Ribosome profiling of selenoproteins in vivo reveals consequences of pathogenic Secisbp2 missense mutations

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3 The abbreviations used are: Sec, selenocysteine; SECIS, selenocysteine insertion sequence; Secisbp2, SECIS-binding protein 2; RPF, ribosome protected fragment; URE, UGA redefinition efficiency; BH, Benjamini–Hochberg.

Mutations in the SECISBP2 gene have been associated with an atypical response to thyroid hormone, which is caused by reduced activity of deiodinase enzymes (1). Deiodinases belong to the class of selenoproteins, proteins carrying the 21st proteinogenic amino acid, selenocysteine (Sec)3 (2). Because Sec is encoded by a UGA codon, recoding as a sense codon depends on the presence of a selenocysteine insertion sequence (SECIS) in the 3′-UTR of the mRNA, which is recognized by SECIS-binding protein 2 (SECISBP2) (3, 4). Selenoprotein expression is generally believed to be highly dependent on SECISBP2, and gene targeting in mice revealed a significantly reduced level of selenoprotein translation in vivo in the absence of functional Secisbp2 (5, 6). Some of the sensitivity toward the lack of Secisbp2 may be due to mRNA degradation following slow decoding or termination at Sec/UGA codons. A number of patients carrying mutations in SECISBP2 have been described and reveal a conspicuous phenotypic heterogeneity among their clinical presentations (7). Several domains in SECISBP2 have been identified that may mediate interactions with the SECIS element or interact with the ribosome and/or elongation factor eEFSEC. Thus, it might be possible that mutations that affect specific functions of SECISBP2 might cause the phenotypic heterogeneity in patients. For example, the first missense mutation identified in homozygosity, R540Q, leads to a rather mild clinical phenotype (1). The changed amino acid lies in the so-called selenocysteine insertion domain of SECISBP2 (8, 9). The mutation has been shown to differentially affect translation of selenoprotein mRNAs (10). Another mutation, C691R, is located in the RNA-binding domain (11). The patient presented with a more severe clinical picture but was compound heterozygous for a presumably hypomorphomic splice site mutation. There are several nonsense or frameshift mutations in the N-terminal part of the protein, and patients carrying SECISBP2 mutations R120X/R770X, R128X/R128X, and K276Kfs*2/K276Kfs*2 show phenotypes of various severity (12–15). It is generally believed that reinitiation 3′ of the premature termi...
nation codons (e.g. at Met-300) rescues some SECISBP2 function in these patients (14, 15).

It was suggested that the affinity of SECISBP2 for individual SECIS elements establishes the observed hierarchy among selenoproteins (16). A systematic study comparing SECISBP2: SECIS binding and SECIS-dependent Sec incorporation in a luciferase reporter system reported that there is no such correlation (17). Because the R540Q mutation differentially affects selenoprotein expression and SECIS binding in vitro (10), we wanted to more systematically assess how this mutation impinges on the translation of different selenoprotein mRNAs in vivo. Using ribosome profiling (18), we aimed to specifically probe the efficiency of UGA/Sec recoding in mice in the same transcript-specific way as we have assessed it previously in Secisbp2-knockout mice (19).

To this end, we have generated mouse models for the R540Q mutation (Secisbp2R540Q) in the selenocysteine incorporation domain and for the C691R mutation (Secisbp2C691R) in the RNA-binding L7Ae domain. We assumed that any residual selenoprotein expression in Secisbp2C691R/− that exceeds the levels in the Secisbp2 knockout may hint toward a potential SECIS binding-independent function of Secisbp2.

Because both homozygous mutations turned out to be embryonic lethal in mice, we combined the missense mutant alleles with a conditional allele and chose to focus on hepatocytes and neurons, the two cell types in which we analyzed selenoprotein expression in the absence of Secisbp2 before (5, 6). Whereas the C696R mutation apparently disrupts RNA-binding activity of Secisbp2 and acts as a complete null in vitro and in vivo, R540Q exhibits thermal instability. Interestingly, while acting as a functional null in liver, it rescues the severe neurological phenotype of neuron-specific Secisbp2 knockout mice (6). The differential activity of the Secisbp2R540Q protein in liver and neurons suggests that the potential function of amino acid 543 within the selenocysteine incorporation domain is being obscured by thermal instability of the protein in vivo. We assessed the effect of the Secisbp2R540Q mutation on selenoprotein translation in detail by ribosome profiling in the cerebral cortex. Despite the general down-regulation of selenoprotein translation, no behavioral phenotype was detected. However, we found a gene expression pattern indicative of a mild inflammatory response in the mutant mouse cortex that was accompanied by obvious astrogliosis (a cellular stress response of astrocytes involving gene expression and shape changes that is readily detected by the up-regulation of the intermediate filament glial fibrillary acidic protein (GFAP)). Thus, different phenotypes of patients carrying different Secisbp2 alleles may not necessarily reflect the functions of individual amino acids in SECISBP2, but may be caused by differences in stability in different cellular contexts.

Results

Generation of mice carrying pathogenic missense mutations in the Secisbp2 gene

Point mutations C696R and R543Q (C691R and R540Q in human SECISBP2, respectively) were introduced into the Secisbp2 locus using recombineering in Escherichia coli and double homologous recombination in embryonic stem cells as detailed under “Experimental procedures.” For the R543Q allele, the mutation was combined with a silent G→A mutation in Leu-540 to produce a diagnostic DraI restriction site (Fig. 1A). The C696R mutation was introduced in exon 14 combined with a silent G>T mutation in Leu-694, creating a diagnostic AfII restriction site (Fig. 1B). Correct homologous recombination of the modified alleles was ensured by Sanger sequencing over the targeted sequence (Fig. 1, A and B) and Southern blotting (Fig. 1, C and D). After germline transmission, the FLP recognition target (FRT)-flanked neomycin resistance cassettes were removed in vivo using FLPe expression in the germline, and deletion of the selection cassette was verified by PCR.

