec}$ is shown in a cloverleaf model, with the location of C65G *TRU-TCA1-1* mutation identified in the patient indicated (circled red). The acceptor stem constitutes paired 5' and 3' terminal bases, with the D arm, the anticodon arm, the variable arm and the TψC arm depicted. Mammalian Sec-tRNA$^{[Ser]Sec}$ undergoes posttranscriptional modification at positions 34 (mcm5U or mcm5Um), 37 (i6A), 55 (ψ) and 58 (m1A). **B.** Schematic of the *TRU-TCA1-1* gene showing the coding region (black box) and regulatory elements (grey boxes) (43) with the location of C65G mutation identified in the patient. **C**. The two Sec-tRNA$^{[Ser]Sec}$ isoforms, containing either mcm5U or mcm5Um modifications of the uracil at position 34 in the anticodon arm, differ from each other by a single methyl group on the 2'-O-ribosyl moiety. This reaction is catalysed by an unknown methylase and abundance of the mcm5Um isoform increases with selenium concentration (19). **D.** Crystal structure showing catalytic and non-catalytic dimers of the SEPSECS tetramer bound to tRNA$^{[Ser]Sec}$ (71), with the position

of the C65 nucleotide indicated. Other nucleotides in $tRNA^{[Ser]Sec}$ (cyan) and amino acids in SEPSECS (yellow) involved in RNA-protein interaction are also highlighted, as are the nucleotides (red) towards the position of Sec and the pyridoxal-5-phosphate substrate (green) within the catalytic domain. **E.** A close-up of the structure around C65, showing H-bonds (dashed green lines) formed between C64 and the C64-C65 backbone with residues (E37 and K38) situated in helix 1 (H1) of the non-catalytic dimer of SEPSECS. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

Figure 4. A. The organisation of human *SEPSECS* gene (top) and schematic of SEPSECS protein (bottom) with the location of human mutations superimposed. Arrowhead denotes the location of the ATG start codon. **B.** Crystal structure showing a single catalytic – non-catalytic dimer from the complex bound to $tRNA^{[Ser]Sec}$, with the position of all the human SEPSECS point mutations superimposed (71). Mutations associated with early onset (red) or late–onset (yellow) disease and the pyridoxal-5-phosphate (cyan) substrate are highlighted. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

Figure 5. Detailed views comparing wild type SEPSECS crystal structure and mutated amino acids modelled in the SEPSECS crystal structure which cause either early-onset (red) or late onset (yellow) disease (71). Hydrogen bonds (dotted green lines), pyridoxal-5-phosphate substrate (cyan), tRNA (orange) and H_2O (blue) are shown. The helices (H), beta-sheets (β), amino acids and nucleotides involved in hydrogen bond networks or which are part of the active catalytic domain are labelled. Crystal structures for T325S and Y334C SEPSECS mutants are available and in the panels with these mutations an overlay of wild type (grey) and mutant (cyan) is shown (73). For more detailed

information see text. The model was generated using the phyre2 web portal, which predicts and analyses protein structures based on homology/analogy recognition to solved protein crystal structures (51). The figures were generated with MacPyMOL Molecular Graphics System, Schrödinger, LLC. To see this illustration in color, the reader is referred to the online version of this article at www.liebertpub.com/ars.

