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Hepatically-secreted SePP restores Se transport in *Sepp*^{-/-} mice

Hepatic selenoprotein P (Sepp) expression restores selenium transport and prevents infertility and motor-incoordination in *Sepp*-knockout mice

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Hepatically-secreted SePP restores Se transport in *Sepp*^{-/-} mice**Synopsis:**

Selenoprotein P (SePP) is central for selenium (Se) transport and distribution. Targeted inactivation of the *Sepp* gene in mice leads to reduced Se content in plasma, kidney, testis, and brain. Accordingly, activities of selenoenzymes are reduced in *Sepp*^{-/-} organs. Male *Sepp*^{-/-} mice are infertile. Unlike Se deficiency, *Sepp*-deficiency leads to neurological impairment with ataxia and seizures. Hepatocyte-specific inactivation of selenoprotein biosynthesis reduces plasma and kidney Se levels similar to *Sepp*^{-/-} mice, but does not result in neurological impairment, suggesting a physiological role of locally expressed SePP in the brain. In an attempt to define the role of liver-derived circulating SePP in contrast to locally expressed SePP, we generated *Sepp*^{-/-} mice with transgenic expression of human SePP under control of a hepatocyte-specific transthyretin promoter. Secreted human SePP was immunologically detectable in serum from *SEPP1*-transgenic mice. Se content and selenoenzyme activities in serum, kidney, testis, and brain of *Sepp*^{-/-};*SEPP1* mice were increased compared with *Sepp*^{-/-} controls. When a Se adequate diet (0.16-0.2 mg/kg) was fed to the mice, liver-specific expression of *SEPP1* rescued the neurological defects of *Sepp*^{-/-} mice and rendered *Sepp*^{-/-} males fertile. When fed a low-Se diet (0.06 mg/kg), *Sepp*^{-/-};*SEPP1* mice survived 4 weeks longer than *Sepp*^{-/-} mice, but ultimately developed the neurodegenerative phenotype. These results indicate that plasma SePP derived from hepatocytes is the main transport form of Se supporting the kidney, testis, and brain. Nevertheless, local *Sepp* expression is required to maintain Se content in Se-privileged tissues such as brain and testis during dietary Se restriction.

Keywords: trace element, brain, glutathione peroxidase, thioredoxin reductase, nutrition, fertility

Abbreviations: GPx, glutathione peroxidase; Se, selenium; Sec, selenocysteine; SECIS, selenocysteine insertion sequence; SePP, selenoprotein P; *Sepp*, mouse SePP gene; *SEPP1*, human SePP gene; *Trsp*, Sec-specific tRNA^{[Ser]Sec}; Txnrd, thioredoxin reductase.

Hepatically-secreted SePP restores Se transport in *Sepp*^{-/-} mice**Introduction:**

Fifty years ago, selenium (Se) was discovered as an essential trace element [1]. Low plasma Se levels have been associated with several disorders, including cancer, autoimmune disease, and others [2]. It is widely assumed that Se exerts its biological functions in the form of selenocysteine (Sec), the 21st proteinogenic amino acid. This rare amino acid contains a selenol instead of a thiol in the side chain. Sec had initially been overlooked when the genetic code was elucidated, since it is encoded by the "STOP" codon UGA and is present in only a small number of proteins [3]. Selenoproteins are essential for mammals. Targeted inactivation of *Trsp*, the gene encoding the Sec-specific tRNA^{[Ser]Sec}, leads to early embryonic lethality [4,5]. The human and mouse genomes contain 25 and 24 genes encoding selenoproteins, respectively [6]. Gene targeting experiments in mice have shown that at least three of these, thioredoxin reductase 1 and 2 (*Txnrd1*, *Txnrd2*) and glutathione peroxidase 4 (*GPx4*), are essential [7,8,9].

Selenoprotein P (SePP) is a plasma selenoprotein containing the unusual number of 8-17 Sec residues, depending on species [10]. The majority of plasma Se is bound to SePP [11] and it was suggested very early that it could represent a Se transport protein [12]. Targeted inactivation of the *Sepp* gene in the mouse has demonstrated that SePP is central to Se transport and storage [13,14]. Expression of selenoenzymes such as glutathione peroxidase 1 (GPx1) or *Txnrd1* is accordingly reduced in *Sepp*-deficient tissues. Male *Sepp*^{-/-} mice are infertile [15] and, depending on dietary Se supply, *Sepp*^{-/-} mice suffer from premature death before weaning, seizures, and ataxia [16,17]. Recently, the lipoprotein receptor ApoER2 has been characterized as a SePP-receptor in testis [18]. However, *Sepp* mRNA is expressed in most tissues [19] including brain, where SePP immunoreactivity has been found on neurons and oligodendroglial cells [20]. Thus, the relative roles of locally expressed SePP versus circulating SePP remained unclear. When we made use of a recently developed mouse model [5,21] in which biosynthesis of all hepatocytic selenoproteins was specifically abrogated by targeted deletion of the gene encoding tRNA^{[Ser]Sec}, we realized that Se levels in serum and kidneys were reduced to the same levels as those in *Sepp*^{-/-} mice. Nevertheless, neurological dysfunction was not observed [22].

In a further attempt to define the role of liver-derived plasma SePP, in contrast to locally expressed SePP, we have now generated *Sepp*^{-/-} mice with liver-specific expression of human SePP (*SEPP1*). Here we show that transgenic expression of *SEPP1* cDNA under control of a mouse transthyretin promoter construct resulted in *SEPP1* expression in the liver only. Although serum Se levels increased only slightly in *SEPP1*-transgenic *Sepp*^{-/-} mice (*Sepp*^{-/-}; *SEPP1*), Se levels and selenoenzyme activities in the kidney, testis and brain were normalized, indicating improved Se traffic from liver to target tissues via hepatocyte-derived human SePP in plasma. In addition, *Sepp*^{-/-}; *SEPP1* mice did not suffer from neurological defects and the males were fertile and survived on Se-sufficient diet. These results suggest that plasma SePP derived

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from liver is the main transport form for Se supporting vitally important functions of Se in kidney, testis, and brain.

Materials and methods:

Animals: Mice were maintained according to local regulations as described for the *Sepp*-deficient mice generated in our laboratory [14]. All animal experiments were approved by the local authorities in Berlin and Munich. The *SEPP1* minigene was cloned as an XhoI fragment into exon 2 of TTR1 exV3, an expression vector containing a 4 kb fragment of the mouse transthyretin gene including the promoter region, exon 1, intron 1, and exon 2. A cryptic ATG was mutated in exon 1 and a StuI site in exon 2 was used for cDNA insertion [23,24]. The minigene was liberated from the vector by HindIII-digestion, gel-purified, and microinjected into the male pronucleus of C57Bl/6 zygotes. Two oligonucleotides spanning the first SECIS element of human *SEPP1*, e5-3fwd (TGAATTGGCTTCCAGATTT) and e5-3rev (AGAAGAGTATGATACAACATTAG) produced a 491 bp PCR fragment specific for *SEPP1* that was used for PCR-genotyping and as a human-specific hybridization probe. Of the 15 potential founder mice obtained, 4 expressed significant human SePP in serum biopsies (#5, #8, #9 and #12). Founder #8 died before reproduction and offspring from founder #12 did not express human SePP in serum, although the strain remained PCR-positive for *SEPP1*. Thus, two *SEPP1*-transgenic lines were established from founder mice #5 and #9, and were designated Tg(SEPP1)1 and Tg(SEPP1)3. All experiments reported here were performed with animals from Tg(SEPP1)1, unless otherwise indicated. Animals received a torula yeast-based diet with 0.16-0.2 mg Se/kg in the form of sodium selenite (Sniff, Hannover, Germany).