Heterozygous matings of Secisbp2R540Q+/ or Secisbp2C696R/+ mice did not result in viable homozygous offspring (Table 1), but were embryonic lethal around E7, the same time as Secisbp2−/−. We thus chose to combine the missense alleles with the conditional Secisbp2fl allele described before (5, 6) and generated mice in which the missense Secisbp2 allele produced the only translated Secisbp2 mRNA in a cell type–specific manner.

Selenoprotein expression in liver

Liver-specific Secisbp2 mutant mice were generated for both missense alleles by breeding with albumin-Cre transgenic mice: Alb-Cre; Secisbp2R540Q/+ (called subsequently Alb-RQ) and Alb-Cre; Secisbp2C696R/+ (Alb-CR) mice are the desired mutants, and Alb-Cre; Secisbp2R540Q/+ and Secisbp2C696R/+ mice served as controls (WT). In most analyses, the missense mutant mice were compared with Alb-Cre, Secisbp2flfl conditional knockout (Alb-KO) mice. Reverse transcription semiquantitative PCR analysis revealed that in the Secisbp2 missense mutants, all hepatic selenoprotein mRNAs analyzed were reduced to the same level and indistinguishable from the Alb-KO (Fig. 2A). Likewise, on the protein level, Gpx1, Gpx4, Selenot, and Sephs2 were reduced as in the Alb-KO (Fig. 2B).

Txnrd1 protein abundance was not reduced in the absence of functional Secisbp2, because the Sec occupies the penultimate position close to the C terminus. Because we were surprised that both mutants behaved apparently like the Alb-KO, we performed Western blot analysis for Secisbp2 and found that both mutant Secisbp2 proteins were virtually undetectable (Fig. 2C). Thus, both missense mutant proteins are functional nulls in vivo.

In vitro activity of recombinantly expressed Secisbp2 mutants

We speculated that the similar levels of Secisbp2 protein in the liver of conditional missense and null mutants and the apparent complete loss of function in the liver of missense mutants might be caused by protein instability and degradation in vivo. We therefore recombinantly expressed mouse Secisbp2 as WT, R543Q, and C696R variants in E. coli, purified the protein by nickel–nitritolriacetatic acid affinity chromatography using a C-terminal hexahistidine tag, and included the recombinant protein in the SECIS-dependent luciferase reporter in which the codon for Cys-258 is replaced by UGA/Sec (20). To create a consistent experimental system, we cloned several mouse SECIS elements into the reporter plasmid. In a first

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A. Generation of Secisbp2 R543Q

B. Generation of Secisbp2 C696R

C. 5’ probe

D. 5’ probe

Wildtype Allele

Secisbp2 R543Q WT

Eco RI

Secisbp2 R543Q

FLPe

Dral

FLP

C696R

SpeI

E. 1B 5H

BglII

HindIII

F11 57

F11 H7

21226

WT

21226

WT

5148

R543Q

5148

C696R

5148

C696R

5148

R543Q

C696R

R543Q

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expansion, we used 100 ng of reporter mRNA (about 13 nM) and a 160 nM concentration of each Secisbp2 protein (Fig. 3A). There was no appreciable difference in luciferase activity between WT and R543Q protein irrespective of the SECIS element, whereas C696R was completely inactive, comparable with negative controls (no Secisbp2 added or mutation in the AUGA quartet of SECIS).

We reasoned that an excess of Secisbp2 protein might have obscured moderate effects of the R543Q mutation. Further, it was difficult to assess what proportion of the recombinant protein was active in this experiment. We therefore titrated SECIS-dependent changes: 1) the R543Q mutation reduces the UGA-recoding in response to a subset of SECIS elements (Fig. 3D). This explains why the R543Q mutant protein was virtually undetectable in the liver of mutant mice and suggests that the C696R mutant, which is also undetectable in liver, might also be thermally unstable.

### Selenoprotein expression in neurons

Judging from Western blotting experiments, selenoprotein expression is reduced in CamK-Cre; Secisbp2R543Q/+ (CamK-RQ) and CamK-Cre; Secisbp2C696R/+ (CamK-CR) mutants, but not above the level in CamK-Cre; Secisbp2fl/fl (CamK-KO) cortex (Fig. 4A). In line with the above results showing the inactivity of Secisbp2C696R in vitro and in the liver, mice with CamK-KO and CamK-CR mutants show indistinguishable phenotypes (6). On P16–P18, the mean body masses were 7.27 ± 0.56 and 3.82 ± 0.60 g for the control and CamK-CR mice, respectively. Furthermore, CamK-CR mice died before weaning (P16–P18) and showed astrogliosis in the cerebral cortex at P16 similar to CamK-KO mice (not shown). As expected, selenoprotein levels were clearly reduced in the brains of these mice (Fig. 4B). Likewise, mRNAs of sensitive selenoproteins (e.g. Gpx1 and Selenow) were reduced (Fig. 4C). The increase of Gpx4 may be related to the astrocytic response, and likewise decreased Selenow in nonneuronal cells may compensate for a reduction in neurons. In addition, CR mice showed a reduced number of parvalbumin-positive (Pvalb+) GABAergic interneurons in the somatosensory cortex at age P16 (Table 2).

Despite its thermal instability and impaired functionality, the low expression of the mutant Secisbp2 protein, CamK-RQ mice showed an apparently normal phenotype without signs of neurological dysfunction. The number of Pvalb+ interneurons was not reduced in the somatosensory cortex at age P35 (Table 2). Seizures or a movement phenotype were not observed.