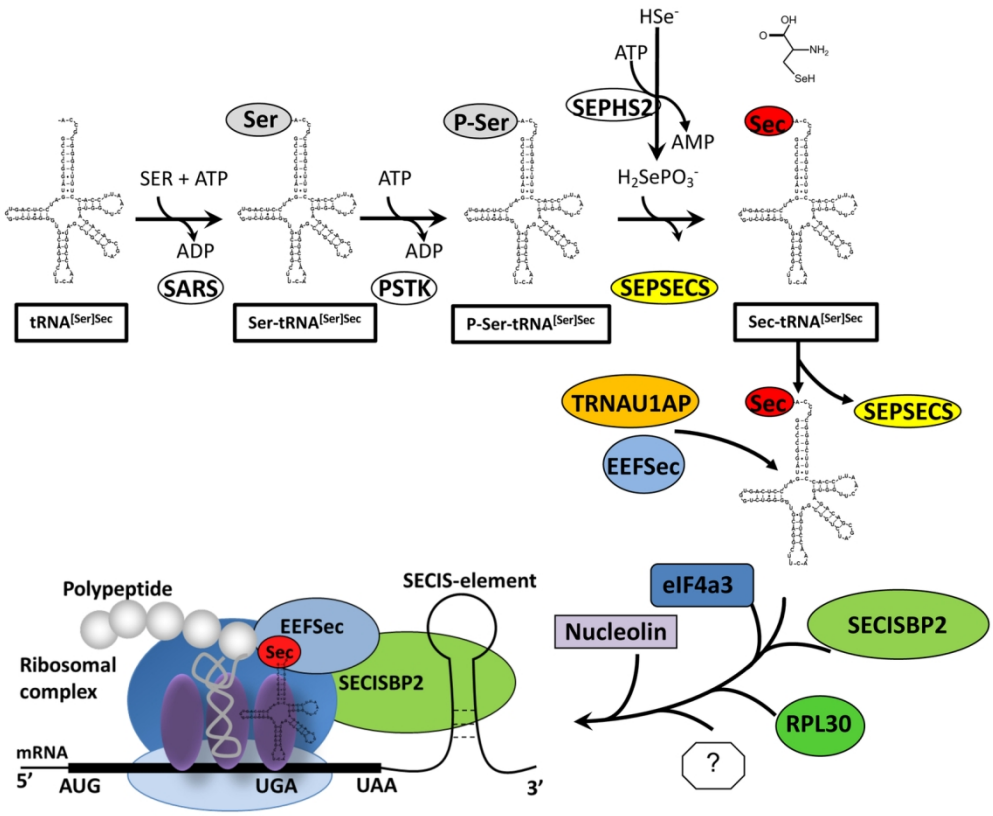

figure 1

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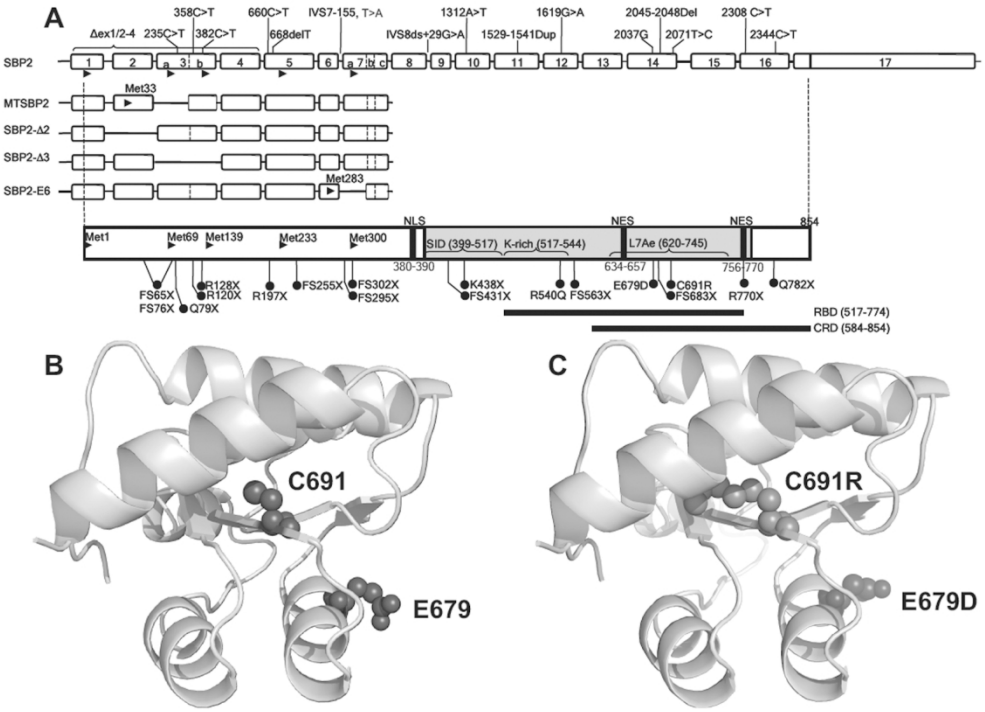