RNA extraction: Tissue was snap-frozen in liquid nitrogen and stored at -80°C until use. For all subsequent sample preparations the tissue was powdered and homogenized under liquid nitrogen.

Total RNA extraction was performed using peqGOLD TriFast (PEQLAB, Erlangen, Germany) according to the instructions of the manufacturer. Isolated total RNA integrity was electrophoretically verified by ethidium bromide staining as described [14].

Semiquantitative RT-PCR: 1 µg of total RNA was reverse transcribed (iScript first strand cDNA synthesis kit, BioRad) and the resulting cDNA was diluted 1:10. 5µl of cDNA-dilution were taken for every single RT-PCR reaction. Absolute QPCR SYBR Green Fluoresceine Mix (Abgene, Epsom, UK) and the iCycler-System (BioRad) were used for transcript quantification.

All reactions were performed with an annealing temperature of 58°C and under standard conditions. Specificity of used primers was verified by melting curve analysis. Each sample was measured in duplicates. Quantitative data were calculated using the Relative Expression Software Tool-Multiple Condition Solver (REST-MCS, version 2, as previously described [25]). Transgene-specific, intron-spanning primers used were TTR-fwd1 (AGATCCACAAGCTCCTGACAGGATC) and SEPP1-rev2

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(GGCTGGGGGTTGCTTACATAAG). 18S-rRNA primers from RTPrimerDB (<http://medgen.ugent.be/rtprimerdb/>) were used for normalization: Fw-m18S-TTGACGGAAGGGCACCACCAG and Rev-m18S-GCACCACCACCCAC GGAATCG.

Northern Blot: cDNA fragments encompassing the complete reading frame of murine *SePP*, and a fragment of the human *SEPP1* 3'UTR were amplified by PCR, subcloned into pGEM-T vectors (Promega, Mannheim, Germany) and verified by DNA-sequencing. The insert was prepared by restriction digest and preparative gel electrophoresis, radioactively labeled with ³²P-dCTP (Hartmann Analytic, Braunschweig, Germany) using a random priming kit (High Prime, Roche Diagnostics GmbH, Mannheim, Germany). Hybridizations were conducted essentially as described [14] and specific signals were quantified employing a phosphoimager (Cyclone, Perkin Elmer, Köln, Germany).

Enzyme assays: GPx activity was determined in tissue homogenates as described [14]. In brief, NADPH consumption by glutathione reductase was monitored at 340 nm as a measure of the GSSG formation catalyzed by GPx. Enzymatic activity was recorded at 37°C in a buffer containing 0.02 M potassium phosphate, pH 7.0, 0.6 mM EDTA, 0.15 mM NADPH, 2 mM GSH and 4 units glutathione reductase (Calbiochem, Darmstadt, Germany). The reaction was started by addition of t-butylhydroperoxide (0.1 mM) as substrate. Background NADPH consumption was determined in the presence of 100 mM mercaptosuccinate and subtracted. Protein concentration was determined by the method of Bradford using IgG as a standard.

Selenium determination: Selenium measurements were performed by a fluorimetric assay as described [16]. A commercial Se standard (Aldrich #247928, Sigma, Munich, Germany) was used to construct a calibration curve and an independent seronorm standard (Sero AS, Billingstad, Norway) served as reference sample.

Rotarod: Rotarod analysis was done as described [16]. Animals were placed on the rotating drum at 4 rounds per min (rpm). After two min of accommodation, the rotation was linearly accelerated during 3 min to 40 rpm. The retention time spent on top of the rotating drum was recorded.

Western Blot: For GPx1 and GPx4 detection in brain, 100µg protein from cytosolic or membrane fraction, respectively, were separated in 12 % SDS-polyacrylamide gels. For GPx4 from testis, 50µg of membrane fractions were used. After electrotransfer, PVDF membranes were stained with Ponceau Red, photographed, and blocked with 5% BSA for 1 h at room temperature. Polyclonal antibodies against GPx1 and GPx4 (both from Abcam) were used at 1:1000 and 1:2000 dilution, respectively. Rabbit polyclonal anti-β-actin antiserum from Sigma was used at 1:1000 dilution. Antibodies against mouse SePP have been described [22]. Antibodies against human SePP were the same as used in the ELISA assay and described before [26].

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SePP ELISA: A human-specific SePP ELISA based on polyclonal sheep antisera was established and is described in detail elsewhere (B. Hollenbach, N.G. Morgenthaler, J. Struck, C. Alonso, A. Bergmann, J. Köhrle, and L. Schomburg, unpublished work).

Sperm motility and morphology: Spermatozoa from the cauda epididymis were released into medium mimicking the osmolality of uterine fluid (330 mmol/kg) containing 12 mg/ml bovine serum albumin. After dispersion, motility was examined at 37°C in 40 µm deep siliconised slide chambers and video-recorded for analysis of kinematics using a computer system (IVOS version 10.8, Hamilton Thorne Research, Beverly, MA, USA). Motile spermatozoa were tracked for 1 s at 25 Hz and swimming velocities and forward progression efficiencies were measured [27]. Aliquots of this suspension were photographed in a Zeiss BX-40 microscope with phase contrast optics and assessed for the percentage of angulated flagella.

Statistics: For all computations, Graph Pad Prism software was used with the tests indicated in the figure legends. Error bars represent the standard error of the mean, SEM.

Results

Generation of mice expressing human SePP specifically in liver

In order to complement *Sepp*^{-/-} mice genetically, we wanted to express human SePP specifically in hepatocytes. The mouse transthyretin promoter has been successfully used to mediate liver-specific expression of cDNAs [24]. Thus, a human *SEPP1* cDNA including the complete open reading frame and the two SECIS elements in the 3'-UTR were cloned into exon 2 of the vector TTR exV3 and the resulting minigene (Fig. 1A) was microinjected into mouse zygotes. Potential founder mice were genotyped via PCR for the presence of the human *SEPP1* 3'-UTR (Fig. 1B). In the next step, founders were screened for human SePP in blood samples using a recently developed human-specific SePP ELISA. Since our antisera are species-specific, we were able to test for human SePP in mice in the presence of the wild-type murine orthologue (Fig. 1C). Four founder mice (#5, #8, #9 and #12) turned out positive for human SePP in the murine serum samples. Next, we asked whether *SEPP1* expression was confined to liver only. Therefore, we tested the species-specificity of our hybridization probes and verified transgene cDNA in offspring from founder mouse #5 (from now on designated Tg(*SEPP1*)1 and mouse #9 (now designated Tg(*SEPP1*)3 (Fig. 1D). Next, we used the human-specific probe to test *SEPP1* expression in several tissues of the transgenic mice. We tested 16 tissues by Northern blotting in both transgenic lines (Fig. 1E). In Tg(*SEPP1*)1, the liver was the only tissue expressing *SEPP1*. In Tg(*SEPP1*)3, low mRNA expression of *SEPP1* was also observed in skeletal muscle. The transgenic *SEPP1* mRNA from mouse liver migrated slightly slower than native human *SEPP1* mRNA from the hepatoma cell line HepG2 providing an additional level of verification and specificity.