Expression of all tested selenoproteins was reduced in the cerebral cortex of CamK-RQ mice, with the exception of Tnnr1, which carries the Sec residue at the penultimate position at the C terminus (Fig. 4D). A low but significant functionality of Secisbp2R543Q is suggested by the fact that Selenow and Selenon proteins were present in the cortex at higher levels than in CamK-CR mice. However, semiquantitative RT-PCR demonstrated that Selenow message is reduced in CamK-RQ mice to a similar extent as in CamK-CR mice. RNA-Seq supported this result and showed on a genome-wide level that selenoprotein mRNA amounts were generally reduced in CamK-RQ mutant brains (Figs. 4E and 5A). We reasoned that this apparent discrepancy between mRNA and protein abundance could be related to differential activity of the Secisbp2...
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A

Gpx1

Gpx4

Selenop

Selenow

Selenot

Sephs2

2 - ΔΔCT ± SD

Ctl KO CR RQ

2 - ΔΔCT ± SD

Ctl KO CR RQ

2 - ΔΔCT ± SD

Ctl KO CR RQ

2 - ΔΔCT ± SD

Ctl KO CR RQ

B

C

Ctl KO CR RQ

25kDa Gpx1

25kDa Gpx4

15kDa Txnrd1

70kDa Selenot

55kDa Sephs2

15kDa β-actin

135kDa Secisbp2

70kDa

40kDa

35kDa

25kDa
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mutants during translational redefinition of the UGA/Sec codon.

UGA recoding in brain assessed by ribosomal profiling

To directly assess the functionality of mutant Secisbp2 during selenoprotein biosynthesis, we probed selenoprotein expression in the brains of Camk-RQ mice by ribosomal profiling. Compared with mRNA abundance, ribosome profiling revealed a more pronounced reduction of ribosome protected fragments (RPFs) associated with selenoproteins, indicating that selenoprotein translation was specifically affected in the mutant brains (Fig. 5A). This was particularly evident for Gpx4, where mRNA abundance did not significantly change in the mutant, but RPF levels dropped more than 2-fold. Plotting the distribution of RPFs over the ORF revealed that 5′ of the UGA/Sec codon, the coverage of Gpx4 was unaltered, whereas 3′ of the UGA/Sec, the ribosomal density (i.e. RPFs/nucleotide mRNA) was significantly decreased (Fig. 5B). Thus, read-through of the UGA/Sec codon is inefficient in Secisbp2<sup>5R43Q</sup> mutants. A similar observation was made for Seltenon. In the case of Seltenon, ribosomal coverage appears unchanged, but this may be due to degradation of the mRNA whenever UGA/Sec recoding fails. Gpx1 shows reduction of RPFs along the entire length of the ORF, but the reduced read-through of the UGA/Sec codon was nevertheless obvious.

We have previously defined UGA redefinition efficiency (URE) as ribosomal density 3′ of the UGA/Sec divided by ribosomal density 5′ of the UGA. Fig. 5C shows that URE is reduced for several selenoproteins that carry the UGA/Sec codon far away from the termination codon. The URE, however, does not account for a reduction of the absolute number of RPFs covering a certain message. To reflect also absolute changes of UGA/Sec read-through, the number of RPFs 3′ of the UGA/Sec per million mapped reads (3′ RPF) was calculated (Fig. 5D). Using this measure, the impairment of selenoprotein translation in Secisbp2<sup>5R43Q</sup> mice was evident. Interestingly, Seltenon was little affected, probably because this selenoprotein is significantly expressed in astrocytes that are not targeted in our Camk-Cre mouse model. Since 3′ RPF is a measure that reflects both translation and mRNA abundance, we also normalized 3′ RPF by mRNA abundance (Fig. 5E).

Taken together, ribosomal profiling reveals that Secisbp2<sup>5R43Q</sup> does not fully support faithful UGA/Sec recoding and selenoprotein biosynthesis in neurons in vivo.

A mild inflammatory response in the brain

Transcriptomic analysis confirmed the significant reduction (20–60%) of selenoprotein mRNA levels, including Seltenon, Gpx1, Seltenon, Gpx3, and Seltenon. Apart from selenoproteins, among the genes up-regulated on the transcriptional (RNA-Seq) or translational (Ribo-Seq) level in Secisbp2<sup>5R43Q</sup> mice, we found a clear signature of genes of inflammatory response pathways. Typically, the up-regulated mRNA levels correlated with a higher translation (RPFs) of the corresponding proteins (Fig. 6A). In particular, we observed an increase in the expression and/or translation of immune cell markers (Itgax, Itgamu, Mpeg1, Cdh48, Cdl180, Cx3cr1, Ly6e, Ly6f, Lag3, Trem2, Adgre1, Tyrobp, and Tlr2), chemokines (Ccl3 and Ccl4), complement (C1qa and C1b), and proteins associated with lysosomes (Lamp2, Lyz1, Lyz2, Laptm5, Hexb, Cts, Ctsd, and Ctsz) and cystatins (Cst3 and Cst7) as well as the antiviral or antimicrobial proteins Bst2, B2m, Vcp, Chill1, and Aif3 (Fig. 6B). Among the down-regulated genes, we observed mainly transcripts of selenoprotein mRNAs (indicated in green) as well as Capn5, a protease located in synapses, and Cdx4, a transcription factor known to act both in the hematopoietic and neural lineage.

The changes in the gene expression observed are indicative of immune infiltration of the brain or, alternatively, induction of these genes in cells present in the brain or brain circulation. Because the astrocytic markers Gfap and App1 were among the up-regulated genes, we performed immunohistochemistry against GFAP and found widespread astrogliosis in Camk-RQ mice, which was limited to lower cortical layers (Fig. 6C). We then asked whether the increase in microglial marker genes (Ctss, Itgam, Cx3cr1, and Gpr34) was related to microglial invasion of the cortex. Immunohistochemical staining for Iba1 did not show any differences in cell density, suggesting that the increased microglial marker gene expression was related to up-regulation of genes in activated microglia (Fig. 6D).

Several neuronal genes were deregulated, including the neu-roproteases Penk, Tac1, Oxt, and Pdyn (Fig. 6A). Genes generally associated with inhibitory interneurons like Gad1, Gad2, GABA transporters, and GABA receptors were not among the significantly deregulated genes, whereas, interestingly, Pvalb and Gpx3 were reduced on the mRNA level, possibly indicating that cells in lower cortical layers are particularly affected (21). Furthermore, Penk and Mgp, markers of layer 6a glutamatergic neurons, were up- and down-regulated, respectively. Finally, astrogliosis was more prominent in deeper cortical layers. Taken together, these gene regulation data suggest that there is a pathophysiological process associated with the dysregulation of selenoprotein expression that starts in lower cortical layers.