figure 2

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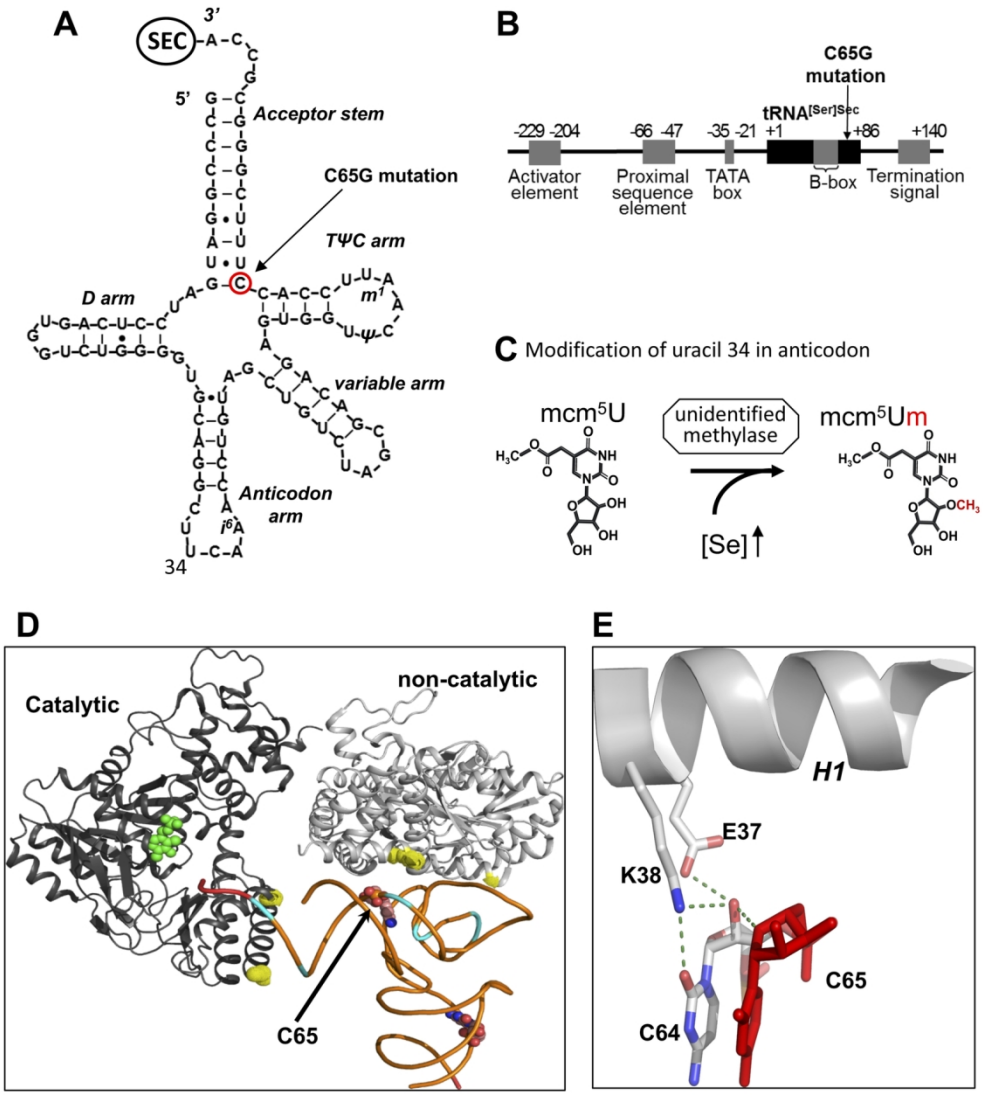