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Selenium transport is restored by transgenic *SEPP1* expression

Transgenic founders were crossed with *Sepp*^{+/-} mice yielding *Sepp*^{+/-}; *SEPP1* offspring. These were crossed with *Sepp*^{+/-} mice generating *SEPP1*-transgenic *Sepp*^{-/-} mice (*Sepp*^{-/-}; *SEPP1*). Serum samples from these mice were tested for transgene expression by Western blot. Using our human-specific antibodies, we detected full-length expression of human SePP in serum of transgenic mice, albeit at lower levels compared with human control serum (Fig. 2A). Although human SePP expression appeared lower in Tg(*SEPP1*)1 mice, this strain was selected for further experiments, since transgene expression was strictly limited to the liver (see Fig. 1E). In order to exclude gene-dose effects, we made sure that in subsequent matings only one parent was *SEPP1*-transgenic. In the next step we wanted to know whether expression of the *SEPP1* transgene increased serum Se content in *Sepp*^{-/-} mice. Therefore, we determined serum Se levels in all six genotypes of our mice. Interestingly, *SEPP1* transgene expression doubled serum Se concentrations in *Sepp*^{-/-} mice, but did not significantly affect Se levels in wild-type or heterozygous mice (Fig. 2B).

We have shown previously that renal Se content and selenoenzyme activities depend on SePP secreted from the liver [22]. We now wanted to know whether transgenic expression of human SePP exclusively in hepatocytes was able to increase Se content and selenoenzyme activities in SePP target tissues from *Sepp*^{-/-} mice. As shown in figure 3, kidney Se content was significantly increased in *SEPP1*-transgenic compared with non-transgenic *Sepp*^{-/-} mice (Fig. 3A). Similarly, renal GPx activity, a sensitive marker of tissue Se status, paralleled Se content and demonstrated partial restoration of selenoenzyme activity in *Sepp*^{-/-}; *SEPP1* mice (Fig. 3B). Moreover, cytosolic Txnrd activity was normalized in *Sepp*^{-/-} kidneys by *SEPP1* expression (not shown). Interestingly, activity of plasma GPx, an enzyme produced in the kidney and secreted into the blood was not restored in *Sepp*^{-/-}; *SEPP1* mice (Fig. 3C). This is in line with the well-established hierarchy of expression among selenoproteins under conditions of limited Se availability.

Restoration of male fertility in *SEPP1* transgenic *Sepp* knockout mice

The testis is another SePP target tissue. Its Se content is reduced in *Sepp*^{-/-} mice causing infertility [13,14]. Recently it has been shown that the lipoprotein receptor ApoER2 functions as a SePP receptor in testis [18]. However, since *Sepp* mRNA is also expressed in the testis [28], it is unclear whether ApoER2 can bind SePP from the circulation only or exclusively from within the testis.

Male infertility is associated with reduced testicular GPx4 expression during dietary Se-deficiency [29,30], *Sepp*-deficiency [15], or *Apoer2*-deficiency [31,18]. We have therefore determined GPx4 protein abundance in testis homogenates from our mice. As expected, GPx4 protein was lost from *Sepp*^{-/-} testis and the *SEPP1* transgene partially restored GPx4 expression (Fig. 4A). We determined GPx activity as a reliable surrogate marker of tissue Se content in the testis. The results were in line with our earlier findings that indicated reduced GPx activity in *Sepp*^{-/-} testis [14]. Hepatic expression of *SEPP1* fully restored

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testicular GPx activity (Fig. 4B). Next, we performed qRT-PCR analysis with transgene-specific primers as indicated in figure 1A. No difference was observed in threshold cycle times whether the animals were *SEPP1*-transgenic or non-transgenic, ruling out ectopic expression of the *SEPP1* transgene in the testis (Fig. 4C). In another attempt to test for ectopic testicular *SEPP1*-transgene expression, we stained tissue sections with an antibody specific for human SePP. Using this method, we were able to demonstrate human SePP in Leydig cells of normal human specimens, but not in *SEPP1*-transgenic murine testes (not shown).

Spermatozoa from the transgenic mice were almost as motile as those from the wild-type mice, swimming with the same vigour and attaining 90% the effectiveness of forward progression of the wild-type cells. The *Sepp*^{-/-} mice had much fewer motile sperm that showed markedly reduced swimming velocities and forward progression (Fig. 5A). Sperm motility was also increased by the *SEPP1* transgene to levels indistinguishable from wild-type sperm. Morphological assessment of spermatozoa from the cauda epididymis showed that *SEPP1* transgene expression prevented the formation of “hairpin-like” turns of the sperm tails (Fig. 5B). *Sepp*^{-/-} mice maintained on a high Se diet (0.6 ppm) served as controls, since *Sepp*^{-/-} mice maintained on Se adequate diet did not survive to reproductive age. For quantitative analysis, spermatozoa were fixed and processed with the Papanicolaou method (4 slides per animal, 200 spermatozoa were assessed). The fraction of spermatozoa with angulated flagella was 52.6±1.4% for *Sepp*^{-/-}, 17.1±2.0% for *Sepp*^{+/+}, and 13.5±1.9% for *Sepp*^{-/-}; *SEPP1* (*Sepp*^{-/-} was different from both other groups as assessed by ANOVA). Consistent with the biochemical, kinematic, and morphological rescue of their sperm phenotype, *Sepp*^{-/-}; *SEPP1* male mice were fertile as demonstrated by normal litter size when mated to *Sepp*^{+/+} dams.

Rescue of neurological dysfunction in *SEPP1*-transgenic *Sepp* knockout mice

Gene targeting of *Sepp* using homologous recombination for the first time clearly revealed a role for Se in brain function [32]. Lack of *Sepp* leads to a reduction of brain cytosolic Gpx and TxnrD activities [13,14,]. Upon transgenic expression of *SEPP1* in the liver, a significant increase in brain GPx activity in *Sepp*^{-/-} mice is observed (Fig. 6A). In parallel, the increase of serum Se concentration in response to expression of *SEPP1* is rather low (see Fig. 2B). These data support the idea that plasma SePP represents the preferred carrier of Se into the brain. Activity of TxnrD, an enzyme that occupies a high rank in the hierarchy among selenoproteins, was fully rescued in *SEPP1* transgenic *Sepp*^{-/-} brains (Fig. 6B). Similarly, expression of the strongly reduced GPx protein levels in *Sepp*^{-/-} mice were partly (in the case of GPx1) or largely (GPx4) restored by hepatic expression of *SEPP1* (Fig. 6C). This differential rescue of GPx isoform expression is again well in agreement with the well-established hierarchical expression of different selenoproteins.

We have reported earlier that *Sepp*^{-/-} mice suffer from movement incoordination that can be quantitatively assessed using the Rotarod apparatus [16]. When *Sepp*^{-/-} mice were tested now along with *SEPP1*-

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expressing *Sepp*^{-/-} littermates, *Sepp*^{-/-;SEPP1} mice behaved indistinguishable from wild-type mice (Fig. 7). When *Sepp*^{-/-} mice are challenged after weaning with a low Se diet, they rapidly deteriorated and died within 7-10 days on low Se diet [17]. Therefore, we challenged a small number of *SEPP1*-transgenic *Sepp* knockouts along with *Sepp*^{-/-} littermates with a low Se diet (0.06 mg Se/kg). In contrast to their non-transgenic littermates, *Sepp*^{-/-;SEPP1} mice survived 4 weeks longer, but ultimately developed the usual *Sepp*^{-/-} phenotype, including neurological dysfunction. We thus selected a more physiological setting and provided Se sufficient diet (0.16-0.2 mg Se/kg) after weaning. Again, we followed the development of the *Sepp*^{-/-} phenotype. Under these conditions, *Sepp*^{-/-} mice developed the neurodegenerative phenotype and had to be euthanized 2 weeks after diet change, while none of the *Sepp*^{-/-;SEPP1} mice showed any signs of neurological damage during the full observation period of 12 weeks after diet change (Fig. 8).

