Published in final edited form as:
Biochem J. 2009 February 15; 418(1): 61–71. doi:10.1042/BJ20081304.

The selenocysteine tRNA STAF-binding region is essential for adequate selenocysteine tRNA status, selenoprotein expression and early age survival of mice

Bradley A. CARLSON[*], **Ulrich SCHWEIZER**[†], **Christine PERELLA**[‡], **Rajeev K. SHRIMALI**[*], **Lionel FEIGENBAUM**[‡], **Liya SHEN**[§], **Svetlana SPERANSKY**[§], **Thomas FLOSS**[‖], **Soon-Jeong JEONG**[*], **Jennifer WATTS**[¶], **Victoria HOFFMANN**[**], **Gerald F. COMBS Jr**[¶], **Vadim N. GLADYSHEV**[††], and **Dolph HATFIELD**[*,1]

[*]Molecular Biology of Selenium Section, Laboratory of Cancer Prevention, Center for Cancer Research, NCI (National Cancer Institute), NIH (National Institutes of Health), Bethesda, MD 20892, U.S.A.

[†]Neurobiology of Selenium, Neuroscience Research Center, Charité-Universitätsmedizin Berlin, 10117 Berlin, Germany

[‡]Laboratory of Animal Science Program, Science Applications International Corporation, NCI, Frederick, MD 21702, U.S.A.

[§]Laboratory of Cellular Carcinogenesis and Tumor Promotion and GMCF (Germline Mutation Core Facility), Center for Cancer Research, NCI, NIH, Bethesda, MD 20892, USA

[‖]Helmholtz Center Munich, Institute of Developmental Genetics, Ingolstäadter Landstrasse 1, D-85764 Neuherberg, Germany

[¶]Grand Forks Human Nutrition Research Center, USDA-ARS, Grand Forks, ND 58201, U.S.A.

[**]Office of the Director, Diagnostic and Research Services Branch, NIH, Bethesda, MD 20189, U.S.A.

[††]Department of Biochemistry and Redox Biology Center, University of Nebraska, Lincoln, NE 68588, U.S.A.

Abstract

STAF [Sec (selenocysteine) tRNA gene transcription activating factor] is a transcription activating factor for a number of RNA Pol III- and RNA Pol II-dependent genes including the *Trsp* [Sec tRNA gene], which in turn controls the expression of all selenoproteins. Here, the role of STAF in regulating expression of Sec tRNA and selenoproteins was examined. We generated transgenic mice expressing the *Trsp* transgene lacking the STAF-binding site and made these mice dependent on the transgene for survival by removing the wild-type *Trsp*. The level of Sec tRNA was unaffected or slightly elevated in heart and testis, but reduced ~60% in liver and kidney, ~70% in lung and spleen and ~80% in brain and muscle compared with the corresponding organs in control mice. Moreover, the ratio of the two isoforms of Sec tRNA that differ by methylation at position 34 (Um34) was altered significantly, and the Um34-containing form was substantially reduced in all tissues examined. Selenoprotein expression in these animals was most affected in tissues in which the Sec tRNA levels were most severely reduced. Importantly, mice had a neurological phenotype strikingly similar to that of mice in which the selenoprotein P gene had been removed and their life span was substantially

1 To whom correspondence should be addressed (hatfield@mail.nih.gov)..

reduced. The results indicate that STAF influences selenoprotein expression by enhancing *Trsp* synthesis in an organ-specific manner and by controlling Sec tRNA modification in each tissue examined.

Keywords

selenium; selenocysteine (Sec) tRNA gene (*Trsp*); selenoprotein; Sec tRNA gene transcription activating factor (STAF); survival rate; tRNA transcription

INTRODUCTION

There are 24 known selenoprotein genes [i.e. genes coding for proteins containing the amino acid Sec (selenocysteine)] in rodents and 25 in humans [1]. One of the key factors required in the expression of selenoproteins is Sec tRNA. Since the biosynthesis of Sec occurs on its tRNA, and Sec synthesis initiates with the aminoacylation of serine, this tRNA has been designated $tRNA^{[Ser]Sec}$ [2]. The $tRNA^{[Ser]Sec}$ population consists of two isoforms that differ by a single methyl group located on the 2′-*O*-hydroxyribosyl moiety at position 34 (designated Um34) (reviewed in [3]). The modified base at position 34 in both isoforms is mcm^5U (5′-methylcarboxylmethyluracil) and the nucleoside at position 34 in the Um34-containing isoform is mcm^5Um (5′-methylcarboxylmethyluridine-2′-*O*-methylribose). Thus the two isoforms are designated mcm^5U and mcm^5Um. Synthesis of Um34 on mcm^5U to form mcm^5Um is the last step in the maturation of $tRNA^{[Ser]Sec}$ and its attachment is dependent on selenium status [3].

We have taken advantage of the fact that selenoprotein synthesis is dependent on the presence of $tRNA^{[Ser]Sec}$ and have thus generated several mouse models that have altered $tRNA^{[Ser]Sec}$ levels as a tool to modulate selenoprotein expression and elucidate the role of this specialized class of proteins in health and development ([4,5] and reviewed in [3]). For example, transgenic mice encoding wild-type or mutant $tRNA^{[Ser]Sec}$ transgenes were generated that resulted in an overexpression of the corresponding wild-type or mutant $tRNA^{[Ser]Sec}$ [3]. Increased levels of wild-type *Trsp* (Sec tRNA gene) caused only minor changes in the selenoprotein population in specific tissues, suggesting that the Sec $tRNA^{[Ser]Sec}$ population in most mammalian cells is not limiting (reviewed in [3]). Transgenic mice encoding mutant *Trsp* transgenes, however, resulted in reduced expression of a subclass of selenoproteins that function primarily in stress-related phenomena [6-8]. One of these mutant transgenic mouse lines has been used to show that these stress-related selenoproteins have a role in preventing colon [9] and prostate [10] cancers.

The *Trsp* gene has also been conditionally deleted in specific tissues and organs using *loxP-Cre* technology [5], resulting in the complete loss of selenoprotein expression after recombination in targeted cells [5,11,12]. The conditional knockout of *Trsp* has been used to show that selenoproteins have a role in proper liver function [11], endothelial cell development and heart disease prevention [12], neuronal development and survival (E. K. Wirth, M. Conrad, B. S. Bharathi, J. Winterer, C. Wozny, B. A. Carlson, S. Roth, D. Schmitz, G. W. Bornkamm, M. Brielmeier, V. Coppola, L. Tessarollo, E. Pohl, L. Schomburg, J. Kohrle, D. L. Hatfield and U. Schweizer, unpublished work) and immune function [13].

Regulation of *Trsp* expression is unique among tRNAs. In contrast with other tRNA genes, *Trsp* transcription is governed by three upstream regulatory regions: the TATA box motif at −30, the *PSE* (proximal sequence element) located at ~−70 [14-17] and a *DSE* (distal sequence element) located at ~−200 in mice [16]. The latter regulatory region is composed of an *AE* (activator element) containing an SPH motif and an octamer sequence [16]. The *DSE* is required

for optimal transcription of *Trsp in vivo* in *Xenopus* oocytes [16], although it does not function in the enhancement of *Trsp* transcription *in vitro* ([18] and reviewed in [19]). The *DSE* has been disrupted in mice by inserting a 3.2 kb fragment between this regulatory region and the *PSE* [20]. Disruption of the *DSE* in this manner was embryonic lethal resulting from loss of *Trsp* transcription and severely decreased amounts of selenoprotein transcripts. A protein factor, designated the STAF (Sec tRNA gene transcription activating factor), binds to the AE. STAF was previously cloned and characterized in *Xenopus* [21] and mice [22-24]. This transcription factor was subsequently found to have roles in the expression of many other genes transcribed by RNA polymerases II and III [25-28].

In the present study, we took an alternative approach in investigating the role of STAF and the *AE* region in *Trsp* transcription. The role of STAF in $tRNA^{[Ser]Sec}$ transcription in an *in vivo* model was examined by generating mutant *Trsp* transgenic mice in which the STAF-binding site of *Trsp*,the *AE* region, was deleted. We then used the deleted *AE* allele to genetically complement *Trsp*-knockout mice [5,29]. The resulting AE^{-} transgenic/*Trsp*-knockout mice expressed $tRNA^{[Ser]Sec}$ in substantially reduced amounts in liver, kidney, brain, lung, spleen and muscle, but in similar or slightly elevated levels in heart and testes. Interestingly, the level of the mcm^5Um isoform was less than that of the mcm^5U isoform in all tissues, and even in heart and testes wherein the Sec $tRNA^{[Ser]Sec}$ population was not reduced. Selenoprotein expression was down-regulated in tissues that were most affected by Sec $tRNA^{[Ser]Sec}$ reduction, and brain, muscle, lung and spleen appeared to be the most affected tissues. AE^{-} animals manifested a neurological phenotype similar to that of mice in which the selenoprotein P gene (*Sepp*) had been targeted for removal [29-33]. These mice had a wide, waddling gait [31,33,34], tremor [35] and hyperexcitability with occasional seizures [32]. The results of these studies are presented and discussed with regard to mechanism of regulation of selenoprotein expression by STAF and the two $tRNA^{[Ser]Sec}$ isoforms.