figure 3

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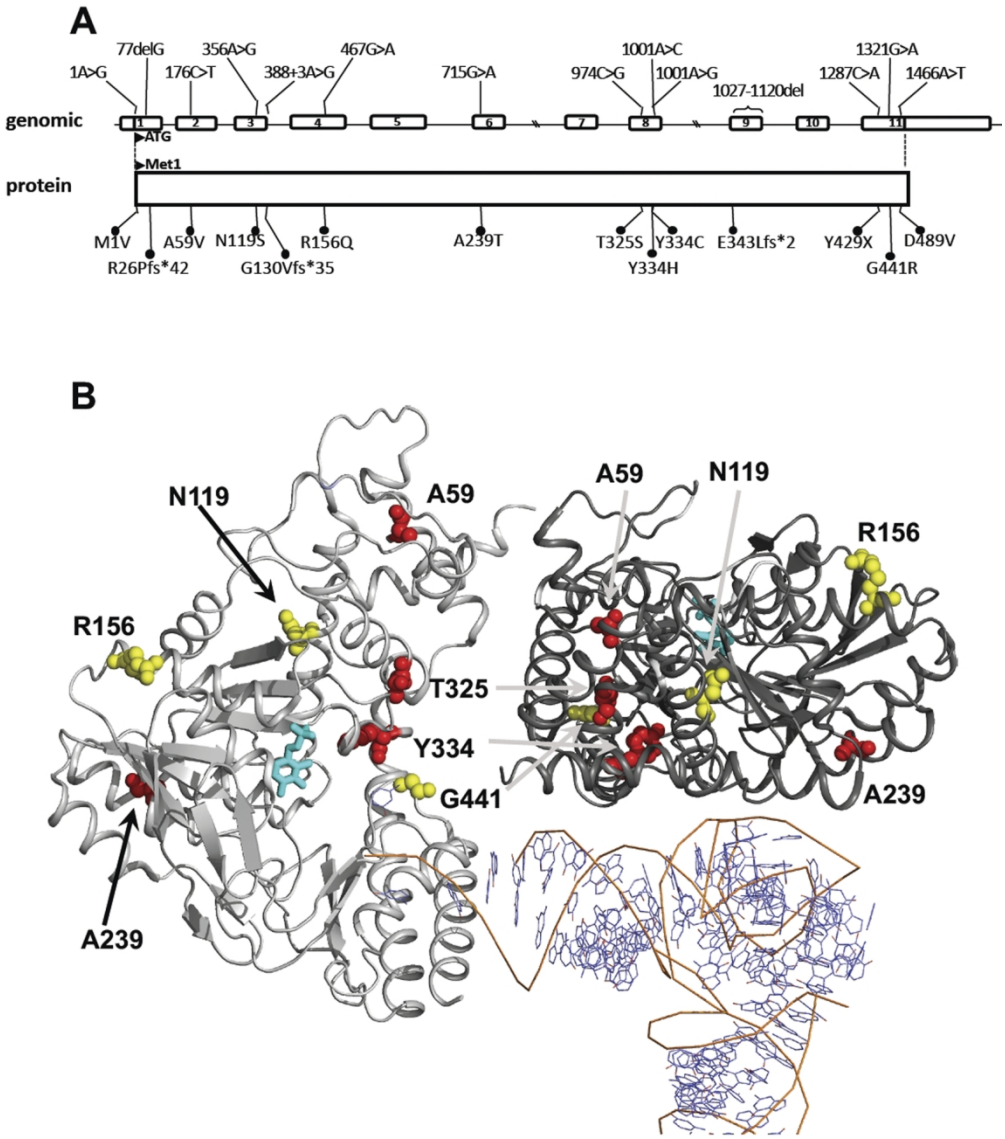