Discussion

Soon after the discovery of SePP, it was postulated that SePP might represent a Se transport protein in plasma [12]. SePP is the only mammalian selenoprotein known to date that contains multiple Sec residues in the polypeptide chain [33]. It is highly expressed in liver, and injection of radioactively labelled SePP into Se-deficient animals leads to accumulation of radioactivity in particular in the brain [11]. However, expression of *Sepp* mRNA is widespread among tissues and sometimes specific for only one cell type within the organ, e.g. for Leydig cells in the testis [28]. Thus, the question remained, why so many tissues should express a plasma Se transport protein, if this was the only function of SePP. It has been proposed that SePP detoxifies heavy metals [34] and protects proteins from oxidation [35], but both hypotheses remain to be supported in vivo. Gene targeting of *Sepp* in mice has revealed that SePP is required for Se distribution within the body [13,14]. In addition, an essential role for SePP in privileged Se supply to the brain was established. Apparently, the top positions of brain and testes in the hierarchy of Se supply to organs [36] were lost in *Sepp*^{-/-} mice. Gene targeting of *Sepp* also demonstrated for the first time that Se was important for normal brain function [16,17]. Interestingly, reducing SePP expression in the liver through targeted deletion of tRNA^{[Ser]Sec}, revealed that the brain is able to retain its Se provided local SePP expression is maintained [22]. Likewise, in dietary Se deficiency, liver SePP secretion is lost, while the brain maintains its Se content [36]. Interestingly, SePP has been purified from serum as a neurotrophic factor for cortical neurons in culture [37,38]. Therefore, it is possible that SePP expression not only provides a Se transport protein, but also generates a local (neuro)trophic molecule. We set out to test our hypothesis of a dual function of SePP [39] by expressing SePP exclusively in liver of *Sepp*^{-/-} mice. Here, we report that the selective transgenic expression of human SePP in hepatocytes only can already restore selenoprotein expression in known SePP target tissues and rescues male fertility, motor coordination, and survival of *Sepp*^{-/-} mice. These findings lend further support to the hypothesis that SePP is an essential Se transport protein from liver to target tissues. However, upon severe dietary Se restriction, the neurological

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phenotype of *Sepp*^{-/-} mice develops even in *SEPP1*-transgenic mice, supporting in turn the postulated second role of SePP as a local Se storage protein in brain [39,40].

If SePP represented a transport protein, a receptor for SePP likely exists. This receptor should be preferentially expressed in SePP target tissues. In fact, ApoER2 has recently been biochemically purified as a SePP receptor from testis [18]. In support of this, *Apoer2*-deficient mice are infertile [31,15] and their sperm phenotype resembles *Sepp*^{-/-} spermatozoa with angulated flagella [31]. Very recently, the group of Burk extended their findings and nicely demonstrated that *Apoer2*^{-/-} mice have reduced testis and brain Se content and display neurological defects similar to those in *Sepp*^{-/-} mice [41]. Thus, biochemical and genetic evidence strongly suggest that ApoER2 is the postulated SePP receptor in the brain and testis. Interestingly, GPx4 expression is more strongly reduced in testicular homogenates than GPx1 activity. It is known that GPx4 resides higher in the hierarchy among selenoproteins than GPx1. We thus speculate that the two proteins do not reside within the same cellular compartment with respect to Se supply, such that GPx4 expression is strictly *Sepp/Apoer2*-dependent, while GPx1 is expressed both in *Apoer2*-dependent and -independent cells in testis. Detailed histological analyses of selenoprotein expression in testicular cell types may thus be required to explain this finding fully. Nevertheless, the restoration of sperm motility and normal sperm morphology in the transgenic mice along with rescued fertility argues for rescued GPx4 expression in spermatozoa, since spermatozoa with angulated flagella do not cross the uterotubal junction in mice [42].

Expression of ApoER2 is not reported in the kidney, a clear SePP target tissue, and kidney Se is not reduced in *Apoer2*^{-/-} mice [41]. Thus, preferential Se supply to the testis and brain is reflected by ApoER2 expression in these organs, but this receptor may not represent the only SePP receptor.

In addition, we have shown here that liver-specific *SEPP1* expression results in increased Se content in the brain and rescues the neurological deficits of *Sepp*^{-/-} mice. Although at first glance this finding supports the transport hypothesis for SePP, new questions have been raised: how does Se contained in SePP cross the blood-brain-barrier (BBB)? Both brain endothelium and neurons are known to express ApoER2, but it is not clear whether SePP taken up at the BBB is transcytosed or whether Se derived from plasma SePP after intracellular degradation is used for de novo synthesis of SePP or another specific Se carrier protein behind the BBB. Since SePP-resynthesis at the BBB is unlikely in *Sepp*^{-/-};*SEPP1* mice, our results favour transcytosis or synthesis of a second Se carrier as the transport mechanism of Se into the brain. Available data from *Apoer2*^{-/-} mice are compatible with both lack of SePP uptake at the BBB and lack of SePP uptake by neurons. It is now clear that dietary (inorganic) Se supplementation restores motor coordination in *Sepp*^{-/-} mice [16], but not testicular Se content and normal sperm morphology. Thus, unlike the testis, the brain can make use of inorganic dietary Se sources to fulfill its essential functions. This notion is also substantiated by the finding that *Apoer2*^{-/-} mice do not show the neurological deficits of *Sepp*^{-/-} mice, as

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long as they are not challenged by low Se diet [41,31]. It remains to be shown which chemical species and which mode of transport establishes this alternative route of Se entry into the brain.

The phenotypic similarities of *Apoer2*^{-/-} and *Sepp*^{-/-} mice suggest that the main function of SePP in vivo is Se transport and storage – otherwise, phenotypic differences between mice lacking ligand and mice lacking receptor should be apparent. Since the phenotype of mice expressing a C-terminally truncated SePP fragment instead of full-length SePP is very similar to *Sepp*^{-/-} mice [43], the current in vivo data do not favour a third prominent function of SePP.

What is the role of Se in the brain? It has been suggested that intractable seizures in children with low Se status depend on Se [44,45]. Considering these data, it may be possible that children expressing low levels of *SEPP1* or *APOER2* in the brain may be particularly sensitive to low dietary Se intake. In fact, polymorphisms in the *SEPP1* gene have recently been reported to impact on SePP biosynthesis and metabolism in humans [46]. Thus it appears as if a peculiar system composed of SePP and ApoER2 is designed to channel Se selectively into brain and testis and thus promote survival of the individual and the species during times of Se shortage.