Discussion

Choice and evaluation of models

Which function the N-terminal domain of Secisbp2 may have remains an open question. Because the N terminus of Secisbp2 is intrinsically disordered (22), this part of the protein is usually omitted when expressing Secisbp2 recombinantly. In vitro experiments pioneered by Paul Copeland and adapted here for murine Secisbp2 and SECIS elements show that selenoprotein translation can be supported by the so-called C-terminal part of Secisbp2, which was as effective as the full-length

Figure 2. Selenoprotein expression in liver-specific Secisbp2 mutant mice. Control mice (WT) were compared with Alb-Cre; Secisbp2<sup>R43Q</sup> (KO), Alb-Cre; secisbp2<sup>R43Q</sup> (CR), and Alb-Cre; Secisbp2<sup>5R43Q</sup> (RO) mice. A, semiquantitative RT-PCR analysis for selected selenoprotein mRNAs. ΔΔCt values are calculated in relation to 18S rRNA. Means are given ± S.D. (error bars), n = 2–8. Western blot analysis for selected selenoproteins. Two individual liver extracts are analyzed for each genotype. C, Western blot analysis for Secisbp2. The nonspecific bands demonstrate equal loading. Images were copied directly from the camera output files and displayed without modifications. The complete image files are available as Fig. S2.
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A

[Bar graph showing luminescence levels for different conditions: control, R543Q, and C696R for Gpx1, Gpx4, Txnrd1, AUGA del, no RNA, no Secisbp2, and Luc_UAA.]

B

[Graphs illustrating luminescence levels for Luc/Gpx1 mRNA and Luc/Txnrd1 mRNA with controls and R543Q mutations.]

C

| Protein | EC_{50} (control) [nM] | EC_{50} (R543Q) [nM] | Significance |
|---------|------------------------|----------------------|--------------|
| Gpx1    | 3.8 +/- 0.6            | 5.8 +/- 1.1          | NS           |
| Gpx4    | 4.3 +/- 0.5            | 5.0 +/- 0.4          | NS           |
| Txnrd1  | 3.7 +/- 0.4            | 5.9 +/- 0.9          | *            |
| Dio1    | 4.8 +/- 0.8            | 8.9 +/- 2.0          | *            |

D

[Graphs showing luminescence levels for Luc/Dio1 mRNA at different temperatures: on ice, at 37°C, and at 40.5°C with controls and R543Q.]
recombinant protein (20). In addition, pathogenic SECISBP2 mutations in humans that involve premature termination codons sometimes lead to a relatively mild clinical phenotype that has been explained by the possibility of initiation of translation at methionine codons 139, 233, and 300 (14, 15).

We have therefore designed the C696R mutation in the mouse to specifically disrupt the L7Ae RNA-binding domain (23). By homology with the crystal structure of the spliceosomal 15.5-kDa protein (24), the Cys is predicted to reside in the central β-sheet of the L7Ae domain, and insertion of a bulky and charged amino acid, as in the pathogenic C691R mutation (11), is expected to fundamentally disrupt protein structure. In fact, no SECIS binding of recombinant Secisbp2C696R was detected in our in vitro experiments, which is compatible with a previous report using rat Secisbp2 (25). The phenotype of CamK-Cre; Secisbp2C696R/− mice was indistinguishable from the phenotype of CamK-Cre; Secisbp2R543Q/− mice described before (6). Similar to PKG-Cre; Secisbp2R543Q/− mice, Secisbp2C696R/C696R mice died during embryogenesis around E7. Finally, Alb-CR mice did not support any selenoprotein expression in liver beyond the levels observed before in the Alb-KO mice (5). We thus conclude that we did not detect in vivo any evidence for a function of Secisbp2 (and its unaltered N terminus), which was independent of the RNA-binding capacity of the L7Ae domain.

The mutant Secisbp2R543Q was designed as a model for the human SECISBP2 mutation R540Q (1), which leads to a relatively mild phenotype in homozygous patients. Interestingly, Secisbp2R543Q/C509Q embryos died in utero like the knockout and homozygous C696R embryos, suggesting that the mutant protein is not fully functional during mouse development. This species difference is reminiscent of the observation that Gpx4 knockout embryos die in utero, whereas a patient with a homozygous nonsense mutation in Gpx4 lived 4 months after birth (26). The R543Q mutation is apparently partially functional in neurons, because clear-cut phenotypes of neuronal selenoprotein deficiency (i.e., loss of Pvalb-positiver interneurons and movement disorders) are not observed (6, 27-31). Yet, Ribo-Seq and RNA-Seq revealed a decreased expression of Pvalb and Satb1, a transcription factor specific for Pvalb+ interneurons. Such a partial deficiency of Secisbp2R543Q is also evident from a reduction by half of RPFs and an altered ribosomal coverage of Secisbp2R543Q (Fig. 7). Whether the altered ribosomal coverage is caused by the R543Q mutation or, inadvertently, by the silent third position base change in the codon for Leu-540 cannot be resolved at present. However, it is known that missense mutations, and even silent base changes, can affect the kinetics and efficiency of translation and cause pathological results (32, 33).

The fact that Secisbp2R543Q is functional in neurons but apparently unstable in hepatocytes suggests that the cellular environment determines the stability of a mutant protein and thus strongly affects its activity. With respect to interpreting the genotype-phenotype relationships in SECISBP2-deficient patients, this means that we cannot easily interrogate, as initially attempted in the present work, the functions of single parts of a protein using point mutations in vivo. Hence, other pathogenic missense mutations not studied here may also depend on the stability of the protein in vivo rather than revealing their immediate impact on a particular binding partner. Second, because the cellular environment determines the activity of a protein, the phenotype in different organs/cell types may be more informative about the variant protein’s stability in different organs than about the specific role of the amino acid. Again, such an unexpected cell type-specific effect is reminiscent of a “silent” pathogenic mutation in cystic fibrosis transmembrane conductance regulator, which is read by a specific rRNA species that is expressed at low levels in airway epithelium but abundant in other tissues (34). Similarly, recently, a polymorphism found in the human DIO2 gene was modeled in a mouse model. Whereas kinetic studies had previously shown only moderate changes in catalytic activity, the mouse model clearly showed a brain-specific ER stress provoked by the missense mutation (35).