figure 4

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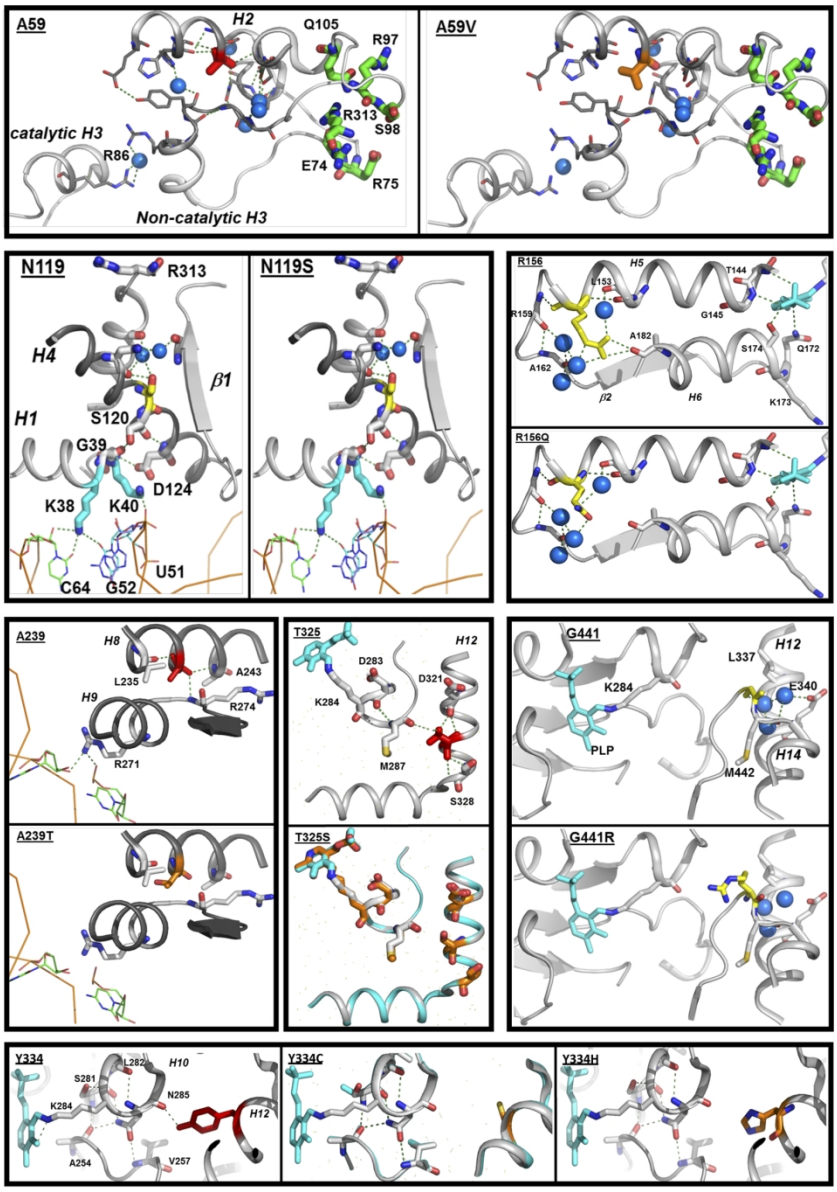

figure 5

144x202mm (300 x 300 DPI)

Table 1 Selenoproteins

Selenoprotein	main known function	Subcellular Localization	expression pattern
GPX1	Oxidoreductase protection against oxidative stress	cytoplasmic	most tissues
GPX2	Oxidoreductase protection against oxidative stress	Nuclear and cytoplasmic	Gastrointestinal tract, bone marrow,immune system,liver,galbladder,kidney, urinary bladder
GPX3	Oxidoreductase protection against oxidative stress	secreted	most tissues , high in kidney, thyroid, adipose
GPX4	Oxidoreductase protection against oxidative stress	Nucleus and mitochondria	most tissues, high in testis, adipose tissue
GPX6	Oxidoreductase protection against oxidative stress	predicted secreted	testis, epididymis, olfactory system
TXNRD1	Oxidoreductase protection against oxidative stress	Nuclear and cytoplasmic	Ubiquitous
TXNRD2	Oxidoreductase protection against oxidative stress	cytoplasmic and mitochondria	Ubiquitous
TXNRD3	Oxidoreductase protection against oxidative stress	Intracellular	most tissues, high in testis
DIO1	Oxidoreductase thyroid hormone metabolism	Intracellular membrane-associated	kidney, liver, thyroid gland
DIO2	Oxidoreductase thyroid hormone metabolism	Intracellular membrane-associated	low in several tissues, high in thyroid, esophagus, cervix,ectocervix,pituitary,endometrium,brain
DIO3	Oxidoreductase thyroid hormone metabolism	Intracellular membrane-associated	several tissues, high in cervix, uterine, placenta, urinary bladder
MSRB1	Oxidoreductase Methionine Sulfoxide Reduction	Nuclear and cytoplasmic	Ubiquitous
SELENOF	Oxidoreductase ER-associated protein folding control	endoplasmic reticulum	Ubiquitous
SELENOH	unknown Oxidoreductase dna/rna binding motif	nuclear	Ubiquitous
SELENOI	Oxidoreductase phospholipid biosynthesis	transmembrane	Ubiquitous
SELENOK	ER-associated protein folding control	ER, plasma membrane	Ubiquitous
SELENOM	unknown	Nuclear and perinuclear	Ubiquitous
SELENON	Oxidoreductase redox-related calcium homeostasis	endoplasmic reticulum	Ubiquitous
SELENOO	Protein AMPylation activity	mitochondria	Ubiquitous
SELENOP	transport / Oxidoreductase	secreted, cytoplasmic	most tissues, high in liver and small intestine
SELENOS	Oxidoreductase ER-associated protein folding control	endoplasmic reticulum	Ubiquitous
SELENOT	unknown Oxidoreductase	endoplasmic reticulum	Ubiquitous
SELENOV	unknown	Intracellular	thyroid, parathyroid, testis, brain
SELENOW	Oxidoreductase protection against oxidative stress	Intracellular	Ubiquitous
SEPHS2	selenophosphate synthesis	Intracellular	Ubiquitous, high in liver kidney