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Hepatically-secreted SePP restores Se transport in *Sepp*^{-/-} mice**Figure legends:****Figure 1:** Generation of *SEPP1* transgenic mice

(A) Schematic representation of the transgene construct. A human *SEPP1* cDNA including the 3'-UTR with both SECIS elements was inserted as an XhoI fragment into exon 2 of pTTR^{exV3} vector. The human-specific hybridization or PCR probe (491 bp) spanning the first SECIS element is indicated. The location of primers used for qRT-PCR is indicated as arrows. (B) Transgenic founder mice were screened by genomic PCR with primers spanning the first human SECIS element. (C) Expression of transgenic SePP in serum of founder animals was assessed by human-specific ELISA assay. Only 4 out of 15 mice expressed detectable amounts of SePP in serum. RLU, relative light units. (D) Specificity of hybridization probes. Northern hybridization of total liver RNA with probes directed against mouse *Sepp* (*Sepp*), human *Sepp* (*SEPP1*), and *18S* rRNA. (E) Multiple tissue Northern blot analysis. 10-15 µg of total RNA from 16 tissues were prepared from F2 animals from lines Tg(*SEPP1*)1 and Tg(*SEPP1*)3. Transgenic mRNA was visualized with the *SEPP1*-specific probe. *18S* rRNA served as loading control. Controls: human hepatoma HepG2 cells; Ht heart; Li liver; Ki kidney; Lu lung; Mu skeletal muscle; Ctx cerebral cortex; Crb cerebellum; Sp spleen; Th thymus; Co colon; Du duodenum; Cae caecum; St stomach; Ut uterus; Sa salivary gland; Pa pancreas.

Figure 2: Human SePP is secreted into mouse serum.

(A) Western blot on serum samples using antibodies specific for mouse and human SePP, respectively. Human serum (hum.) and serum from a non-transgenic *Sepp*^{+/+} mouse (+/+) served as controls. Human SePP from transgenic mouse serum co-migrates with SePP from regular human serum. Genotypes are indicated at the bottom. Tg1, Tg3, transgenic lines Tg(*SEPP1*)1 and Tg(*SEPP1*)3, respectively. The sizes of marker proteins are indicated at the right. One representative of three individual experiments is shown. (B) Serum Se content in transgenic mice. Expression of the *SEPP1* transgene leads to significantly increased Se levels in *Sepp*^{-/-} mice (n=5-17 for each genotype). Statistical significance versus wild-type was calculated with ANOVA followed by Bonferroni's post-hoc test. The horizontal bar indicates the comparison between *Sepp*^{-/-} and *Sepp*^{-/-;SEPP1}. **p<0.01;***p<0.001.

Figure 3: Se transport to kidney is partially restored in *Sepp*^{-/-} by liver-specific expression of *SEPP1*.

(A) Kidney Se content. Se content is significantly increased in *Sepp*^{-/-;SEPP1} kidneys as compared to *Sepp*^{-/-} kidneys, n=5-16. (B) Cytosolic GPx activity. GPx activity, a sensitive marker of tissue Se status, is increased in *Sepp*^{-/-} kidney by transgenic expression of *SEPP1* in liver, n=10-17. (C) Plasma GPx activity. GPx activity in serum is not increased in *Sepp*^{-/-;SEPP1} mice as compared to *Sepp*^{-/-}, n=7-15. Error bars indicate SEM. Statistical significance of differences between *Sepp*^{-/-} and *Sepp*^{-/-;SEPP1} was calculated with ANOVA followed by Dunnett's post-hoc test as indicated by the horizontal bars. The difference between

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wild-type and *Sepp*^{-/-} mice has been described in our earlier work [14, 16]. **p*<0.05; ***p*<0.01; n.s. non significant.

Figure 4: Hepatic *SEPP1* expression restores fertility in *Sepp*^{-/-;SEPP1} mice.

(A) GPx4 protein in testis. GPx4 expression in testis correlates with fertility of male mice. Western Blot demonstrates partial restoration of GPx4 expression in *Sepp*^{-/-;SEPP1} testis. β -actin (β -Act.) served as loading control. Samples from three different animals were loaded per genotype and one representative of two experiments is shown. (B) Cytosolic GPx activity in testis homogenates. GPx activity, a sensitive probe of Se content, is restored in *Sepp*^{-/-;SEPP1} mice, *n*=3 per genotype. Statistical significance was calculated with ANOVA followed by Dunnett's post-hoc test as indicated by the horizontal bars. **p*<0.05. (C) qRT-PCR with testis homogenates. The *SEPP1* transgene is not expressed in testis from transgenic mice. Threshold cycle numbers (*C_T*) using testicular cDNAs are not different between *SEPP1*-transgenic and non-transgenic mice. cDNA from *SEPP1*-transgenic liver served as a standard in serial dilutions as indicated. 18S-rRNA served as control.

Figure 5: Analysis of sperm phenotype.

(A) Kinematic data of motile spermatozoa isolated from the cauda epididymis. Liver-specific *SEPP1* expression in *Sepp*^{-/-} mice partially restores sperm swimming velocity and linearity of progression. Motility in % (range) was 69 (66-72) for *Sepp*^{+/+}, 18 (14-20) for *Sepp*^{-/-}, and 57 (41-72) for *Sepp*^{-/-;SEPP1}, respectively. * different from wild type; § different from wild type and transgenic mice as calculated by One Way ANOVAR on Ranks followed by Dunn's test on all pair-wise comparisons. (B) Morphology of spermatozoa isolated from the cauda epididymis. Morphology was assessed by phase contrast microscopy in *Sepp*^{+/+}, *Sepp*^{-/-}, and *Sepp*^{-/-;SEPP1} mice. Hepatic expression of *SEPP1* rescues the structural defects of spermatozoa from *Sepp*^{-/-} mice. The percentage of angulated flagella ("hairpin" spermatozoa; white arrows) in *Sepp*^{-/-;SEPP1} mice (13.5%) was similar to that in wild-type *Sepp*^{+/+} mice (17.1%), whereas the percentage of aberrant spermatozoa was 52.6% in non-transgenic *Sepp*^{-/-} mice. Scale bars, 40 μ m (*upper panels*), 10 μ m (*lower panels*).

Figure 6: Brain selenoprotein expression is increased in *SEPP1*-transgenic *Sepp*^{-/-} mice.

(A) Cytosolic GPx activity. Hepatic expression of *SEPP1* partially restores brain GPx activity in *Sepp*^{-/-;SEPP1} mice as compared to *Sepp*^{-/-} mice, *n*=10-15. (B) Cytosolic Txnrd activity. Brain Txnrd activity in *Sepp*^{-/-} mice is less affected than brain GPx activity in *Sepp*^{-/-} mice. Txnrd activity in brains of *Sepp*^{-/-} mice is fully restored upon hepatic *SEPP1* expression, *n*=9-13. Error bars indicate the SEM. Statistical significance was calculated with ANOVA followed by Dunnett's post-hoc test as indicated by the horizontal bars. **p*<0.05; ***p*<0.01. (C) Western blot analysis of GPx1 and GPx4 expression. GPx1

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expression is partly restored by the *SEPP1*-transgene in *Sepp*^{-/-} mice. GPx4 protein levels are fully restored in *Sepp*^{-/-;SEPP1} mice as compared to *Sepp*^{-/-} mice. β -actin (β -Act.) served as loading control. Samples from two different animals were loaded per genotype and one representative of two experiments is shown.

Figure 7: Motor impairment of *Sepp*^{-/-} mice is prevented by hepatic *SEPP1* expression.