EXPERIMENTAL

Materials and animals

^{75}Se (specific radioactivity 1000 Ci/mmol) was obtained from the Research Reactor Facility, University of Missouri (Columbia, MO, U.S.A.), [α-^{32}P]dCTP (specific radioactivity ~6000 Ci/mmol) from PerkinElmer and [^{3}H]serine (specific radioactivity 30 Ci/mmol), Ready-to-Go DNA Labelled Beads and Hybond Nylon N^+ membranes from GE Healthcare. NuPAGE 10% polyacrylamide gels, 10% TBE (Tris/borate/EDTA; 1 × TBE = 45 mM Tris/borate and 1 mM EDTA)/urea gels, PVDF membranes, TRIzol® and SeeBlue Plus2-protein markers were purchased from Invitrogen. SuperSignal West Dura Extended Duration Substrate was obtained from Pierce, the GeneEditor In Vitro Site-Directed Mutagenesis System from Promega, antibodies against GP×1 (glutathione peroxidase 1) from Abcam, antibodies against GP×4 (glutathione peroxidase 4), SelR (selenoprotein R) and SelT (selenoprotein T) from our stock supplies and anti-rabbit-HRP (horseradish peroxidase)-conjugated secondary antibodies from Sigma. All other reagents were obtained commercially and were products of the highest grade available. Heterozygous *Trsp*-knockout mice ($Trsp^{+/-}$) in a C57Bl/6 genetic background were from a previous study in our laboratory [5]. Mice were maintained on standard rodent diet, NIH-31A, containing adequate amounts of selenium as described in [36] and the care of animals was in accordance with the National Institutes of Health Institutional Guidelines under the expert direction of Dr Kyle Stump (NCI, NIH, Bethesda, MD, U.S.A.).

Preparation of mutant transgene, generation of experimental mouse lines, breeding, genotyping, survival rates and pathology

A 2158 bp DNA fragment encoding *Trsp* [4] with a 14 bp deletion of the *AE* region (bases −213 to −226) was used to generate transgenic mice by zygote injection as described elsewhere

[4]. The 14 bp deletion was prepared using the GeneEditor In Vitro Site-directed Mutagenesis System according to the manufacturer's instructions. Three independent founder transgenic mice carrying the 2158 bp transgene with a deletion of the *AE* allele were established in strain FVB/N and designated $Trsp^{tAE-}$. DNA was extracted from mouse-tail clippings and the $Trsp^{tAE-}$ copy number initially assessed in the three founder mice by Southern blotting of DNA that was digested with XhoI, probed with [^{32}P]CTP-labelled probe and estimated to be one copy in one founder and five copies in the other two founders by procedures described previously [4,37]. The low-copy-number $Trsp^{tAE-}$ founder and one of the higher number copy founders were bred to obtain the corresponding homozygous mice for further examination. The presence or absence of *Trsp* or $Trsp^{tAE-}$ was determined in all subsequent studies by PCR and the primers used have been described in detail previously [6,7]. These primers were designated CKNO2 (forward primer) and RES1 and VP1 (reverse primers). CKNO2-RES1 yields a 980 bp PCR *Trsp* fragment and a 500 bp PCR $Trsp^{-}$ fragment and CKNO2-VP1 yields a 1058 bp PCR $Trsp^{tAE-}$ fragment.

Survival rates of $Trsp^{tAE-/-}/Trsp^{-/-}$ mice carrying two or ten copies of $Trsp^{tAE-/-}$ and control mice were assessed as described in the legend to Figure 1.

$Trsp^{tAE-/-}/Trsp^{-/-}$ homozygous mice (five males and three females encoding two copies of $Trsp^{tAE-/-}$) and control mice (two males and three females), 1−2 months old, were subjected to pathological analysis. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned 5-μm-thick, stained with H/E (haematoxylin/eosin), coverslipped and examined using an Olympus BX41 microscope.

Isolation, aminoacylation, fractionation and quantification of tRNA

Total tRNA was isolated from mouse tissues (see Figure 2 for tissues examined) of $Trsp^{tAE-/-}/Trsp^{-/-}$ mice carrying two or ten copies of $Trsp^{tAE-/-}$ and control mice, and the amounts of $tRNA^{Ser}$ were quantified after gel electrophoresis and hybridization with the appropriate ^{32}P-labelled probe by Northern-blot analysis using a PhosphorImager (Molecular Dynamics) [5]. Only $Trsp^{tAE-/-}$ mice carrying two copies of the mutant transgene and control mice were examined in all subsequent studies. Total tRNA from tissues of $Trsp^{tAE-/-}/Trsp^{-/-}$ mice and control mice was aminoacylated with [^{3}H]serine and 19 unlabelled amino acids in the presence of rabbit reticulocyte synthetases [38]. The resulting aminoacylated tRNA was fractionated on an RPC-5 column [39], first in the absence and then in the presence of Mg^{2+} as given in [4,5], the amounts of $tRNA^{[Ser]Sec}$ relative to the total Ser tRNA population, and of the distributions of the two isoforms, mcm^{5}U and mcm^{5}Um, were determined as described in detail previously [4,5].

Labelling of selenoproteins and selenium levels

Mice were given intraperitoneal injections of ^{75}Se (50 μCi/g) and killed 48 h after injection. Tissues and organs were excised, frozen in liquid nitrogen and stored at −80°C until use. Tissues were homogenized, the protein extracts were electrophoresed on NuPAGE 10% polyacrylamide gels, along with molecular mass markers, gels were stained with Coomassie Brilliant Blue, dried and exposed to a PhosphorImager and band intensities were assessed as described in [11].

The amount of selenium in extracts of liver, kidney, brain, heart, testes, lung, spleen and plasma was determined as described previously [3,40]. Statistical analysis was performed by a Student's *t* test (unpaired, two-sided) using GraphPad Prism 4.0.

Western-blot analysis

Protein extracts were prepared from liver, kidney, brain, testes, heart, lung, spleen and muscle from three $Trsp^{tAE-/-}/Trsp^{-/-}$ mice and three control mice, all 4–5 weeks old, were electrophoresed on NuPAGE 10% polyacrylamide gels, and the resulting fractionated proteins were transferred to PVDF membranes and immunoblotted with antibodies against GP×1 (1:1000 dilution), GP×4 (1:1000), SelR (1:1000) and SelT (1:800). Anti-rabbit-HRP-conjugated secondary antibody (1:20000) was used in all Western blots. After the incubation with the secondary antibody, membranes were washed with 0.1% TBS-T (20 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20), incubated in SuperSignal West Dura Extended Duration Substrate and then exposed to an X-ray film. Band intensities were assessed by PhosphorImager analysis [11]. Separate gels were used for each antibody, and blots were stripped and probed with β-tubulin (1:1000) to serve as a loading control.

Immunohistology

Brains of 5-week-old $Trsp^{tAE-/-}/Trsp^{-/-}$ and control mice were immersion-fixed in 4% (w/v) paraformaldehyde/0.1 M sodium phosphate buffer, pH 7.4 (PB). After cryoprotection in 30% sucrose in PB, brains were frozen on dry ice and stored at −80°C. Sections of 35 μm thickness were cut on a cryostat. Nissl staining was done as described in [41]. Immunostaining on free-floating sections was performed with the following antibodies: rabbit anti-PV (parvalbumin) (1:5000; Swant, Bellinzona, Switzerland) and mouse anti-GFAP (glial fibrillary acidic protein; Sigma, Munich, Germany) (1:1000). Immunoreactivity was visualized with HRP using the Vectastain reagents (Vector, Burlingame, VT, U.S.A.) and the MOM kit (Vector) with diaminobenzidine as the substrate.

RESULTS

Generation of $Trsp^{tAE-/-}/Trsp^{-/-}$ mice and survival rates

To examine the role of STAF in *Trsp* transcription, we deleted the STAF activating (*AE*) region in a *Trsp* fragment and generated transgenic mice carrying two or ten copies of the mutant transgene (homozygous animals designated $Trsp^{tAE-/-}$). We then used these transgenic mice to genetically complement *Trsp*-knockout ($Trsp^{-/-}$) mice with the mutant transgenes. The resulting mice were viable, and therefore, the transgenic *AE*-lacking Sec $tRNA^{[Ser]Sec}$ rescued the lethal phenotype of $Trsp^{-/-}$.

Since mice encoding $Trsp^{-/-}$ were dependent on the mutant transgene for survival, this provided us with a model for examining the role of the *AE* region in $tRNA^{[Ser]Sec}$ expression in various tissues. The breeding scheme used in obtaining the $Trsp^{tAE-/-}/Trsp^{-/-}$ and control mice is shown in Table 1. The survival rates of high- and low-copy-number mutant and control transgenic mice were compared (see Figure 1). Most $Trsp^{tAE-/-}/Trsp^{-/-}$ mice died prematurely (Figure 1). By 46 and 48 days, half of the $Trsp^{tAE-/-}/Trsp^{-/-}$ mice carrying two and ten copies of mutant transgene respectively died. There were no differences observed between survival rates of males and females. The high death rate was characteristic of young mice, whereas those who survived to adulthood, whether they carried two or ten copies of the mutant transgene, had much better chances of long-term survival.

Sec $tRNA^{[Ser]Sec}$ expression and isoform distribution in $Trsp^{tAE-/-}/Trsp^{-/-}$ and control mice

We examined the expression and distribution of $tRNA^{[Ser]Sec}$ and its isoforms, mcm^5U and mcm^5Um, in $Trsp^{tAE-/-}/Trsp^{-/-}$ mice. Total tRNA was isolated from liver, kidney, brain, heart, testes, lung, spleen and muscle of $Trsp^{tAE-/-}/Trsp^{-/-}$ and control mice and the expression of $tRNA^{[Ser]Sec}$ was examined by Northern-blot analysis in control and $Trsp^{tAE-/-}/Trsp^{-/-}$ mice carrying two or ten copies of the mutant transgene (Figure 2). The analysis was repeated in

triplicate with the control and low-copy-number transgenic mice. The levels of $tRNA^{[Ser]Sec}$ from tissues of control mice were averaged and assigned a value of 1.0 (legend to Figure 2). The levels of $tRNA^{[Ser]Sec}$ from tissues of $Trsp^{tAE-/-}/Trsp^{-/-}$ mice carrying two copies of transgene relative to the wild-type control are shown in the upper panel of Figure 2. With the exception of heart and testes, which had similar levels of $tRNA^{[Ser]Sec}$ in control and mutant transgenic mice, the levels of $tRNA^{[Ser]Sec}$ were substantially reduced in all other tissues of mutant transgenic mice compared with the control wherein liver, brain, lung spleen and muscle manifested less than 50% expression. In addition, $tRNA^{[Ser]Sec}$ levels were examined in thymus of mice carrying two copies of mutant transgene and found to be approx. 50% of the wild-type level. Although the Northern-blot analysis was only carried out with $tRNA^{[Ser]Sec}$ from tissues of a single mouse carrying the higher number of mutant transgenes, the pattern of $tRNA^{[Ser]Sec}$ reduction was similar in the two mouse lines in that the reduction of $tRNA^{[Ser]Sec}$ occurred in all tissues except heart and testes, and muscle showed the lowest level relative to the control.