Table 2 Human *SECISBP2* mutations: genetics and effect

Family	Gene Mutation	Predicted protein change	Alleles Affected	Suggested Mechanism	Ethnicity	Ref
A	c.1619 G>A	R540Q	homozygous	predicted to affect SECIS & ribosome binding	Saudi Arabian	31
B	c.1312A>T c.IVS8ds+29 G>A	K438X fs431X	compound heterozygous	premature stop, no/decreased full length protein	Irish/ Kenyan	31
C	c.382 C>T	R128X	homozygous	premature stop, no full-length protein	Ghanaian	28
D	c.358 C>T c.2308 C>T	R120X R770X	compound heterozygous	premature stop, no full-length protein premature stop, no full-length protein	Brazilian	9
E	c.668delT c.IVS7 -155, T>A	F223fs255X fs295X+fs302X	compound heterozygous	premature stop, no full-length protein premature stop, no or decreased full length protein	British	75
F	c. 2017T>C 1-5 intronic SNP's	C691R fs65X + fs76X	compound heterozygous	predicted to affect SECIS & ribosome binding, increased degradation premature stop, no full-length protein /splice variants affected	British	75
G	c.1529_1541dup CCAGCGCCCCACT c.235 C>T	M515fs563X Q79X	compound heterozygous	premature stop, no full-length protein	Japanese	43
H	c.2344 C>T c.2045-2048 delAACA	Q782X K682fs683X	compound heterozygous	premature stop, no full-length protein	Turkish	32
I	c.660 C>T c.2108 G> T or C	R197X E679D	compound heterozygous	premature stop, no full-length protein /splice variants affected predicted to affect SECIS & ribosome binding	Argentinian	37
J	c.800_801insA	K267Kfs*2	homozygous	premature stop, no full-length protein	Turkish	20
K	c.283delT c.589C>T	T95Ifs31* R197X	compound heterozygous	premature stop, no full-length protein	N/A	55

N/A not available

Table 3 Human *SECISBP2* mutations: A multisystem disorder with a thyroid signature