In vitro studies provide valuable additional information in such situations. Our Secisbp2:SECIS interaction assays suggest a decreased affinity of R543Q for several mouse SECIS elements. The concentration of SECIS associated with half-maximal luciferase activity may be interpreted as an apparent $K_D$ value if no other component in the system is limiting. We found an apparent $EC_{50}$ in the range of 3–4 nM for the WT protein and lower $EC_{50}$ of up to 8 nM (for Dio1 SECIS) for the R543Q mutant protein. These values are close to the rat Secisbp2:rat Gpx4 SECIS $K_D$ of 17 nM determined in the Driscoll laboratory by REMSA (10) and the nanomolar $K_D$ for human Sec (MSRB1) and Gpx3 SECIS (17). Our assay detected differences in SECIS affinity but did not show a loss of SECIS binding in the case of Gpx1 and Dio1 as was previously reported (7). The differences between the ability of mouse and rat Secisbp2 R531Q mutants to interact with Dio1 and Gpx1 SECIS may represent a species difference, be related to different methods used for the analysis, or reflect the mutant protein’s instability.

Selenoprotein expression

The relative stability of the Secisbp2R543Q mutant protein in neurons allowed us to assess the impact of the mutation on UGA/Sec recoding by ribosomal profiling (Ribo-Seq). Neurons

Figure 3. In vitro functional analysis of recombinantly expressed mouse Secisbp2 R543Q and C696R. Reporter constructs containing a Sec-dependent luciferase cDNA and SECIS elements cloned into the 3′ UTR as given are in vitro translated, and the amount of luciferase produced was measured by an activity assay. A, Secisbp2 R543Q is functional, whereas C696R is not functional at all. Recombinant Secisbp2 at 160 nM was incubated with constructs containing murine Gpx1, Gpx4, and Tendr1 SECIS elements, and luciferase activity was measured. A rat Gpx4 SECIS element lacking the AUGA sequence of the essential kink-turn served as negative control. Further negative controls were no mRNA added, no Secisbp2 protein added, and a luciferase carrying a UAA codon at the position of the UGA/Sec codon. A log$_2$ scale is used to better appreciate the small increment of luciferase activity in the controls lacking Secisbp2 or the SECIS quartet over lack of mRNA or a definite stop codon indicating a small SECIS/Secisbp2-independent UGA read-through. 8, kinetics of Secisbp2R543Q:SECIS binding compared with Secisbp2WT for several murine SECIS elements. The concentration of recombinant Secisbp2 was 80 nM, and 0.1–20 nM mRNA was used. The apparent $K_D$ of Secisbp2R543Q was significantly reduced for Dio1 and Gpx1 SECIS only, and the maximal amount of luciferase was decreased when using the mutant as compared with Secisbp2WT. C, thermal instability of Secisbp2R543Q in vitro. Recombinant Secisbp2 protein at 160 nM concentration was incubated for 30 min on ice, at 37°C, and at 40.5°C and then used for in vitro translation with the luciferase/Gpx4 SECIS construct. The luciferase activity values of the respective Secisbp2WT incubated on ice was defined as 100%. The experiment was done twice in triplicates. Error bars, S.D.
Table 2
Parvalbumin-positive interneuron density in the primary somatosensory barrel field cortex (518F)

|                      | Control                        | Mutant                        | Age at analysis |
|----------------------|--------------------------------|--------------------------------|-----------------|
| CamK-Cre; Secisbp2C696R mice | 107 ± 9 (3)                    | 75 ± 11 (3)**                 | P16             |
| CamK-Cre; Secisbp2R543Q mice    | 197 ± 18 (4)                   | 215 ± 9 (4)                   | P35             |


eXpress a large number of selenoproteins and thus are ideally suited to address this question (36). The Secisbp2


gene into bacterial artificial chromosome (BAC) vectors were purchased from Source Biosciences. pDTA contains an ampicillin resistance gene and a diphtheria toxin A fragment (DTA) for positive and negative selection in bacteria and ES cells, respectively. pDTA Dual Neo vector includes a neomycin phosphotransferase gene for ES cell selection, flanked by two FRT sites that allow the cassette removal. 5′ (exon 10) and 3′ (exon 16) homologous arms were amplified by PCR using a BAC vector as template and were subcloned into pGEMT-Easy vector (Promega). See Table S1 for primer details. Restriction sites were added by PCR (Xhol and HindIII for exon 10 and HindIII and XbaI for exon 16). Both homologous arms were subsequently cloned stepwise into pDTA vector.

**Experimental procedures**

**Construction of targeting vectors by homologous recombination in bacteria**

Chloramphenicol-resistant

E. coli DH10B bacteria clones containing mouse Secisbp2 gene into bacterial artificial chromosome (BAC) vectors were purchased from Source Biosciences. pDTA contains an ampicillin resistance gene and a diphtheria toxin A fragment (DTA) for positive and negative selection in bacteria and ES cells, respectively. pDTA Dual Neo vector includes a neomycin phosphotransferase gene for ES cell selection, flanked by two FRT sites that allow the cassette removal. 5′ (exon 10) and 3′ (exon 16) homologous arms were amplified by PCR using a BAC vector as template and were subcloned into pGEMT-Easy vector (Promega). See Table S1 for primer details. Restriction sites were added by PCR (Xhol and HindIII for exon 10 and HindIII and XbaI for exon 16). Both homologous arms were subsequently cloned stepwise into pDTA vector.