The above observations on survival rates (Figure 1) and $tRNA^{[Ser]Sec}$ expression (Figure 2) suggested that mice carrying either the high- or low-copy-number mutant transgenes, wherein the mice were dependent on the transgene for survival, produced similar effects on mice. Thus only the low-copy-number $Trsp^{tAE-/-}/Trsp^{-/-}$ mice were further examined.

$tRNA^{[Ser]Sec}$ expression in $Trsp^{tAE-/-}/Trsp^{-/-}$ control mice was also examined by RPC-5 chromatography, and the levels of the total $tRNA^{[Ser]Sec}$ population relative to the total seryl-$tRNA^{Ser}$ population were determined (Table 2). The amount of the $tRNA^{[Ser]Sec}$ population appeared to be slightly elevated in heart and was approx. 1.4 times higher in testes of $Trsp^{tAE-/-}/Trsp^{-/-}$ animals compared with controls (Table 2, column 3). However, $tRNA^{[Ser]Sec}$ levels were substantially lower in liver (64% reduction), kidney (56% reduction), brain (79% reduction), lung (69% reduction), spleen (67% reduction) and muscle (83% reduction) of $Trsp^{tAE-/-}/Trsp^{-/-}$ animals.

The distributions of the mcm^5U and mcm^5Um isoforms within the $tRNA^{[Ser]Sec}$ population were assessed by RPC chromatography in liver, kidney, brain, heart, lung, testes, muscle and spleen (Table 2). The percentage of mcm^5U and mcm^5Um within the total $tRNA^{[Ser]Sec}$ population is shown in columns 4 and 5 respectively. The distributions were dramatically altered in each tissue from $Trsp^{tAE-/-}/Trsp^{-/-}$ animals compared with those of control animals in that the mcm^5Um isoform was reduced substantially relative to the mcm^5U isoform. The percentage change of both isoforms in each of these tissues in $Trsp^{tAE-/-}/Trsp^{-/-}$ compared with control mice is shown in columns 6 and 7 of Table 2. These data show the reduced amount of mcm^5Um in each tissue and either the increase (heart and testes) or decrease (liver, kidney, brain, muscle and spleen) of mcm^5U. The most pronounced reduction in both isoforms occurred in brain, lung, muscle and spleen, whereas large losses of mcm^5U occurred in all tissues except heart and testes. The lowest levels of mcm^5Um expression occurred in brain and muscle.

^{75}Se-labelling in $Trsp^{tAE-/-}/Trsp^{-/-}$ and control mice

Since the level of $tRNA^{[Ser]Sec}$ is not limiting in tissues of normal mice (reviewed in [3]), but the levels of this tRNA were reduced substantially in most of the tissues examined in the present study, we analysed selenoprotein expression by ^{75}Se-labelling of $Trsp^{tAE-/-}/Trsp^{-/-}$ and control mice (Figure 3A). Coomassie Brilliant Blue-stained SDS/PAGE gels revealed no visible differences in total protein levels in the corresponding tissues of $Trsp^{tAE-/-}/Trsp^{-/-}$ and control animals (results not shown). In the PhosphorImager analyses that visualize ^{75}Se-labelled proteins, those selenoproteins that were identified previously [6,42] are indicated in the Figure along with the molecular mass markers on the liver and plasma panels (Figure 3). Although the labelling was weakest in brain, the overall selenoprotein labelling pattern appeared to be highly affected in this tissue and possibly in lung and spleen of $Trsp^{tAE-/-}$/

Trsp$^{-/-}$ mice. GPx1 in liver appeared to be slightly reduced relative to other selenoproteins in tissues from mutant mice. Selenoprotein labelling in muscle was not examined as selenoproteins were very poorly labelled in this tissue in earlier studies [4]. The intensity of the various bands relative to that found in the corresponding control proteins was quantified by PhosphorImager analysis (Figure 3B). Values obtained for labelling of the selenoproteins analysed were assigned a value of 1.0 in each control tissue. The levels of GP×1, GP×4 and TR1 appeared to be lowest in spleen, lung and brain and less affected in the other tissues. GP×3 was slightly reduced in plasma and SelP was somewhat more reduced in this tissue.

Analyses of selenoprotein expression by Western blotting

Metabolic labelling of selenoproteins may be influenced by protein turnover, differential delivery of the isotope to specific organs and the level of available selenium in respective tissues. We therefore placed greater emphasis on analysing selenoproteins by Western blotting to assess changes in their levels in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice. Several selenoproteins were selected that were readily detectable by immunoblotting and are affected by the methylation status of tRNA$^{[Ser]Sec}$ [6,7,11] for further analysis (Figure 4). Western blots were carried out in triplicate from three individual *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice and a representative blot from one of the affected and control animals is shown in Figure 4(A). However, since the Western blots were done in triplicate, we quantified the results from the tissues of the three *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice and did a statistical analysis on the three runs (Figure 4B). GP×1 levels were reduced statistically in all tissues examined except kidney compared with controls, while GP×4 was reduced statistically only in brain, lung and spleen. SelR levels were down in liver, brain, testes, heart and muscle and the variations were too extreme in lung and spleen to assess whether SelR levels were down statistically in these tissues compared with controls. SelT levels were reduced only significantly in lung, spleen and muscle compared with the controls. Possible reasons for the variations in levels of these selenoproteins in the different tissues are further considered in the Discussion section.

Influence of the STAF-binding region on selenium status of mouse tissues

Selenium amounts were determined in liver, kidney, brain, heart, testes, lung, spleen, plasma and muscle of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control animals (Table 3). Although selenium levels were numerically lower in each tissue from *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ animals compared with controls, statistical analysis showed that Se contents were significantly lower in liver, brain, lung and muscle in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice, but not in the other tissues.

Phenotypic characteristics and pathology of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice

Phenotypically, the *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice were smaller in size than their normal littermates, which was readily noticeable after approx. 3 weeks of age. Pups were weighed at 3–5 weeks after birth and the average weights of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice during this time frame were 71% of those of control mice.

Several pathological abnormalities were observed in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice (Figure 5). Six of eight *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice had smaller spleens with little haematopoietic activity and smaller follicles, which also lacked a marginal zone compared with control mice (Figure 5A). Five of eight *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice exhibited acute cerebral neuronal necrosis, while none of the five control mice had this lesion (Figure 5B). Less frequent findings in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice included an observation that three of eight mice had either single cell necrosis/apoptosis or larger areas of coagulative hepatic necrosis (Figure 5C). One control had mild hepatocellular single cell necrosis. Three of eight *AE* mutant mice had gastric ulcers and/or gastritis. In addition, mineralization of skeletal muscle, tongue, heart and adipose tissue was present in six of eight mutant mice, whereas only one control mouse had mild mineral deposition in the heart.

Reduced survival, growth retardation, mineralization of muscle, small spleens and liver abnormalities were all previously found in *Sepp*$^{-/-}$ mice ([30,31,33,34,43]; and U. Schweizer, unpublished work). Moreover, the *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice appeared hyperactive, with tremor, periodically exhibited seizures (which could be induced by handling) and a wide waddling gait, and occasional backward movement, which are also phenotypes observed in *Sepp*$^{-/-}$ mice ([30-33]; U. Schweizer, unpublished work).

Immunohistological analysis of the brain

In order to investigate the neurological phenotype of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice in more detail, we performed further histological studies of their brains. As judged by Cresyl Violet staining, the brains of these mice appeared structurally normal (Figure 6A). However, if neurons die by apoptosis and at a low rate, their remnants will be cleared from the brain parenchyma within days. Therefore we probed brain sections for increased expression of GFAP, which is a marker of reactive astrocytes and an established indicator of neurodegenerative processes. In *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice, GFAP immunoreactivity was massively increased throughout the brain, in particular in the cerebral cortex grey matter where GFAP is normally very low and confined to astrocytes surrounding blood vessels (Figure 6B). We speculated that the hyperexcitable phenotype of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice may be related to an impaired inhibitory system. In the cerebral cortex and hippocampus, GABA (γ -aminobutyric acid) is the predominant inhibitory neurotransmitter. Most of the GABAergic neurons within the cerebral cortex express the small calcium-binding protein PV, which can be used as a marker for these neurons [44]. In *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice, the number of PV-immunopositive neurons was greatly reduced both in the cerebral cortex and in the hippocampus (Figure 6C). Thus the neurological phenotype of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mutant mice may be partially caused by a disturbance of the GABAergic inhibitory system. On the other hand, it has been shown that epilepsy leads to the loss of GABAergic interneurons [45]. Thus we cannot be certain at present whether the loss of PV cells is the cause or consequence of the observed phenotype. Nevertheless, increased and widespread astrogliosis as shown by GFAP immunostaining argues for a mechanism of diffuse neurodegeneration throughout the brain.