Family	[Se]	TT4	FT4	TT3	rT3	TSH	Growth & Skeletal	Musculoskeletal	Neurocognitive	Hearing	other
A	L	H	H	L	H	H	Short stature, DBA	N/A	Normal mental development	normal	N/A
B	L	H	H	N/L	H	N	Short stature, DBA	N/A	N/A	normal	N/A
C	L	H	H	N/L	H	N	Short stature, DBA	N/A	N/A	N/A	
D	L	H	H	N/L	H	H	Short stature, DBA, Kyphoscoliosis	hypotonia, hip girdle weakness, Spirometry: reduced expiratory & inspiratory flow, fatty infiltration of muscle	Impaired mental development and motor coordination	bilateral sensorineural loss	Failure to thrive, Bilateral clinodactyly, Asymmetric leg length, Peripheral sensory neuropathy, Increased fat mass
E	L	N/A	H	N	N/A	N	short stature, Genu valgus	Lumbar spinal rigidity, reduced axial & neck strength, Spirometry: reduced vital capacity, nocturnal hypoventilation, Fatty infiltration of muscle	Developmental delay	Bilateral sensorineural loss, Vertigo	Azoopsermia, Raynauds disease, Photosensitivity, mild lymphopenia and reduced red cell mass, Low insulin and high adiponectin levels, favourable blood lipid profile, low intrahepatic lipid
F	L	N/A	H	L	N/A	N	Short Stature	Proximal & axial myopathy, lumbar rigidity, fatty infiltration of adductor muscles	Developmental delay	bilateral sensorineural loss	Failure to thrive, eosinophilic colitis, Increased fat mass, high adiponectin levels, hypoglycaemia with low fasting insulin
G	L	N/A	H	N/L	N/A	N/H	Short Stature, DBA	Fatty infiltration of muscles	Delayed motor & intellectual development, IQ 70	Bilateral mild conductive loss Rotatory vertigo	Failure to thrive, hypoplastic thyroid gland, no photosensitivity, increased fat mass
H	L	H	H	L	H	N/H	Short Stature	N/A	Mental and motor retardation, IQ 50	N/A	N/A
I	N/A	H	H	L	H	N	DBA	N/A	N/A	N/A	Failure to thrive
J	L	H	H	L	H	N/H	decreased growth velocity from 13y	muscle weakness, fatty infiltration of the muscles	Normal early development	normal	Right-eye ptosis, attention-deficit disorder with poor school performance, impaired growth hormone response, obese (BMI 29.5), impaired OGTT
K	N/A	H	H	L	H	N	Short Stature, DBA	N/A	Developmental delay	N/A	Failure to thrive
L	L	H	H	L	H	N	delayed growth	hypotonia, myopathy with fatty infiltration of muscles, ataxic gait	Developmental delay	bilateral sensorineural loss	dysarthic speech, tapered fingers, seizures, Increased fat mass

DBA, delayed bone age; Se, selenium; T3, triiodothyronine; T4, thyroxine; TT4 total T4; FT4 free T4; TT3 total T3; rT3 reverse T3; TSH thyroid stimulating; hormone; L, low; H high; N normal; N/L low normal; N/H, high-normal; N/A not available; IQ, intelligence quotient; OGTT oral glucose tolerance test

Table 4 Human *SEPSECS* mutations: genetics and effect

Family	Gene Mutation	Predicted protein change	Alleles Affected	Suggested Mechanism	Ethnicity	Phenotype	Ref
A,B	c.1001A>G	Y334C	homozygous	affects folding and reduced catalytic activity	Jewish/ Iraqi	PCH2D	1
C, D	c.715G>A	A239T	compound heterozygous	affects folding and reduced catalytic activity	Iraqi/ Moroccan	PCH2D	1
	c.1001A>G	Y334C					
E, F, G	c.974C>G	T325S	compound heterozygous	affects folding and reduced catalytic activity	Finnish	PCH2D	6
	c.1287C>A	Y429X		premature stop, no full length protein			
H	c.1001A>C	Y334H	homozygous	predicted to affect folding and reduce catalytic activity	Arabian	PCH2D	72
I	c.77delG	R26Pfs*42	compound heterozygous	premature stop, no full length protein	Japanese	late onset PCH2D	50
	c.356A>G	N119S		predicted reduced catalytic activity/RNA binding			
J	c.356A>G	N119S	compound heterozygous	predicted reduced catalytic activity/RNA binding	Japanese	late onset PCH2D	50
	c.467G>A	R156Q		predicted reduced catalytic activity			
K	c.1A>G	M1V	compound heterozygous	No ATG start, absence of full length protein	N/A	PCH2D	97
	c.388+3A>G	G130Vfs*35		premature stop, no full length protein			
L	c.1466A>T	D489V	homozygous	Likely pathogenic	Jordan	Developmental Delay/Intellectual Disability	61
M	c.1027-1120del	E343Lfs*2	homozygous	premature stop, no full length protein	N/A	Neurodegenerative disease	3
N	c.176C>T	A59V	homozygous	predicted to affect folding, dimerisation and reduce activity	N/A	EOEE-BS; PCH2D	67
O	c. 1321G>A	G441R	homozygous	predicted to reduce catalytic activity	N/A	late onset PCH2D	86
P	N/A	N/A	N/A	N/A	N/A	PCH2D	8

EOEE-BS: early onset epileptic encephalopathy with burst suppression; PCH2D: pontocerebellar hypoplasia type 2D; N/A not available