**Inflammatory response**

The induction of astrogliosis has been reported in several mouse models of selenoprotein deficiency, also if the selenoprotein deficiency was limited to neurons (28), which is supported by our observations here. Thus, astrogliosis represents a general response to neuronal selenoprotein deficiency. Interestingly, a similar limitation of astrogliosis to deeper cortical layers was observed in the tRNA[Ser]Sec hypomorph mouse model (29). The primary target cells of selenoprotein deficiency might be Pvalb-positive GABAergic interneurons (28), and our transcriptomic analysis shows a down-regulation of Pvalb and Satb1. In the CamK-RQ model, the dysfunction is not sufficiently severe for a loss of these cells, in contrast to the CamK-CR or CamK-KO models. We also observed a reduction of the expression of several nuclear encoded genes for components of the mitochondrial electron transport chain. Why these genes are down-regulated is unclear, but Gpx4 and Txnrd2 are known to be present in mitochondria, and their reduced activity may lead to mitochondrial damage. We did not observe an increase in Iba1-positive cells, indicating that no invasion of microglial Iba1-positive cells occurred in the CamK-RQ mice. The increased expression of a wide range of immunity-related genes indicates that a mild inflammatory response occurs in CamK-RQ mice. The set of up-regulated immunity-related genes in our experiment is very similar to the genes induced in Npc1 mutant mice (37). Deficiency of Npc1 causes Niemann–Pick disease type C, a lysosomal storage disorder, in which cholesterol accumulates in lysosomes that are unable to export cholesterol. In humans and mice, this disorder leads to neurodegeneration. In our mice, the induction of inflammatory genes is lower than in the Npc1 model, a finding that is compatible with a lack of obvious neurodegeneration in CamK-Cre-RQ mice. Neurodegeneration may occur during aging of our mice but was not the focus of the present study.

**Figure 4. Selenoprotein expression in neuron-specific Secisbp2 mutant mice.** A, Western blotting against Secisbp2. 100 μg of total cortical protein from two individual mice of each genotype. B, Western blotting against selenoproteins in the cortex of CamK-Cre; Secisbp2<sup>CamK-CR</sup> (CR) and littermate control mice. C, semiquantitative RT-PCR analysis of selected selenoprotein mRNAs, n = 2; ***, p < 0.001, Student’s t test. D, Western blotting against selenoproteins in the cortex of CamK-Cre; Secisbp2<sup>CamK-CR</sup> (CR) and littermate control mice. E, semiquantitative RT-PCR analysis of selected selenoprotein mRNAs, n = 3; ***, p < 0.001, Student’s t test. Images were copied directly from the camera output files and displayed without modifications. The complete image files are available as Fig. S3. Error bars, S.E.
EDITORS' PICK: Assessing missense mutations in Secisbp2

A

B

C

D

E

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the corresponding mutations, R543Q in exon 12 and C969R in exon 14, were introduced, respectively, by site-directed mutagenesis following the manufacturer’s instructions (QuikChange II site-directed mutagenesis kit, Agilent Technologies). To facilitate the posterior screening, DraI or AflII restriction sites were also created upstream of the corresponding Secisbp2 mutation (R543Q or C696R) by site-directed mutagenesis. In both cases, nucleotide sequence was modified creating a silent mutation at Leu-540 and Leu-694, respectively. Finally, these arms were cloned stepwise into pFRT Dual Neo vector to create pFRT Dual Neo_R543Q and pFRT Dual Neo_C696R vectors, respectively.

A defective bacteriophage λ (mini λ Tet) provides a temperature-inducible system, which expresses transiently the phage recombination genes and contains a tetracycline resistance gene. After induction at 42 °C of bacteria containing mini λ/H9261Tet plasmid, excised plasmid particles were purified by the PureYield™ Plasmid Midiprep System kit (Promega). Electrocompetent DH10B bacteria containing Secisbp2 BAC were electroporated with mini λ/H9261Tet plasmid. Selected tetracycline- and chloramphenicol-resistant bacteria were induced at 42 °C and made electrocompetent. These bacteria were electroporated with HindIII-linearized pDTA vector. A first homologous recombination event rescued the Secisbp2 gene sequence.

Figure 5. Selenoprotein expression in neuron-specific Secisbp2R543Q mice assessed by ribosomal profiling and RNA-Seq in cerebral cortex. A, relative abundance of selenoprotein-related reads in Ribo-Seq (RPF) and RNA-Seq (RNA) in RQ mice compared with controls. n = 2 per genotype. *, q < 0.05, BH correction. Significant changes are highlighted in red. B, RPF coverage of selected selenoprotein mRNAs. The position of the Sec/UGA codon is indicated by a red bar. Reads are plotted in blue for controls (Ctl) and in orange for CamK-Cre; Secisbp2 R543Q/fl (RQ) mice. C, URE calculated for selenoproteins with UGA/Sec far from the termination codon. URE is calculated as (3′RPFmutant/5′RPFmutant)/(3′RPFcontrol/5′RPFcontrol). URE is not a good measure if mRNA levels or initiation rates change. D, 3′ RPM (reads 3′ of UGA/Sec per million mapped reads) calculated for selenoproteins. This measure gives a measure for the actual translation of full-length selenoproteins. E, 3′ RPM/mRNA is the same measure as in D, but normalized to mRNA abundance. C–E, *, p < 0.05, Student’s t test. Error bars, S.D.

Figure 6. Mild inflammatory response in neuron-specific Secisbp2R543Q mice. A and B, brain transcriptomic analysis reveals induction of immunity-related genes (red) on mRNA (A) and RPF levels (B). Selenoproteins are labeled in green and neuronal genes in blue. Only significant regulated genes are shown. n = 2 animals/genotype, *, q < 0.05, BH correction. C, pathway analysis shows that immunity-related pathways were induced on the transcriptional level. D, astrogliosis assessed by GFAP staining in the somatosensory cortex. E, Iba1 staining in the somatosensory cortex. Mean cell density was 375 and 378 cells/mm² for control and CamK-RQ mice, respectively.