DISCUSSION

In the present study, the role of STAF in *Trsp* transcription was investigated *in vivo* by genetically complementing *Trsp*$^{-/-}$ mice with *Trsp*$^{tAE-/-}$ alleles, which rescued these knockout mice from lethality. The loss of STAF responsiveness of transgenic *Trsp* expression in two mutant mouse lines carrying two or ten copies of *Trsp*$^{tAE-/-}$ resulted in tissue-specific *Trsp* transcription changes wherein testes and heart appeared to have similar or slightly enriched levels of Sec tRNA$^{[Ser]Sec}$ and liver, kidney, brain, lung, muscle and spleen had reduced levels. The low- and high-copy-number *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mouse lines had reduced but similar rates of survival compared with control animals, and the levels of tRNA$^{[Ser]Sec}$ expression in the various tissues examined by Northern-blot analysis were similar. In light of these observations and the fact that the *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mouse carrying two copies of the mutant transgene would more naturally mimic a mutation in the AE region precluding STAF attachment, further studies were only pursued with the low-copy-number transgenic mouse. In addition, the possibility that the effect of the deleted AE region may be influenced by the position of insertion of the mutant fragment in the genome rather than due solely to the loss of this regulatory site seems unlikely, since another founder mouse encoding a higher copy number of mutant transgenes manifested a similar survival rate and synthesized similar levels of tRNA$^{[Ser]Sec}$. Surprisingly, mice carrying two or ten copies of *Trsp*$^{tAE-}$ generated similar levels of tRNA$^{[Ser]Sec}$ transgene product (see Figure 2), whereas previous studies had shown that the amount of tRNA$^{[Ser]Sec}$ synthesized from such genes is proportional to the number of transgenes [4,37]. The synthesis of similar amounts of tRNA$^{[Ser]Sec}$ from single and multiple

Trsp^{tAE-} transgenes may be related to the reduced expression of the mcm^{5}Um isoform as selenium-deficient cells and tissues synthesize less of this isoform and less tRNA$^{[Ser]Sec}$ overall (reviewed in [3]). Furthermore, Secp43 is known to have a role in Um34 synthesis (and possibly a role in shuttling SecS from the nucleus to the cytoplasm) [46]. Whether Secp43 may be involved in the overall level of tRNA$^{[Ser]Sec}$ expression from multiple (trans)genes remains to be established, but its role in Um34 synthesis and the reduced expression of the mcm^{5}Um isoform observed in the present study in *AE*$^{-}$ mice would seem to be more than just coincidental.

The above observations raise a question of whether the effects seen by the removal of the STAF-binding region is related to selenium status and may be only indirectly related to the loss of STAF binding. This seems unlikely as the levels of selenium were reduced significantly in liver, brain, lung and muscle, but not in kidney, heart, testes, spleen or plasma, whereas the levels of mcm^{5}Um were reduced dramatically in all tissues examined regardless of whether the overall levels of tRNA$^{[Ser]Sec}$ and selenium were, or were not, reduced. Clearly, the overall levels of tRNA$^{[Ser]Sec}$ appeared to be increased in heart and testes, while the levels of selenium were not significantly changed in affected compared with control mice.

We have used both Northern blotting and RPC-5 chromatography to assess tRNA$^{[Ser]Sec}$ levels in mammalian cells (e.g. see [5] and the present study), while other investigators have used Northern blotting exclusively [20,29]. Although the two methods appear to yield similar results, a major advantage of RPC chromatography is that it permits an assessment of the distributions of the two isoforms, whereas Northern blotting can be used on much smaller amounts of tissue.

Kelly et al. [20] generated a mouse with a disrupted *DSE* wherein a 3.2 kb fragment was inserted between this region and the *PSE* regulatory region. Sec tRNA$^{[Ser]Sec}$ transcription was severely impaired in the mouse carrying the insertion on both alleles and embryo death occurred at day 7.5. The heterozygous mouse survived normally and, despite reduced levels of Sec tRNA in kidney and liver, this animal expressed selenoprotein mRNAs and the major mammalian selenoprotein, GP×1, in normal amounts. It is not surprising that the disrupted *DSE* generated by Kelly et al. [20] had a more pronounced effect on reducing *Trsp* transcription than the *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mouse, since the entire *DSE* was disrupted in their study, while our present study focused on the STAF-binding region by deleting only *AE*.

It is not clear why *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ animals were smaller and their survival rates were reduced compared with their normal littermates. Most likely, reduced selenoprotein expression in those tissues most affected, e.g. brain and muscle, played a role in these abnormalities. The phenotypic similarity between *Sepp*$^{-/-}$ mice and *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice was, however, striking. Both mouse models are characterized by growth retardation, small spleen, tissue calcification, liver and brain defects [31-35]. The exact mechanism why reduced selenoprotein expression in a particular cell type eventually leads to a growth defect in the mice is not yet understood. One possibility is that brain appears to be among the most affected organs in both models. Therefore we investigated brain at the molecular level and in more detail. We did not find evidence for developmental abnormalities within the brains of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice. Rather, the alterations that we observed pointed towards degenerative processes as evidenced by widespread astrogliosis and hypereosinophilic neurons in several brain regions. The most specific finding was the apparent reduction of PV expressing interneurons in cerebral cortex and hippocampus. This finding may be related to the observed neurological phenotype, in particular to the hyperexcitability, the abnormal gait and impaired righting reflex. However, a more detailed study would be required to elucidate the mechanism by which reduced selenoprotein expression causes neurodegeneration. Moreover, a dedicated neuropathological

study would be needed to determine whether $Trsp^{tAE-/-}/Trsp^{-/-}$ mice also suffer from axonal degeneration [35].

One of the more intriguing findings in the present study was the observed alteration in mcm^5U and mcm^5Um distribution in $Trsp^{tAE-/-}/Trsp^{-/-}$ mice wherein the latter isoform was more dramatically reduced in the tissues examined. In liver, kidney, brain, heart and lung of control animals, the ratio of mcm^5Um/mcm^5U was 1−1.5. However, upon reduction of *Trsp* expression in $Trsp^{tAE-/-}/Trsp^{-/-}$ mice, the ratio fell to ≤1. In heart and testes, mcm^5U was increased in the mutant, but mcm^5Um decreased even at similar or slightly higher levels of the $tRNA^{[Ser]Sec}$ population. In both the latter tissues of the $Trsp^{tAE-/-}/Trsp^{-/-}$ mice, selenoprotein expression was partially disrupted. Thus it appears as if the bulk levels of $tRNA^{[Ser]Sec}$ isoforms are not governing selenoprotein expression alone. Possibly, additional cellular compartments exist in these organs that respond differently to disruption of the activation element in *Trsp*. These compartments could express different sets of selenoproteins and thus a selenoprotein may appear reduced in spite of increased $tRNA^{[Ser]Sec}$ levels. In the case of testis, there is evidence for such a possibility. In $Sepp^{-/-}$ mice, testicular GP×4 expression is undetectable, while GP×1 is almost normal [47]. Usually, GP×4 is less dependent on Se supply than GP×1. Thus GP×4 may be expressed in an SePP (selenoprotein P)-dependent compartment, whereas GP×1 is not. It is otherwise difficult to explain how deletion of the *AE* region and/or the failure of STAF to properly recognize this region may be involved in the methylation of the mcm^5U isoform unless Secp43 may be involved as noted above.

GP×1 was not detected in muscle by Western blotting. In liver, the levels of GP×1 and SelR, but not SelT and GP×4, were reduced significantly in $Trsp^{tAE-/-}/Trsp^{-/-}$ mice compared with control mice as assessed by Western blotting. In other tissues, GP×1 was also statistically reduced in brain, heart, testes, lung, spleen and muscle, while SelR appeared to be reduced in these same tissues, but SelT only in lung, spleen and muscle and GP×4 in brain, lung and spleen in affected compared with control mice. mcm^5Um amounts were reduced more substantially than mcm^5U amounts in all tissues examined. These results provide interesting insights into the complex nature of expression of two subclasses of selenoproteins at the level of translation that is carried out by two separate isoforms.

We have also rescued $Trsp^{-/-}$ mice with a mutant *Trsp* transgene [7] wherein the tRNA gene product was incapable of generating the mcm^5Um isoform due to a mutation at position 37 [48]. Several selenoproteins serving stress-related functions, such as GP×1, GP×3, SelR, SelT and SelW, were poorly expressed in the rescued mouse, but other selenoproteins serving largely housekeeping functions, such as TR1, TR3 and GP×4, were expressed in normal or slightly reduced amounts [7]. Mice dependent on the mutant transgene for survival were phenotypically normal with the exception that males had defective sperm and produced fewer offspring than their wild-type littermates, while rescued females appeared to have some aberration with regard to fertility and/or carrying pregnancies to term. Although rescued $Trsp^{-/-}$ mice in the present study also expressed mcm^5Um poorly, they additionally expressed mcm^5U poorly in several tissues that apparently resulted in several housekeeping selenoproteins being synthesized in reduced but sufficient levels to permit the resulting mice to survive at least to an early age, as shown in Figure 1. The significance of the transgenic mouse model described in the present study over those used previously in which the $tRNA^{[Ser]Sec}$ levels were also altered resulting in differential expression of the subclasses of selenoproteins (reviewed in [3]) is that the present model down-regulates both $tRNA^{[Ser]Sec}$ isoforms in specific organs and tissues. The present study also emphasizes the more important role of housekeeping selenoproteins in the day-to-day function with regard to overall health and life span than stress-related selenoproteins.

FUNDING

This work was supported by the Intramural Research Program of the Center for Cancer Research, NCI, NIH, to D.L.H., NIH grants to V.N.G. and Deutsche Forschungsgemeinschaft grants [DFG SFB665, Scho849/1-2] to U.S.