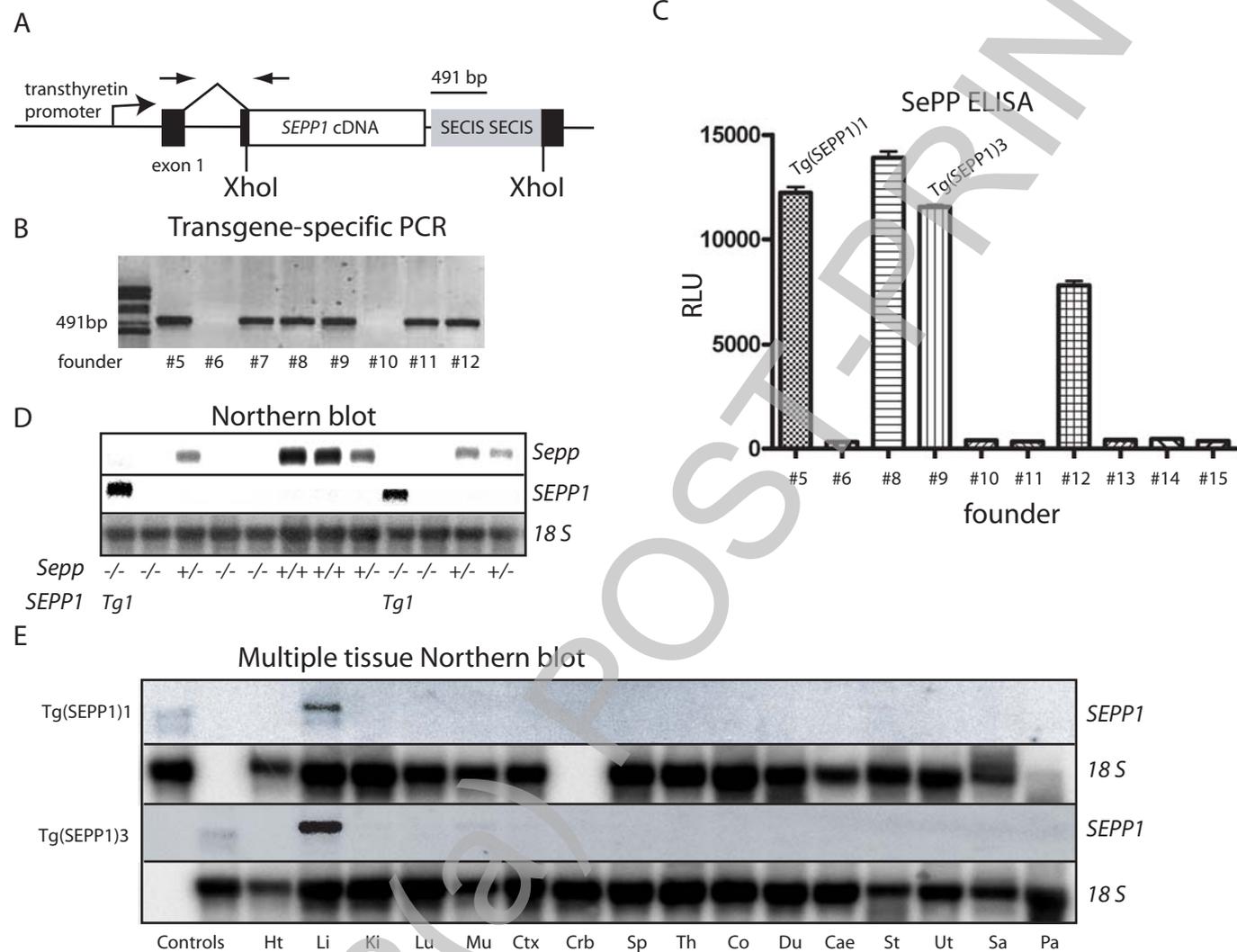
Motor coordination was quantified with the Rotarod apparatus. When compared with *Sepp*^{+/+} mice (n=13), movement coordination is completely normal in *Sepp*^{-/-;SEPP1} mice (n=9), while *Sepp*^{-/-} mice are not able to remain for more than one min on the rotating drum (n=14). Three *Sepp*^{-/-} mice completely unable to remain on the rotating drum for one revolution were excluded from the analysis. Rotarod analysis was done on postnatal day 35.

Figure 8. Rescue of neurological dysfunction of *Sepp*^{-/-} by the *SEPP1* transgene depends on dietary Se availability.

Sepp^{-/-;SEPP1} mice survive without apparent phenotype for at least 12 weeks on Se-sufficient diet, but develop the neurological phenotype with atactic gait and occasional seizures after 4 weeks on Se-deficient diet. Mice were maintained either on Se-sufficient diet (RDA; 0.16-0.2 mg Se/kg) or on Se-deficient diet (low Se; 0.06 mg Se/kg) and a Kaplan-Meier plot was created demonstrating the time at which mice had to be euthanized, because of their severe neurological phenotype. Numbers of animals: low Se, *Sepp*^{-/-;SEPP1} (n=5); *Sepp*^{-/-} (n=9); *Sepp*^{+/+} (n=14). Adequate Se, *Sepp*^{-/-;SEPP1} (n=13); *Sepp*^{-/-} (n=14); *Sepp*^{+/+} (n=32).

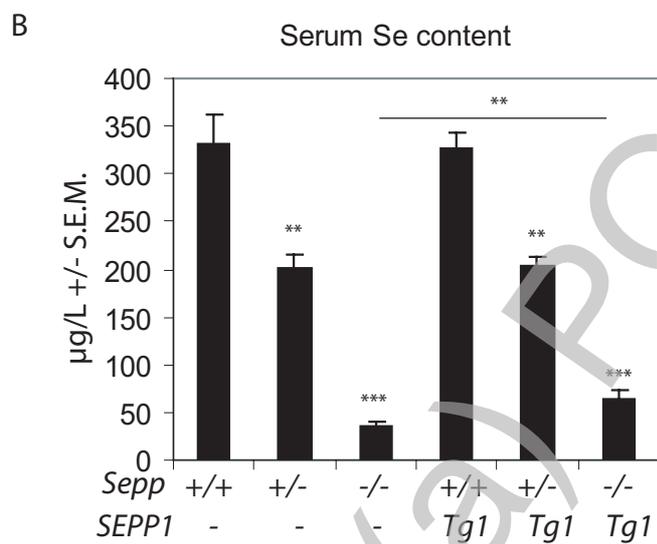
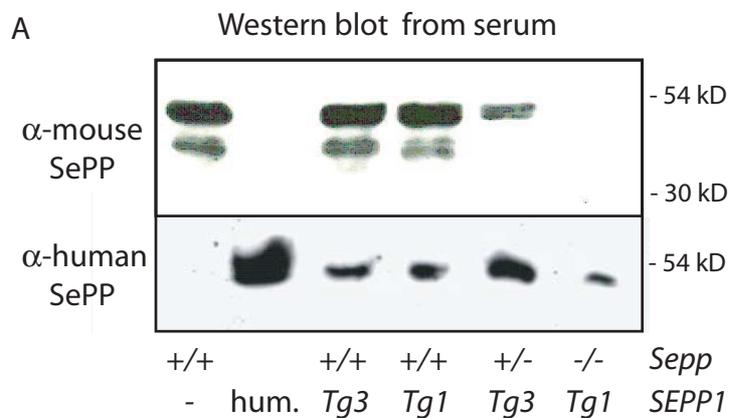
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Figure 1