Figure 7. Ribosomal coverage of Secisbp2 in cortex. The site of the R543Q mutation is marked in red. Although the ribosomal coverage pattern is similar in mutant and control, it is obvious that the mutant is translated at a lower level, in particular 3′ of the site of the mutation indicated in red.
between exon 10 and 16 into the BAC vector, which was introduced in pDTA vector. See Fig. S1. Positive colonies were confirmed by restriction analysis and exon PCR. Electrocompetent DH10B bacteria containing pDTA_e10-e16 and mini λ vectors were induced and electroporated with Xhol-NotI linearized pFRT Dual Neo_C696R or pFRT Dual Neo_R540Q, respectively. A second homologous recombination step between homologous sequences (exon 12 and intron 12 for R540Q and exon 14 and intron 14 for C696R) introduced the corresponding point mutation and the neo-cassette into pDTA_e10-e16 vector. After confirmation of correct vector sequence by restriction analysis and sequencing, these targeting vectors (pDTA_e10-e16_FRT Dual Neo_R540Q and pDTA_e10-e16_FRT Dual Neo_C696R) were linearized by NotI and used to create the corresponding mouse models.

**Generation of mouse models**

Mouse studies were approved by the authorities in Berlin and Nordrhein-Westfalen: approval numbers G 0468/09 and T0458/09 and approval number 84-02.04.2012.A146, respectively. A second homologous recombination step between homologous sequences (exon 12 and intron 12 for R540Q and exon 14 and intron 14 for C696R) introduced the corresponding point mutation and the neo-cassette into pDTA_e10-e16 vector. After confirmation of correct vector sequence by restriction analysis and sequencing, these targeting vectors (pDTA_e10-e16_FRT Dual Neo_R543Q and pDTA_e10-e16_FRT Dual Neo_C696R) were linearized by NotI and used to create the corresponding mouse models.

Mouse studies were approved by the authorities in Berlin and Nordrhein-Westfalen: approval numbers G 0468/09 and T0458/09 and approval number 84-02.04.2012.A146, respectively. 30 µg of linearized targeting vectors, pDTA_e10-e16_FRT Dual Neo_R543Q and pDTA_e10-e16_FRT Dual Neo_C696R, were used for homologous recombination in 129 Ola Hsd ES cells and HM1, respectively. After electroporation, 50 neomycin-resistant ES clones were grown and posteriorly analyzed for correct insertion by Southern blot. Two ESC positive clones, which showed an acceptable karyotype, were microinjected into blastocysts from the C57Bl/6 female mouse strain and transferred to a foster mother. Male chimeras were backcrossed with C57Bl/6 females for germline transmission, which was confirmed by PCR. Heterozygous mice for the transgene were backcrossed with the FLP-deleter mouse to remove the selection cassette. Homozygous mice for both transgenes were never born from transgene heterozygous mouse matings (Table 1). To analyze these Secisbp2 mutant proteins specifically in hepatocytes or neurons, heterozygous mutant (Secisbp2^{R543Q/+} or Secisbp2^{C696R/+}) mice were bred with cell-specific Secisbp2^{R543Q/+} (Alb-Cre; Secisbp2^{R543Q/+} or CamKII-Cre; Secisbp2^{R543Q/+}) mice (5, 6). Thus, Alb-Cre; Secisbp2^{mut/β} or CamK-Cre; Secisbp2^{mut/β} mice were considered as mutant mice, and their Secisbp2^{mut/β} and Alb-Cre; Secisbp2^{R543Q/+} or CamK-Cre; Secisbp2^{R543Q/+} littersmates as control animals for the experiments. Animals were killed for experiments at different ages: Alb-Cre; Secisbp2^{mut/β} mice were killed 7 weeks after birth; CamK-Cre; Secisbp2^{C696R/+} mice were killed after 16 days, and CamK-Cre; Secisbp2^{R543Q/+} mice were killed 5 weeks after birth, respectively.

**Southern blot**

Ten µg of genomic DNA was extracted from different ESC clones using phenol/chloroform/isoamyl alcohol (Sigma). ESC clones were subjected to restriction enzyme digestion and then to electrophoresis in 0.8% agarose gel. Depurination followed by denaturation and neutralization steps were performed before capillary transfer overnight using a positively charged nylon membrane (Hybond-N+, GE Healthcare). Membrane was UV-cross-linked and prehybridized with Church buffer (0.25 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, 7% SDS) at 65°C for 1 h. Probes were labeled with 50 µCi of [α-32P]dCTP (PerkinElmer Life Sciences) using the Prime-It RmT random primer labeling kit (Agilent Technologies). Illustra microspin G-50 columns (GE Healthcare) were used to remove unincorporated labeled nucleotides. Labeled probes were combined with 10 µg of unlabeled genomic DNA and Church buffer, denatured at 99°C, and incubated at 65°C for 1 h. Membrane hybridization was performed overnight at 65°C. Washing steps (30 min at 65°C) using buffer I (1× SSC and 1% SDS), buffer II (1× SSC and 0.1% SDS), and buffer III (0.5× SSC and 0.1% SDS) were carried out. The membrane was exposed for 2 days and visualized using a BAS 1800 II Phosphorimager (FujiFilm).

**Mouse genotyping**

Primers used for genotyping Secisbp2^{R543Q/+} and Secisbp2^{C696R/+} mice were displayed in Table S1. Alb-Cre; Secisbp2^{R543Q/+} or CamK-Cre; Secisbp2^{R543Q/+} were genotyped as described (5, 6).

**RT-PCR**

Real-time PCR was carried out as described before (5, 6) with minor modifications. RNA was extracted from mouse tissue (liver or cortex) according to the instructions for TRizol reagent (Invitrogen). cDNA was prepared following the protocol of the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed on a Mastercycler epgradient S realplex (Eppendorf) using Absolute qPCR SYBR Green Fluorescein Mix (Thermo Fisher Scientific). Primers and PCR conditions were described previously (5, 6). 18S rRNA was used as a housekeeping gene for normalization.

**Western blotting**

50 µg of liver or 100 µg of cortex protein in radiolabeled precipitation buffer were electrophoresed in a SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (GE Healthcare). After Ponceau staining, membranes were blocked in 5% skim milk in TBST. Antibodies and dilutions used for this study were already published before (5, 6) with the exception of rabbit polyclonal anti-Sepsh2 (Rockland) used at 1:2000. Detection was performed by Fusion Solo imaging system (Vilber Lourmat Deutschland GmbH) using horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibodies (Jackson Immunotech) and the enhanced horseradish peroxidase chemiluminescence substrate SuperSignalWest Dura (Thermo Fisher Scientific).