Abbreviations used

AE, activator element
DSE, distal sequence element
GABA, γ-aminobutyric acid
GFAP, glial fibrillary acidic protein
GPx1, glutathione peroxidase 1
H/E, haematoxylin/eosin
HRP, horseradish peroxidase
mcm^{5}U, 5′-methylcarboxylmethyluracil
mcm^{5}Um, 5′-methylcarboxylmethyluridine-2′-*O*-methylribose
PSE, proximal sequence element
PV, parvalbumin
Sec, selenocysteine
Trsp, Sec tRNA gene
SelR, selenoprotein R
SelT, selenoprotein T
STAF, Sec tRNA gene transcription activating factor

REFERENCES

1. Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigo R, Gladyshev VN. Characterization of mammalian selenoproteomes. Science 2003;300:1439–1443. [PubMed: 12775843]
2. Hatfield, DL.; Choi, IS.; Ohama, T.; Jung, J-E.; Diamond, AM. Selenocysteine tRNA[Ser]Sec isoacceptors as central components in selenoprotein biosynthesis in eukaryotes.. In: Burk, RF., editor. Selenium in Biology and Human Health. Springer-Verlag; New York: 1994. p. 25-44.
3. Hatfield DL, Carlson BA, Xu XM, Mix H, Gladyshev VN. Selenocysteine incorporation machinery and the role of selenoproteins in development and health. Prog. Nucleic Acid Res. Mol. Biol 2006;81:97–142. [PubMed: 16891170]
4. Moustafa ME, Carlson BA, El-Saadani MA, Kryukov GV, Sun QA, Harney JW, Hill KE, Combs GF, Feigenbaum L, Mansur DB, et al. Selective inhibition of selenocysteine tRNA maturation and selenoprotein synthesis in transgenic mice expressing isopentenyladenosine-deficient selenocysteine tRNA. Mol. Cell. Biol 2001;21:3840–3852. [PubMed: 11340175]
5. Kumaraswamy E, Carlson BA, Morgan F, Miyoshi K, Robinson GW, Su D, Wang S, Southon E, Tessarollo L, Lee BJ, et al. Selective removal of the selenocysteine tRNA [Ser]Sec gene (Trsp) in mouse mammary epithelium. Mol. Cell. Biol 2003;23:1477–1488. [PubMed: 12588969]
6. Carlson BA, Xu XM, Gladyshev VN, Hatfield DL. Selective rescue of selenoprotein expression in mice lacking a highly specialized methyl group in selenocysteine tRNA. J. Biol. Chem 2005;280:5542–5548. [PubMed: 15611090]
7. Carlson, BA.; Xu, XM.; Gladyshev, VN.; Hatfield, DL. Fine-tuning of RNA Functions by Modification and Editing. Springer; Berlin: 2005.
8. Carlson BA, Moustafa ME, Sengupta A, Schweizer U, Shrimali R, Rao M, Zhong N, Wang S, Feigenbaum L, Lee BJ, et al. Selective restoration of the selenoprotein population in a mouse hepatocyte selenoproteinless background with different mutant selenocysteine tRNAs lacking Um34. J. Biol. Chem 2007;282:32591–32602. [PubMed: 17848557]
9. Irons R, Carlson BA, Hatfield DL, Davis CD. Both selenoproteins and low molecular weight selenocompounds reduce colon cancer risk in mice with genetically impaired selenoprotein expression. J. Nutr 2006;136:1311–1317. [PubMed: 16614422]

10. Diwadkar-Navsariwala V, Prins GS, Swanson SM, Birch LA, Ray VH, Hedayat S, Lantvit DL, Diamond AM. Selenoprotein deficiency accelerates prostate carcinogenesis in a transgenic model. Proc. Natl. Acad. Sci. U.S.A 2006;103:8179–8184. [PubMed: 16690748]

11. Carlson BA, Novoselov SV, Kumaraswamy E, Lee BJ, Anver MR, Gladyshev VN, Hatfield DL. Specific excision of the selenocysteine tRNA[Ser]Sec (Trsp) gene in mouse liver demonstrates an essential role of selenoproteins in liver function. J. Biol. Chem 2004;279:8011–8017. [PubMed: 14660662]

12. Shrimali RK, Weaver JA, Miller GF, Starost MF, Carlson BA, Novoselov SV, Kumaraswamy E, Gladyshev VN, Hatfield DL. Selenoprotein expression is essential in endothelial cell development and cardiac muscle function. Neuromuscul. Disord 2007;17:135–142. [PubMed: 17142041]

13. Shrimali RK, Irons RD, Carlson BA, Sano Y, Gladyshev VN, Park JM, Hatfield DL. Selenoproteins mediate T cell immunity through an antioxidant mechanism. J. Biol. Chem 2008;283:20181–20185. [PubMed: 18487203]

14. Lee BJ, de la Peña P, Tobian JA, Zasloff M, Hatfield DL. Unique pathway of expression of an opal suppressor phosphoserine tRNA. Proc. Natl. Acad. Sci. U.S.A 1987;84:6384–6388. [PubMed: 3114749]

15. Lee BJ, Kang SG, Hatfield DL. Transcription of Xenopus selenocysteine tRNA Ser (formerly designated opal suppressor phosphoserine tRNA) gene is directed by multiple 5′-extragenic regulatory elements. J. Biol. Chem 1989;264:9696–9702. [PubMed: 2524488]

16. Myslinski E, Krol A, Carbon P. Optimal tRNA((Ser)Sec) gene activity requires an upstream SPH motif. Nucleic Acids Res 1992;20:203–209. [PubMed: 1311068]

17. Park JM, Choi IS, Kang SG, Lee JY, Hatfield DL, Lee BJ. Upstream promoter elements are sufficient for selenocysteine tRNA[Ser] Sec gene transcription and to determine the transcription start point. Gene 1995;162:13–19. [PubMed: 7557401]

18. Park JM, Yang ES, Hatfield DL, Lee BJ. Analysis of the selenocysteine tRNA[Ser]Sec gene transcription *in vitro* using *Xenopus* oocyte extracts. Biochem. Biophys. Res. Commun 1996;226:231–236. [PubMed: 8806619]

19. Hatfield, D.; Gladyshev, V.; Park, J.; Park, S.; Chittum, H.; Huh, J.; Carlson, B.; Kim, M.; Moustafa, M.; Lee, BJ. Comprehensive Natural Products Chemistry. Vol. 4. Elsevier Science; Oxford, U.K.: 1999. Biosynthesis of selenocysteine and its incorporation into protein as the 21st amino acid.; p. 353-380.

20. Kelly VP, Suzuki T, Nakajima O, Arai T, Tamai Y, Takahashi S, Nishimura S, Yamamoto M. The distal sequence element of the selenocysteine tRNA gene is a tissue-dependent enhancer essential for mouse embryogenesis. Mol. Cell. Biol 2005;25:3658–3669. [PubMed: 15831471]

21. Schuster C, Myslinski E, Krol A, Carbon P. Staf, a novel zinc finger protein that activates the RNA polymerase III promoter of the selenocysteine tRNA gene. EMBO J 1995;14:3777–3787. [PubMed: 7641696]

22. Adachi K, Saito H, Tanaka T, Oka T. Molecular cloning and characterization of the murine Staf cDNA encoding a transcription activating factor for the selenocysteine tRNA gene in mouse mammary gland. J. Biol. Chem 1998;273:8598–8606. [PubMed: 9535833]

23. Adachi K, Tanaka T, Saito H, Oka T. Hormonal induction of mouse selenocysteine transfer ribonucleic acid (tRNA) gene transcription-activating factor and its functional importance in the selenocysteine tRNA gene transcription in mouse mammary gland. Endocrinology 1999;140:618–623. [PubMed: 9927285]

24. Adachi K, Katsuyama M, Song S, Oka T. Genomic organization, chromosomal mapping and promoter analysis of the mouse selenocysteine tRNA gene transcription-activating factor (mStaf) gene. Biochem. J 2000;346:45–51. [PubMed: 10657238]

25. Schaub M, Myslinski E, Schuster C, Krol A, Carbon P. Staf, a promiscuous activator for enhanced transcription by RNA polymerases II and III. EMBO J 1997;16:173–181. [PubMed: 9009278]

26. Schaub M, Myslinski E, Krol A, Carbon P. Maximization of selenocysteine tRNA and U6 small nuclear RNA transcriptional activation achieved by flexible utilization of a Staf zinc finger. J. Biol. Chem 1999;274:25042–25050. [PubMed: 10455183]

27. Schaub M, Krol A, Carbon P. Flexible zinc finger requirement for binding of the transcriptional activator Staf to U6 small nuclear RNA and tRNA(Sec) promoters. J. Biol. Chem 1999;274:24241–24249. [PubMed: 10446199]
28. Barski OA, Papusha VZ, Kunkel GR, Gabbay KH. Regulation of aldehyde reductase expression by STAF and CHOP. Genomics 2004;83:119–129. [PubMed: 14667815]
29. Bösl MR, Takaku K, Oshima M, Nishimura S, Taketo MM. Early embryonic lethality caused by targeted disruption of the mouse selenocysteine tRNA gene (Trsp). Proc. Natl. Acad. Sci. U.S.A 1997;94:5531–5534. [PubMed: 9159106]
30. Hill KE, Zhou J, McMahan WJ, Motley AK, Atkins JF, Gesteland RF, Burk RF. Deletion of selenoprotein P alters distribution of selenium in the mouse. J Biol. Chem 2003;278:13640–13646. [PubMed: 12574155]
31. Schomburg L, Schweizer U, Holtmann B, Flohé L, Sendtner M, Köhrle J. Gene disruption discloses role of selenoprotein P in selenium delivery to target tissues. Biochem. J 2003;370:397–402. [PubMed: 12521380]
32. Schweizer U, Bräuer AU, Köhrle J, Nitsch R, Savaskan NE. Selenium and brain function: a poorly recognized liaison. Brain Res. Rev 2004;45:164–178. [PubMed: 15210302]
33. Schweizer U, Michaelis M, Köhrle J, Schomburg L. Efficient selenium transfer from mother to offspring in selenoprotein-P-deficient mice enables dose-dependent rescue of phenotypes associated with selenium deficiency. Biochem. J 2004;378:21–26. [PubMed: 14664694]
34. Hill KE, Zhou J, McMahan WJ, Motley AK, Burk RF. Neurological dysfunction occurs in mice with targeted deletion of the selenoprotein P gene. J. Nutr 2004;134:157–161. [PubMed: 14704310]
35. Valentine WM, Abel TW, Hill KE, Austin LM, Burk RF. Neurodegeneration in mice resulting from loss of functional selenoprotein P or its receptor apolipoprotein E receptor 2. J. Neuropathol. Exp. Neurol 2008;67:68–77. [PubMed: 18172410]
36. Irons R, Carlson BA, Hatfield DL, Davis CD. Both selenoproteins and low molecular weight selenocompounds reduce colon cancer risk in mice with genetically impaired selenoprotein expression. J. Nutr 2006;136:11311–11317.
37. Moustafa ME, El-Saadani MA, Kandeel KM, Mansur DB, Lee BJ, Hatfield DL, Diamond AM. Over-production of selenocysteine tRNA$^{[Ser]Sec}$ in Chinese hamster ovary cells following transfection of the mouse tRNA$^{[Ser]Sec}$ gene. RNA 1998;4:1436–1443. [PubMed: 9814763]
38. Hatfield D, Matthews CR, Rice M. Aminoacyl-transfer RNA populations in mammalian cells chromatographic profiles and patterns of codon recognition. Biochim. Biophys. Acta 1979;564:414–423. [PubMed: 259017]
39. Kelmers AD, Heatherly DE. Columns for rapid chromatographic separation of small amounts of tracer-labeled transfer ribonucleic acids. Anal. Biochem 1971;44:486–495. [PubMed: 4943341]
40. McShane LM, Clark LC, Combs GF Jr, Turnbull BW. Reporting the accuracy of biochemical measurements for epidemiologic and nutrition studies. Am. J. Clin. Nutr 1991;53:1354–1360. [PubMed: 2035462]
41. Schomburg L, Riese C, Michaelis M, Griebert E, Klein MO, Sapin R, Schweizer U, Köhrle J. Synthesis and metabolism of thyroid hormones is preferentially maintained in selenium-deficient transgenic mice. Endocrinology 2006;147:1306–1313. [PubMed: 16322066]
42. Gladyshev VN, Stadtman TC, Hatfield DL, Jeang KT. Levels of major selenoproteins in T cells decrease during HIV infection and low molecular mass selenium compounds increase. Proc. Natl. Acad. Sci. U.S.A 1999;96:835–839. [PubMed: 9927654]
43. Schweizer U, Streckfuss F, Pelt P, Carlson BA, Hatfield DL, Köhrle J, Schomburg L. Hepatically derived selenoprotein P is a key factor for kidney but not for brain selenium supply. Biochem. J 2005;386:221–226. [PubMed: 15638810]
44. Kawaguchi Y, Kondo S. Parvalbumin, somatostatin and cholecystokinin as chemical markers for specific GABAergic interneuron types in the rat frontal cortex. J. Neurocytol 2002;31:277–287. [PubMed: 12815247]
45. Gorter JA, van Vliet EA, Aronica E, Lopes da Silva FH. Progression of spontaneous seizures after status epilepticus is associated with mossy fibre sprouting and extensive bilateral loss of hilar parvalbumin and somatostatin-immunoreactive neurons. Eur. J. Neurosci 2001;13:657–669. [PubMed: 11207801]