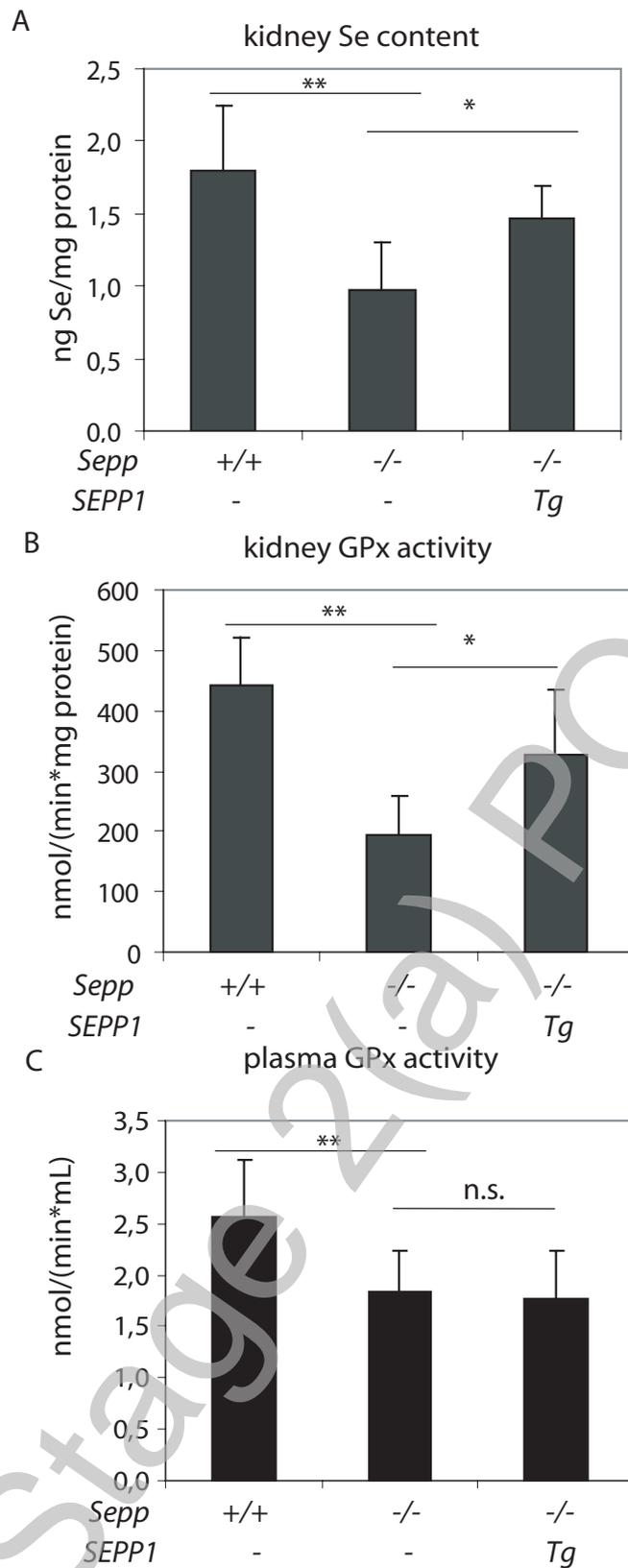
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Figure 2



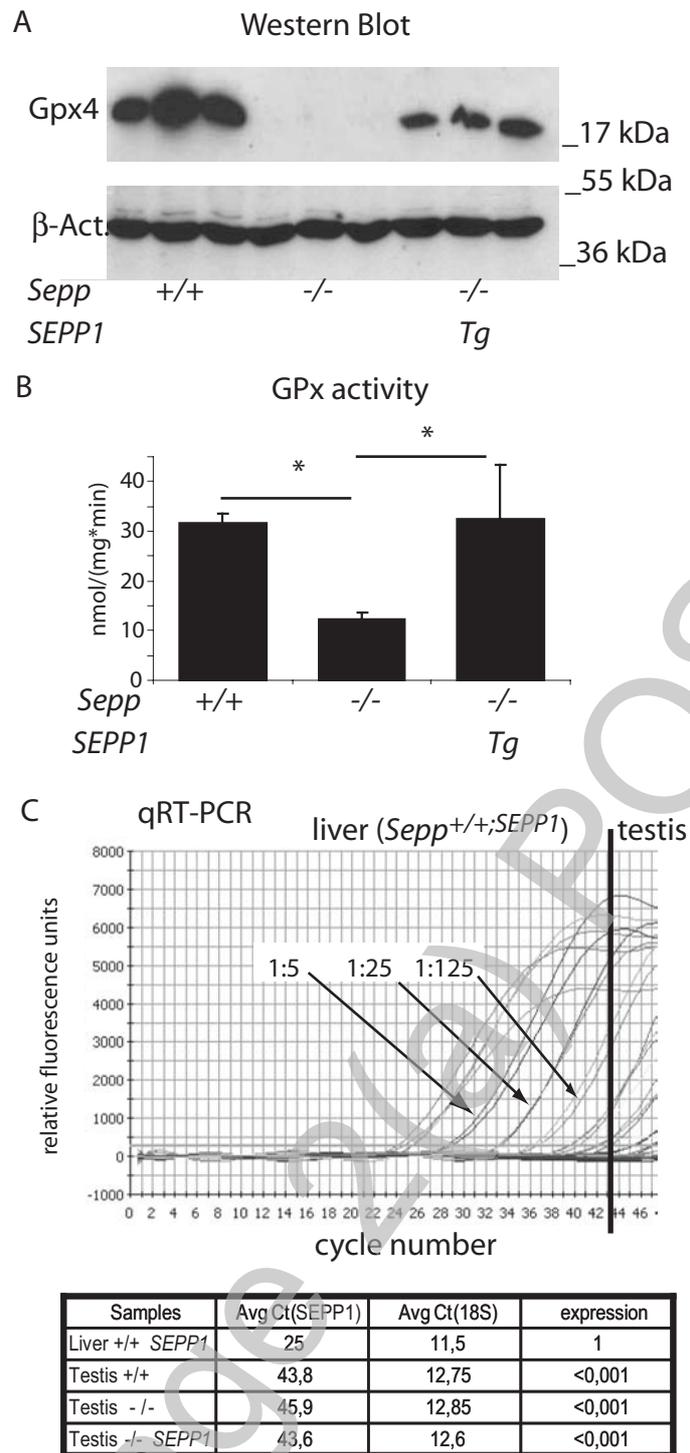
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Figure 3



