SUPPLEMENTARY MATERIAL

N-Myc and Sp Regulate Phosphatidylserine Synthase-1 Expression in Brain and Glial Cells

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Supplementary Tables

Suppl. TABLE S1: Primers for amplification of -1408/+197 bp region of the $Pss1$ promoter. Numbering is relative to the transcriptional start site. A reverse primer (+197 bp at the 5’-position) was used with indicated forward primers to generate eight promoter fragments with restriction sites for HindIII and Smal at the 5’ and 3’ positions, respectively.

| Forward primer 5’-position: | Sequence (sense orientation: 5’→3’) |
|-----------------------------|-----------------------------------|
| -1408                       | 5’-TCC CCC GGG GGA ATG TCA CTG TAG CCC ACG ATG-3’ |
| -1008                       | 5’- TCC CCC GGG GGA CCA CCT TCC CAG GGT CTT TTC-3’ |
| -808                        | 5’- TCC CCC GGG GGA AGT AGG GTA CGC CAG CTC TCA-3’ |
| -508                        | 5’- TCC CCC GGG GGA CCA CGG ATC GAG CCC GAA CCT-3’ |
| -398                        | 5’- TCC CCC GGG GGA GGT CTC GCG CTC CTG CCT CAC-3’ |
| -309                        | 5’- TCC CCC GGG GGA GGC CGG CAC CGC CCC CCA CGT-3’ |
| -208                        | 5’- TCC CCC GGG GGA GGG GTT TGC AGG CCC TGG AGC-3’ |
| -99                         | 5’- TCC CCC GGG GGA GGC TGC CTT CTC CCC CTG CTC-3’ |

Reverse primer 5’- CCC AAG CTT GGG CCA CTT GCT GCT CGT TGA TCA-3’
Suppl. TABLE S2: Site-directed mutagenesis of promoter binding motifs. Binding sequences for Sp (mSp), Myc (mMyc), Tal1/E47 (mE), AP-2 (mAP2), AML-3 (mAML3) and IK-1 (mIK1) in the -398/+197 Pss1 promoter region were mutated and cloned into pGL3-Basic vector. Location of mutated motif is shown in Suppl. Fig. 2A.

| Construct name | Modifications into core binding motifs |
|----------------|----------------------------------------|
| -398_mSp       | 5'-CCGCC-3' replaced by 5'-CCGAGC-3'   |
| -398_mMyc      | 5'-CACGTG-3' replaced by 5'-CTCGAG-3'  |
| -398_mE        | 5'-CATCTG-3' replaced by 5'-GCTCCC-3'  |
| -398_mAP2      | 5'-GCCCTGGG-3' replaced by 5'-GTTTGGG-3' |
| -398_mAML3     | 5'-TGGGGTT-3' replaced by 5'-TGAAGTT-3' |
| -398_mIK1      | 5'-TTC-3' replaced by 5'-TTAAC-3' }
Supplementary Figure Legends

**Suppl. FIG 1: Binding of nuclear proteins from C3H10T1/2 cells to the promoter.** Nuclear extracts (NE) from C3H10T1/2 cells were used in EMSAs with $^{32}$P-labeled promoter fragments: -356/-302, -308/-250, -260/-198) and -213/-150 (10 µg protein/reaction). Protein-DNA complexes were resolved on 6% non-denaturing polyacrylamide gels and visualized by autoradiography. For competition assays, prior to addition of probe, extracts were pre-incubated with corresponding unlabeled oligonucleotide [10- and 100-fold molar excess (10x s and 100x s, respectively)] or non-specific oligonucleotide (100x ns) [100-fold molar excess relative to labeled probe]. Unlabeled -213/-150 bp fragment was used as non-specific oligonucleotide competitor with -356/-302 bp and -308/-250 bp probes. Unlabeled -356/-302 bp fragment was used as non-specific competitive oligonucleotide with -260/-198 bp and -213/-150 bp probes. The arrows indicate specific protein-DNA complexes. Results are representative of at least 3 independent experiments with similar results.

**Suppl. FIG 2: Functional cis-elements in the Pss1 promoter.** (A): nucleotide sequence of the mouse Pss1 proximal promoter with transcriptional start site (+1) and initiation codon ATG (underlined/bold). Predicted binding sites for transcription factors are indicated. Mutations in Sp1 and non-canonical E-box binding sites are indicated with asterisks. (B): nuclear extracts from C3H10T1/2 cells were used in EMSAs with $^{32}$P-labeled -308/-278 bp fragment (5 µg protein/reaction). Protein-DNA complexes were resolved by 5% non-denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Lane 1: probe alone; lane 2: nuclear extract+probe; lanes 3-5: nuclear extract incubated for 10 min with unlabeled -308/-278 bp fragment prior to addition of probe. For competition assays, wild-type Sp1 consensus
oligonucleotide (wtSp1cs) or mutated Sp1 consensus oligonucleotide (mSp1 cs) were added at 200-fold molar excess relative to probe. Lanes 6-8 (supershift): nuclear extract incubated with anti-
Sp antibodies (1 μg/reaction) for 20 min prior to addition of probe; arrow indicates supershifted
Sp1/DNA complex. Data are representative of 3 independent experiments with similar results. (C):
C3H10T1/2 cells were transiently transfected with pGL3-Basic vector containing wild-type (WT)
or mutated -398/+197 bp sequences upstream of the luciferase coding region. Mutations were
introduced to disrupt binding to Sp1 (mSp), the non-canonical E-box (mE), AP-2 (mAP2), AML3
(mAML) and IK-1 (mIK1) response elements. Luciferase activity was measured 48 h after
transfection relative to β-galactosidase activity [relative luciferase units (RLU)]. Data are means ±
S.D. of triplicate measurements of one experiment representative of 3 independent experiments. *,
P<0.01 v. WT.