46. Xu X-M, Mix H, Carlson BA, Grabowski PJ, Gladyshev VN, Berry MJ, Hatfield DL. Evidence for direct roles of two additional factors, SECp43 and SLA, in the selenoprotein synthesis machinery. J. Biol. Chem 2005;280:41568–41575. [PubMed: 16230358]

47. Renko K, Werner M, Renner-Müller I, Cooper TG, Yeung CH, Hollenbach B, Scharpf M, Köhrle J, Schomburg L, Schweizer U. Hepatic selenoprotein P (SePP) expression restores selenium transport and prevents infertility and motor-incoordination in Sepp-knockout mice. Biochem. J 2008;409:741–749. [PubMed: 17961124]

48. Kim LK, Matsufuji T, Matsufuji S, Carlson BA, Kim SS, Hatfield DL, Lee BJ. Methylation of the ribosyl moiety at position 34 of selenocysteine tRNA[Ser]Sec is governed by both primary and tertiary structure. RNA 2000;6:1306–1315. [PubMed: 10999607]

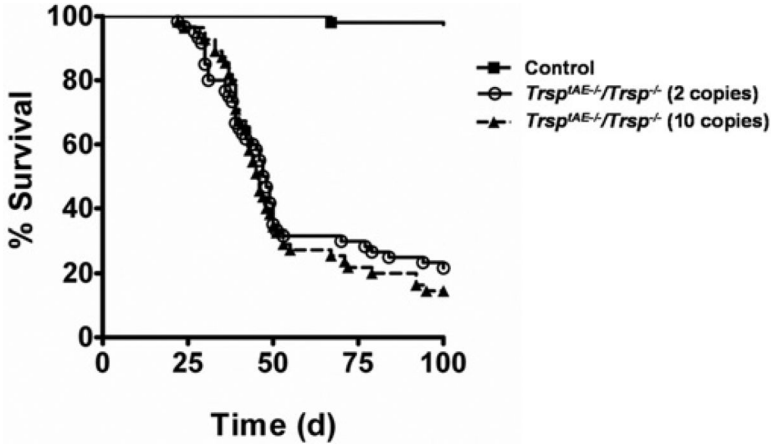

Figure 1. Survival rates of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice

Survival rates of transgenic mice carrying two (n = 60) or ten copies (n = 54) of the mutant transgene were measured and found to be significantly different from control mice ($P < 0.0001$, the log-rank test) as shown in the Figure. The median survival rate of both *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mouse lines was determined to be 48 days in mice carrying two and 46 days for mice carrying ten copies of *Trsp*$^{tAE-/-}$. Survival analysis was performed using GraphPad Prism 4.0.

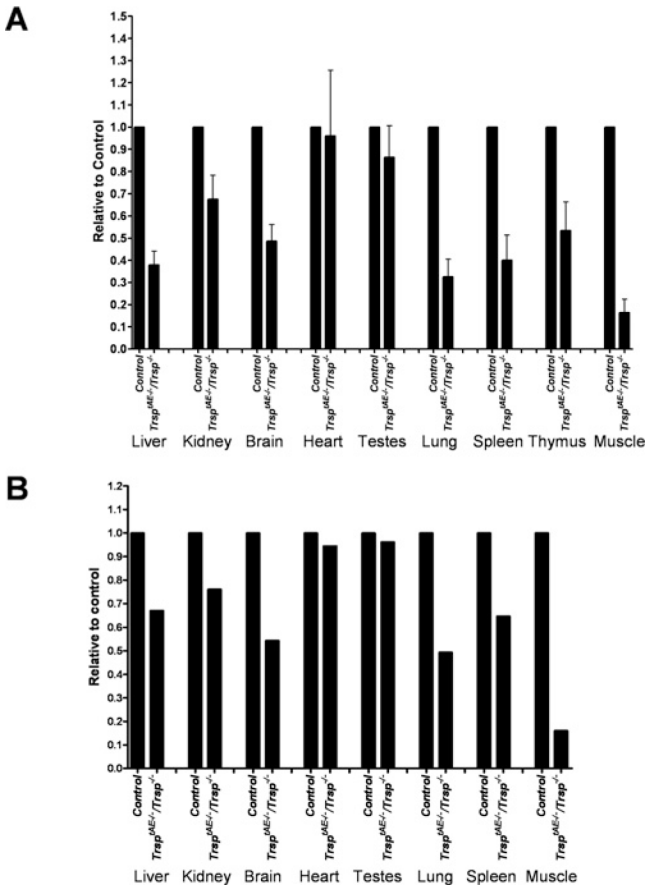

Figure 2. Northern-blot analysis of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice

Bulk tRNA was isolated from the various tissues shown in the Figure, prepared for electrophoresis and electrophoresed on gels and the levels of tRNA$^{[Ser]Sec}$ in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice carrying two (**A**) or ten copies (**B**) of mutant transgene relative to control mice were determined as described in the Experimental section. Levels of tRNA$^{[Ser]Sec}$ in tissues from mice carrying two copies of mutant transgene and the corresponding controls were carried out on three different mice from both mouse lines. The amounts of tRNA$^{[Ser]Sec}$ were averaged in control mice and assigned a value of 1.0, while the error bars from the three assays with mutant mice are shown (**A**). Levels of tRNA$^{[Ser]Sec}$ in tissues from mice carrying ten copies of mutant transgene were carried out on a single mouse and compared with the averaged levels from three control mice (**B**).