**Immunohistochemistry**

Mice were perfused with 4% paraformaldehyde in 1× PBS. After dissection, brains were post-fixed in 4% paraformaldehyde overnight and washed in 1× PBS before sectioning. 70-µm brain sections were cut using a vibratome (VT 1000S, Leica). After blocking in 5% BSA, staining of floating sections was performed with the indicated antibodies at 4°C overnight. Anti-Pvalb and anti-GFAP antibody dilutions were published before (5, 6). Rabbit anti-Iba1 (Wako) was used at 1:1000 dilution. Fluorescence was detected using Alexa 488 anti-mouse (Thermo Fisher Scientific) or Cy3 anti-rabbit (Jackson Immunotech).
**Cloning, expression, and purification of CSecisbp2**

We followed the procedures described previously (39) with minor modifications. The C terminus of the mouse Secisbp2 gene was cloned into pTrcHis2 vector (Invitrogen) using BstBI and NcoI restriction sites. The R543Q and C696R mutations were introduced into the pTrcHis2-CCisisbp2 vector using the QuikChange II site-directed mutagenesis kit (Agilent Technologies). Sanger sequencing was used to check for success. pTrcHis2-CCisisbp2 plasmids were transformed into BL21 star E. coli. Bacteria grown in lysogeny broth medium to a density of ~1.0 A_{\text{600}} were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside. Bacteria pellets were collected by centrifugation, resuspended in buffer XH1 (50 mM sodium phosphate, pH 8, 1 M NaCl, 1% Tween 20, 20 mM imidazole, 1 X Complete EDTA-free protease inhibitor (Roche Applied Science)), and lysed by sonication. After centrifugation, the supernatants were mixed with 2 ml of equilibrated nickel-nitrilotriacetic acid agarose beads (Qiagen) into gravity flow columns (Bio-Rad) and incubated for 30 min on ice or at 37/40.5 °C before in vitro translation. For titration experiments, reporter mRNA was diluted in nuclease-free water to obtain nine different concentrations shown in the figure before in vitro translation.

**Ribosome profiling and RNA-Seq**

Treatment of the samples was performed as described previously (19) with the exception of the RNase I incubation time, which was reduced to 20 min for the cortex tissue. Raw sequence data and raw counts can be obtained from the NCBI GEO repository entry GSE119681.

**Quality control and preprocessing of deep-sequence data**

Quality of sequencing data was controlled via FastQC version 0.11.8 (40) before and after trimming and rRNA removal. Adapter sequences and low-quality bases were trimmed using TrimGalore 0.4.3 with cutadapt 1.12 (41, 42). Sequences from ribosome profiling data were first aligned with bowtie2 2.3.4.3 (43) (maximum of 2 mismatches allowed) against rRNA and tRNA sequences obtained from the UCSC Genome Browser via the Table Browser tool (43–45). Unaligned reads (reads that are neither tRNA nor rRNA) were then aligned with bowtie2 against a UCSC mm10 RefSeq mouse transcriptome, containing the longest isoform of corresponding genes with 5’-UTR, CDS, and 3’UTR regions. Results were filtered after unique reads with samtools 1.9 (– F 4 – q 4) for downstream analysis (46). The size distribution was calculated, and all reads with length within mean ± S.D. were used for further analysis. Trimmed sequences of the RNA-Seq data were aligned with STAR aligner 2.6.0 against the GRCh38 (release 95) mouse genome retrieved from the Ensembl database via the biomaRt tool (47–49). Samtools was used to sort and index the resulting files containing the aligned sequences. The amount of sequences aligned to transcripts was counted with HTSeq 0.6.0 for following differential analysis with the DESeq2-package version 1.14.1 in R 3.3.1 (50–52). For ribosome profiling, reads with length between 28 and 32 nucleotides were used for differential expression analysis. GO enrichment analysis for significant overexpressed genes was done with PantherDB 14.1 (53–55).

**Offset to ribosome P-site**

Distribution of read sizes was calculated, and the sizes in the area of the mean ± S.D. (28–32 nucleotides for all samples) were used for downstream analysis. Offsets from 5’ ends of the reads to corresponding ribosome P-sites were calculated length- and sample-specific. Therefore, reads in an area of 20 nucleotides 5’ of start codons to 150 nucleotides 3’ of start
codons were extracted, and the relative positions of the 5’ ends in this area, according to the start codon, were set. The highest peak 5’ of the start codon shows the highest density of ribosomes at the translation initiation codon. This peak was set as preliminary offset to P-site, and the amount of ribosomes within this periodicity was calculated for the first 50 codons. It was done in the same way for the positions before and after this peak. The position with the highest periodicity value was set as offset to the P-site. Size-specific offsets were the same in all samples. Sizes 28, 29, and 30 had an offset of 12 nucleotides, whereas sizes 31 and 32 showed an offset of 13 nucleotides.

Statistics

Means are reported with S.D. unless otherwise indicated. The numbers of biological replicates are given in the figure legends. Statistical tests employed were usually Student’s t test. Statistics and curve fittings were calculated using GraphPad Prism. In RNA-Seq experiments, Benjamini–Hochberg (BH) test was performed. In S.e.m. experiments, the start codon, were set. The highest peak 5’ in this area, according to the start codon, were set. The highest peak 5’ of the start codon shows the highest density of ribosomes at the translation initiation codon. This peak was set as preliminary offset to P-site, and the amount of ribosomes within this periodicity was calculated for the first 50 codons. It was done in the same way for the positions before and after this peak. The position with the highest periodicity value was set as offset to the P-site. Size-specific offsets were the same in all samples. Sizes 28, 29, and 30 had an offset of 12 nucleotides, whereas sizes 31 and 32 showed an offset of 13 nucleotides.

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