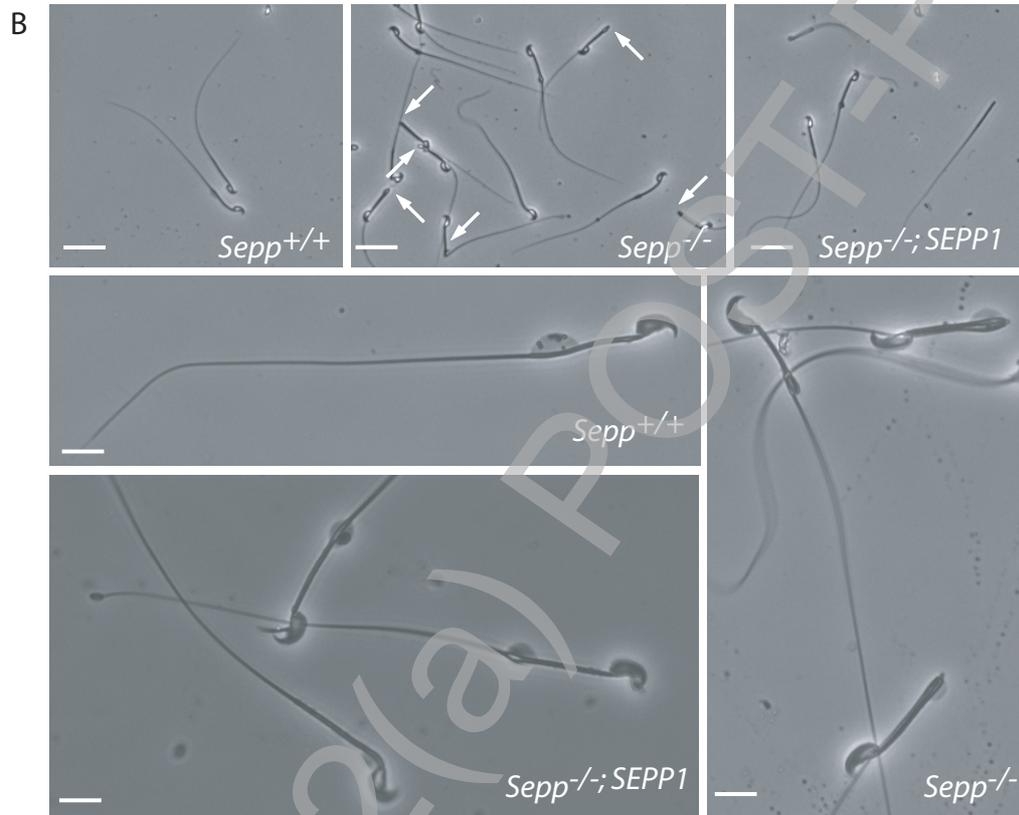
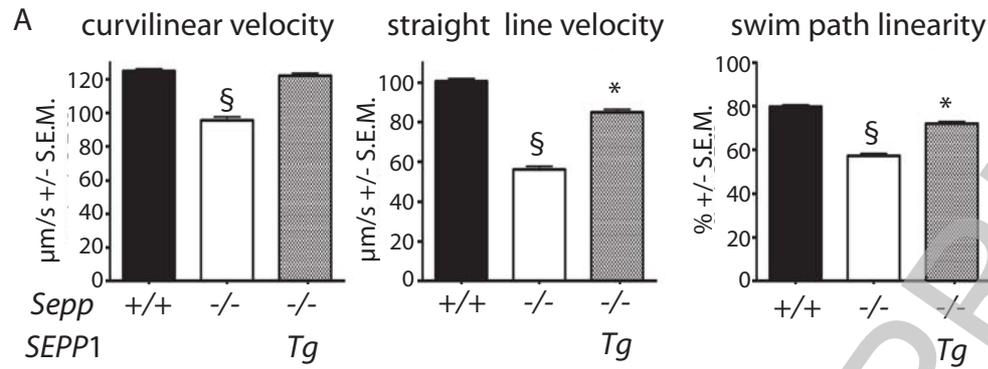
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Figure 4



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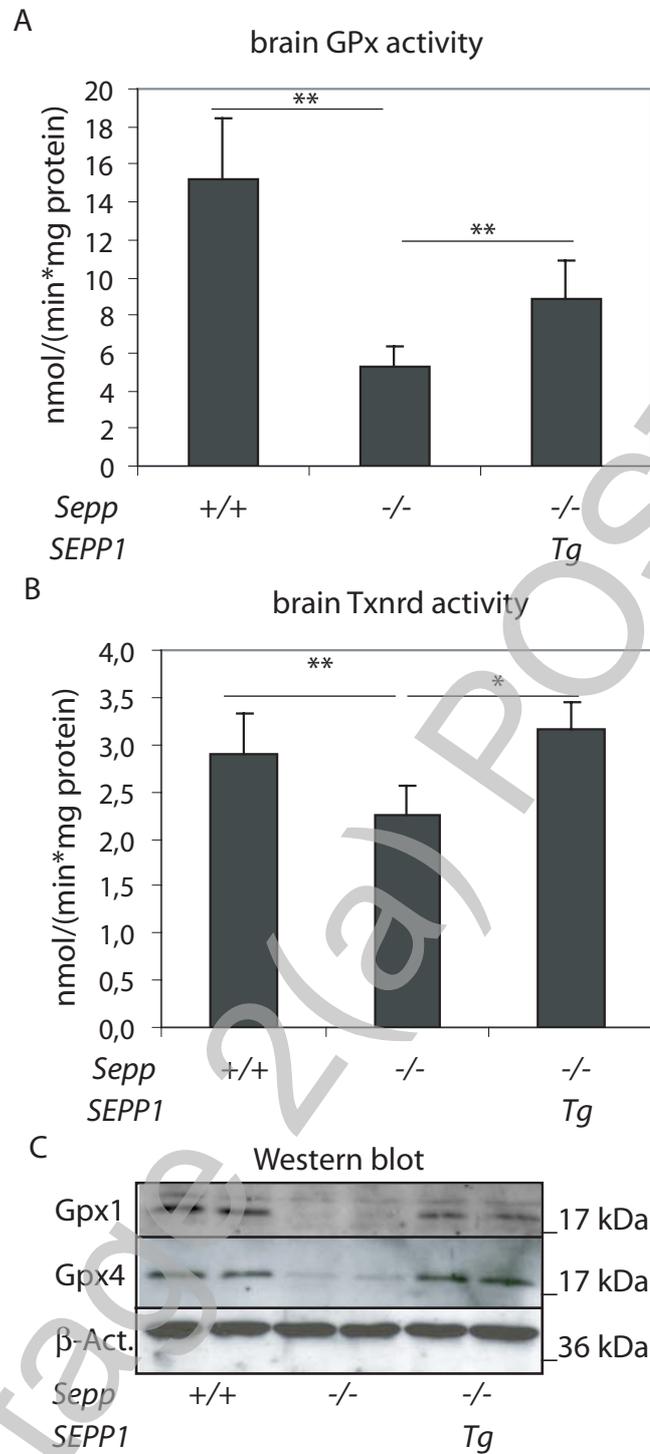
Figure 5



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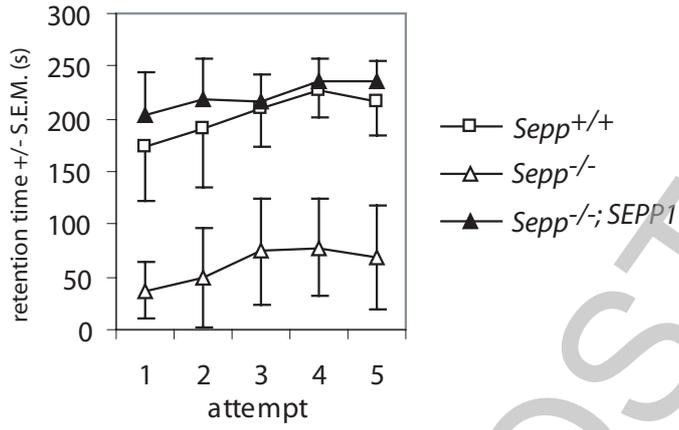
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Figure 6



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Figure 7



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Figure 8