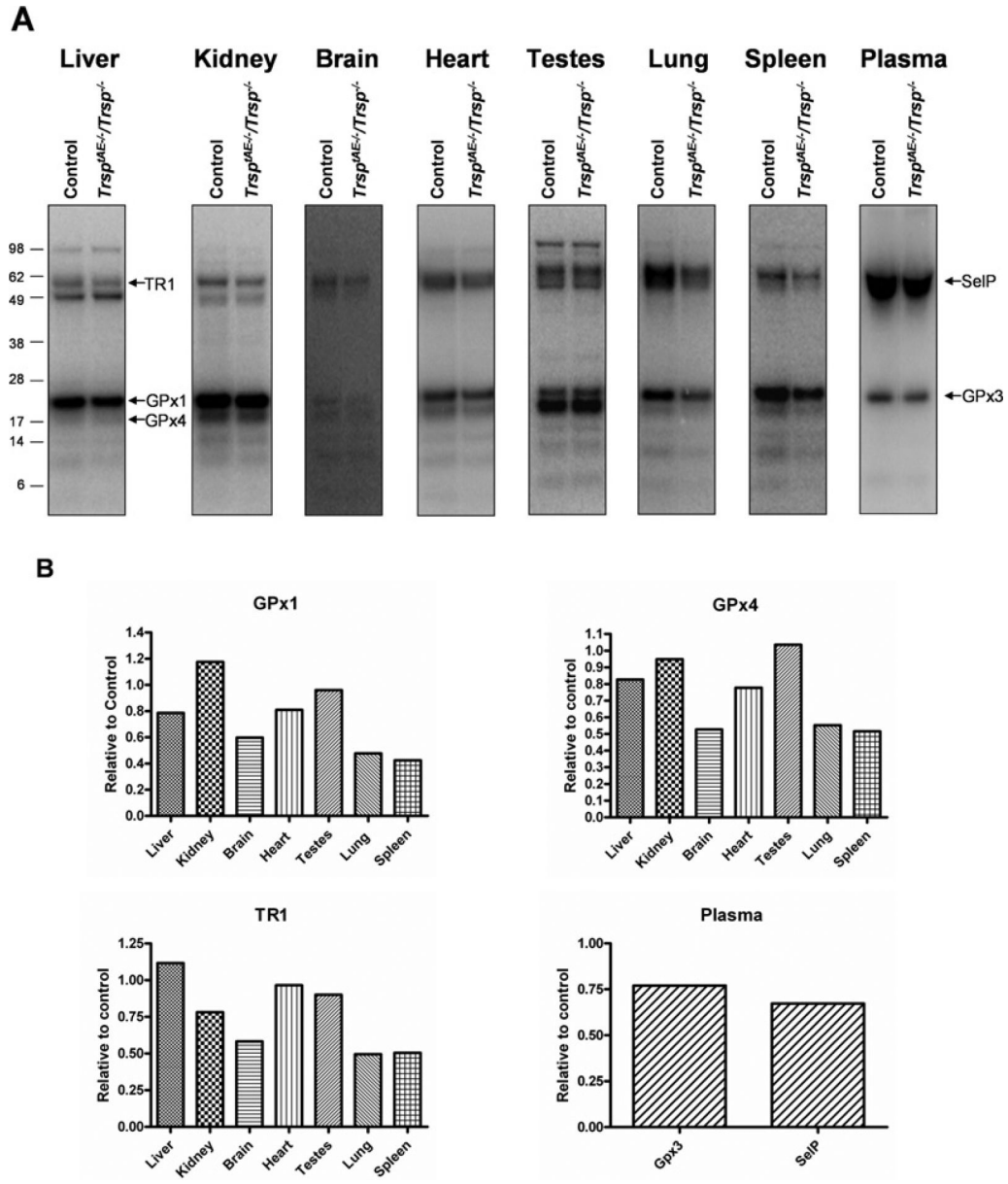

Figure 3. Metabolic ^{75}Se-labelling of selenoproteins in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice

Mice were injected with ^{75}Se; after 48 h, proteins were extracted and electrophoresed and the gels were stained with Coomassie Brilliant Blue and then exposed to a PhosphorImager to detect ^{75}Se-labelled proteins. Coomassie Brilliant Blue-stained total protein from each tissue served as a loading control wherein the amounts of proteins observed from the Coomassie Brilliant Blue-stained gels were virtually identical in the same tissue from both *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control animals (results not shown). (**A**) ^{75}Se-labelling of selenoproteins. Labelled selenoproteins observed in each tissue examined are shown along with molecular mass markers on the left side of the liver panel and selenoproteins identified in previous studies [6,42] are shown on the right sides of the liver and plasma panels. (**B**) Quantification of band intensities relative to corresponding control proteins. Band intensities of each selenoprotein evaluated in control tissues were assigned a value of 1.0 and the relative intensities of the corresponding bands in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice are shown. Band intensities were assessed by PhosphorImager analysis as given in the Experimental section.

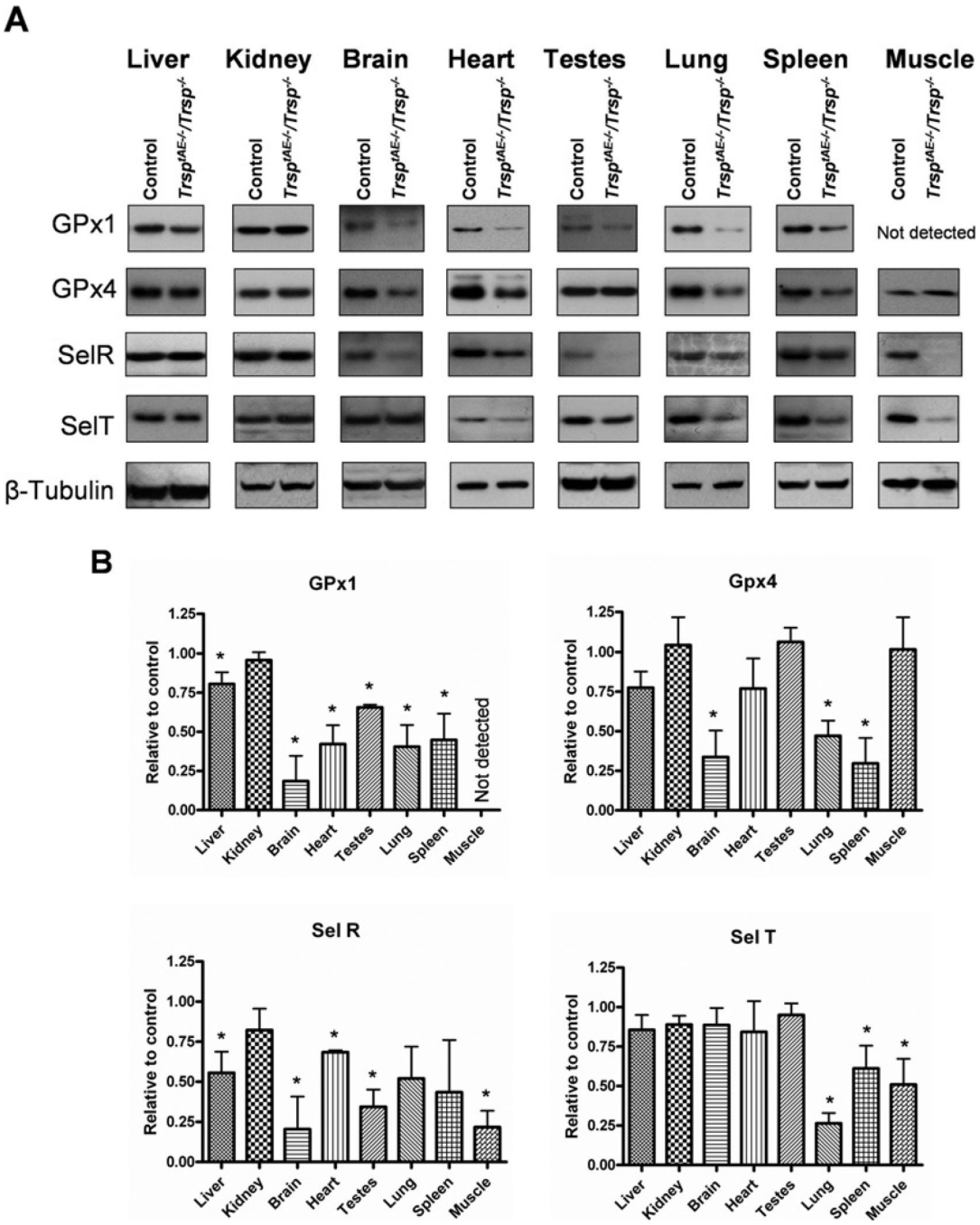

Figure 4. Western-blot analysis of selenoproteins affected by reduction of the mcm^{5}Um isoform

(**A**) Protein extracts were prepared from *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice in the tissues shown in the Figure, electrophoresed, transblotted on to membranes and treated with antibodies as detailed in the Experimental section. The blots shown were from tissues of a single mouse and are representative of the selenoproteins from the corresponding tissues of three different animals. GP×1 was not detected in muscle. Tubulin was used as a loading control. (**B**) Levels of analysed selenoproteins from the tissues of three mice relative to the corresponding selenoprotein levels from control tissues. The asterisk indicates that the differences in selenoprotein levels were significant ($P < 0.05$) between control and affected tissues as

determined by the Student's *t* test (unpaired, two-sided) using GraphPad Prism 4.0 as described in the Experimental section.

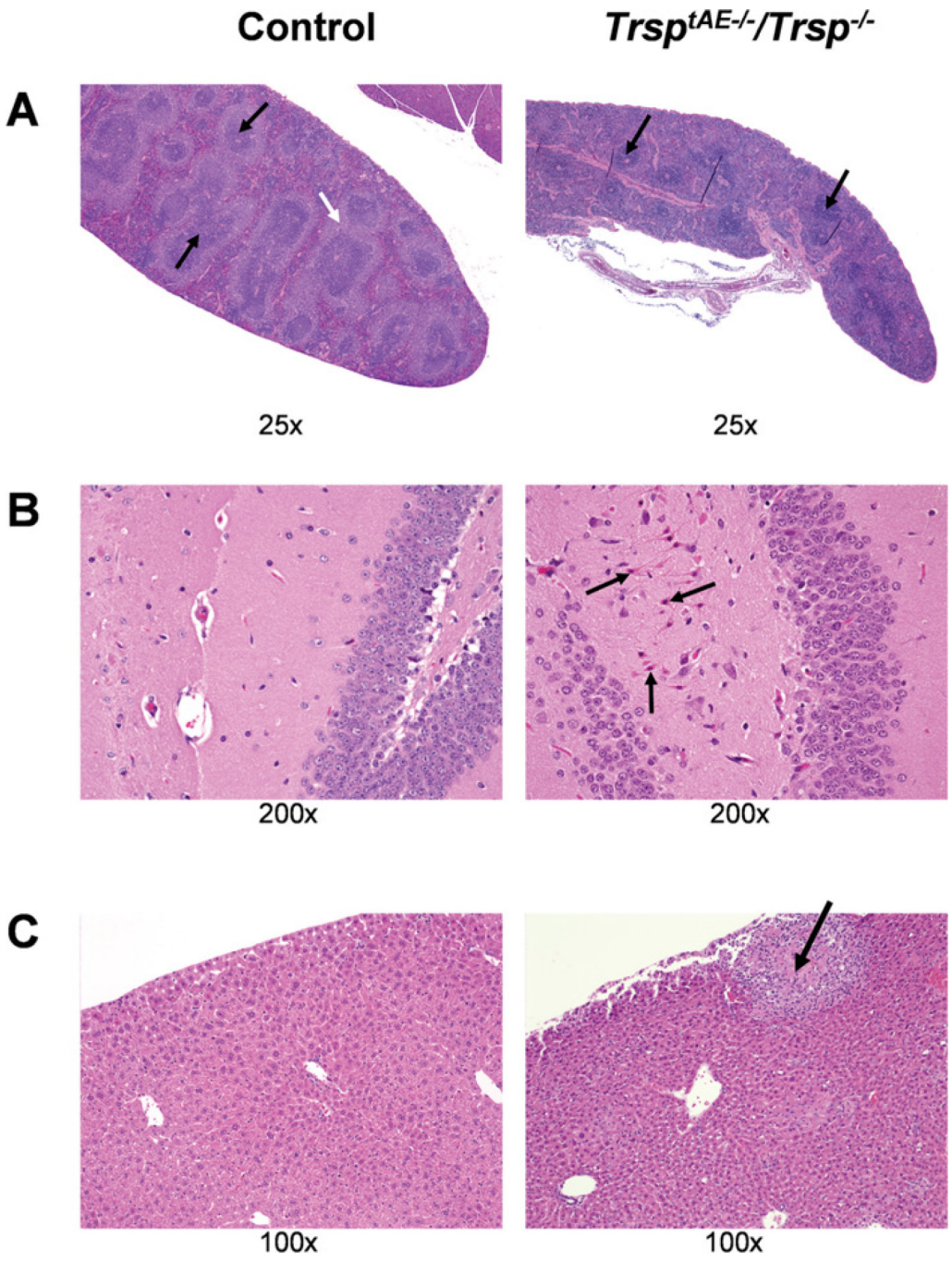

Figure 5. Pathological analysis of spleen, brain and liver in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice

Panels show H/E-stained tissues of control (left side) and *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ (right side) mice. (**A**) Spleens of six of eight *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice were smaller in size with smaller splenic follicles (black arrows show comparisons between control and mutant spleen), wherein the follicles also lacked a marginal zone (note the white arrow in the left panel showing marginal zone in control spleen), when compared with control mice. (**B**) Brains of five of eight *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice manifested acute neuronal necrosis characterized by hypereosinophilic shrunken neurons (marked by arrows) compared with control mice. (**C**) Livers of three of eight *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice had foci of necrosis (marked by arrow) and hepatocytes were smaller than in control mice.

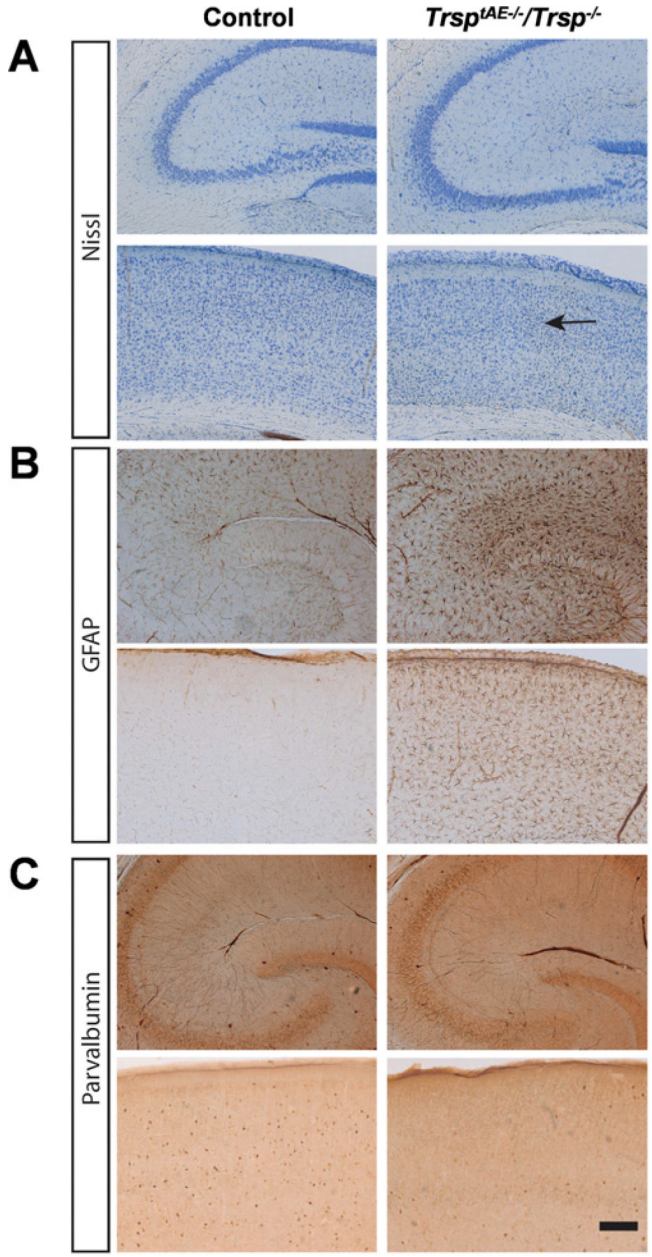

Figure 6. Histological analysis of brain tissues from *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice

Analysis of control and *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice is shown in the left and right panels respectively. (**A**) Cresyl Violet (Nissl) staining of hippocampus (upper panel) and somatosensory cortex (lower panel). The arrow points to a region of diffuse neuronal death with a high fraction of pyknotic cells. (**B**) Immunohistochemistry of GFAP. Antibodies directed against GFAP revealed massive astrogliosis in hippocampus and cerebral cortex grey matter in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ compared with control mice. (**C**) Immunohistochemistry for PV. Antibodies directed against PV demonstrated a decreased number of PV-immunopositive GABAergic interneurons in hippocampus and somatosensory cortex in *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ mice compared with control mice. $N = 5$ per genotype. Scale bar, 200 μm.

Table 1

Breeding scheme for obtaining *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice

	Matings	
1st breeding	*Trsp*$^{tAE-/-}$* × *Trsp*$^{+/-}$†	
F_1 (2nd breeding)	*Trsp*$^{tAE-}$/*Trsp*$^{+/-}$ × *Trsp*$^{tAE-}$/*Trsp*$^{+/-}$	
F_2 (3rd breeding)‡	*Trsp*$^{tAE-/-}$/*Trsp*$^{+/-}$ × *Trsp*$^{tAE-/-}$/*Trsp*$^{+/-}$	*Trsp*$^{+/+}$ × *Trsp*$^{+/+}$
F_3	*Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$§	*Trsp*$^{+/+}$//

*$Trsp^{tAE-/-}$ transgenic mice carrying either two or ten copies of the mutant transgene wherein superscript *t* denotes that *Trsp* is a transgene and superscript $tAE^{-/-}$ denotes that both transgene alleles encode deleted AE.

†$Trsp^{+/-}$ mice (strain C57BL6) were obtained as described in [5].

‡Oniy those offspring that were used for further breeding are shown.

§Only those offspring that were used experimentally are shown.

//Control mice.

Table 2

Levels and distributions of tRNA$^{[Ser]Sec}_{mcmU}$ and tRNA$^{[Ser]Sec}_{mcmUm}$ isoforms in tissues of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice*

Tissue	Genotype	% (Total)†	% (Total)‡		% of control mouse§	
			mcm^{5}U	mcm^{5}Um	Mcm^{5}U	mcm^{5}Um
Liver	Control	3.46	1.38	2.08		
	Trsp$^{tAE-/-}$/*Trsp*$^{-/-}$	1.24	0.64	0.59	46	29
Kidney	Control	3.45	1.54	1.91		
	Trsp$^{tAE-/-}$/*Trsp*$^{-/-}$	1.52	0.85	0.66	55	35
Brain	Control	2.12	0.85	1.26		
	Trsp$^{tAE-/-}$/*Trsp*$^{-/-}$	0.44	0.22	0.22	26	17
Heart	Control	3.28	1.52	1.76		
	Trsp$^{tAE-/-}$/*Trsp*$^{-/-}$	3.50	2.36	1.14	155	65
Lung	Control	3.16	1.49	1.67		
	Trsp$^{tAE-/-}$/*Trsp*$^{-/-}$	0.97	0.51	0.46	34	28
Testes	Control	5.38	3.74	1.64		
	Trsp$^{tAE-/-}$/*Trsp*$^{-/-}$	7.45	6.20	1.24	166	76
Muscle	Control	1.80	0.99	0.81		
	Trsp$^{tAE-/-}$/*Trsp*$^{-/-}$	0.31	0.18	0.13	18	16
Spleen	Control	3.02	1.58	1.44		
	Trsp$^{tAE-/-}$/*Trsp*$^{-/-}$	0.99	0.59	0.40	37	28

*Total tRNA was isolated from the tissues listed (column 1) and fractionated and then the amounts of total tRNA$^{[Ser]Sec}$ (column 3) and the percentage of tRNA$^{[Ser]Sec}_{mcmU}$ and tRNA$^{[Ser]Sec}_{mcmUm}$ isoforms (columns 4 and 5) determined as described in the Experimental section.

†Percentage of tRNA$^{[Ser]Sec}$ population within the total seryl-tRNA population.

‡Percentage of tRNA$^{[Ser]Sec}_{mcmU}$ and tRNA$^{[Ser]Sec}_{mcmUm}$ isoforms within the total seryl-tRNA population.

§Percentage of tRNA$^{[Ser]Sec}_{mcmU}$ and percentage of tRNA$^{[Ser]Sec}_{mcmUm}$ were determined by dividing the percentage of the *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ isoform by the percentage of the respective control isoform.

Table 3

Selenium levels in tissues of *Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$ and control mice

	Selenium levels (ng/g)*	
Tissue	**Control**	***Trsp*$^{tAE-/-}$/*Trsp*$^{-/-}$**
Liver	1140 ± 76	837 ± 42†
Kidney	977 ± 60	860 ± 48
Brain	185 ± 3	135 ± 14†
Heart	252 ± 5	204 ± 17
Testes	440	384
Lung	342 ± 10	224 ± 26†
Spleen	347 ± 18	298 ± 27
Muscle	167 ± 11	124 ± 3†
Plasma	297 ± 43	178 ± 72

*Selenium levels were measured as described in the Experimental section. Values represent the mean ± S.E.M. for three different mice of each genotype with the exception of testes for which the experiment was carried out in duplicate.

†This symbol indicates that the differences in selenium levels were significant ($P < 0.05$) between control and affected tissues as determined by the Student's *t* test (unpaired, two-sided) using GraphPAD Prism 4.0 as described in the Experimental section.