Suppl. FIG 3: Correlation of Pss1 transcript levels in mouse tissues with binding of nuclear
factors to -308/-274 region of the Pss1 promoter. (A): nuclear extracts from brain and liver were
isolated from mice between birth (day 0) and day 21. EMSAs were performed with 20 μg protein
and [32P]-labeled -308/-274 bp fragment. Protein-DNA complexes were resolved by 6% non-
denaturing polyacrylamide gel electrophoresis and detected by autoradiography. Data are
representative of 2-3 independent experiments with similar results. (B): Pss1 transcript levels in
brain and liver from neonatal and 21-day-old mice. mRNA levels of Pss1 and four reference genes
cyclophilin A, CypA; beta-actin, ActB; glyceraldehyde 3-phosphate dehydrogenase, Gapdh;
hypoxanthine phosphoribosyltransferase-1, Hprt1) were measured by real-time qPCR. The graph
shows normalized Pss1 transcript levels using geNorm software (http://medgen.ugent.be). Data are
means ± S.D. from at least 3 independent experiments.
Suppl. FIG 4: *Pss1* and *Pss2* mRNA levels in neurons and astrocytes. Primary cortical neurons (white bars) and astrocytes (grey bars) were isolated from 1-day-old rats. *Pss1* and *Pss2* mRNA levels were quantified by qPCR relative to the amount of cyclophilin A mRNA (left) or β-actin mRNA (right). Results are averages ± S.D. of 3 independent experiments.

Suppl. FIG 5: Nuclear extracts from rat and mouse (brain and cerebral cortex) exhibit similar binding patterns with promoter fragments. Nuclear extracts from whole brain and cerebral cortex (B and C, respectively) of 1-day-old rats and mice were used in binding reactions with [32P]-labeled -308/-274 bp or -272/-251 bp fragments of the mouse *Pss1* promoter. Protein-DNA complexes were resolved by 5% (left panel) or 6% (right panel) non-denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Left: 10 µg protein; right: 20 µg protein. Arrows indicate protein-DNA complexes. Data are representative of at least 3 independent experiments.
Suppl. FIG 2

A.  

\[
\begin{align*}
-600 & \text{GCGCGCAGGC GTCGGAGAGG TCTGAGCTAC AGCTGAGCCG GACGTATAAC CCGGTGGAGG} +401 \\
& \text{Sp1(c)} \\
-400 & \text{GCGCGCTGCC CAGGCAGCTA GGGGGGCTGAC AGCTGAGCCG} -341 \\
-340 & \text{GTACCTCTGC AGACGGCGCA TACGGGGGAC GGGGGCCGCC CACGTGGCTGC} -201 \\
& \text{Sp1(c)} \quad \text{NMybShift} \\
-280 & \text{GGCTGGA/GTG GGGAGAAAACGG CCGGGGAGG GACCGGGCGG GGGACGGAGA -221} \\
& \text{non-canonical E-box} \\
-220 & \text{CAGACCTGCAG TGGGATTGGAGA CAGCCGCTGG AGCCGTGTGC CGGGGTCCCA GGATGACCA -161} \\
& \text{AP-2 FAML-3 (+)} \\
-100 & \text{CTCCGCGGTA ATGSGGGAG GGTGAGACT CTCAGATGTG CCGCTACTAG GCTGCTTCTT -101} \\
-100 & \text{GACGCTGCT GTCGGCCGC AGGCGGGCAG GGGGGGGGCG GGGGGGGCG CCGCTCTCGG} +41 \\
+40 & \text{GGGGGTCTC TCTCTGCAG ACGACCTTC TACGGGGC ACGCCGGC CGCGGTACAG +20} \\
& \text{NF-kB} \\
+21 & \text{GACACCTGCAG TGGGATTGGAGA CAGCCGCTGG AGCCGTGTGC CGGGGTCCCA GGATGACCA -69} \\
+81 & \text{CAGAGGCGAGCAGGAGAGAAGAGATGATTGCTCTCTCTCCGAGGAGAGAGAGCTCAGG} \\
& \text{CAAGATGATATGACTGAGGATAGTGATTCGAGTTCCGCGATCATACGAGGAGAGAGAGAGATGAGA} +197
\end{align*}
\]

B.  

Nuclear Extract: 

\[\text{Sp1 supershift}\]

Lane: 1 2 3 4 5 6 7 8

C.  

Luciferase Activity (RLU)

|       | WT | mSp | mE | mSp/mE | mE/mK1 | mSp/mK1 | mE/mK1 |
|-------|----|-----|----|--------|--------|---------|--------|
|       |    |     |    |        |        |         |        |
|       | 60 | 50  | 40 | 30     | 20     | 10      | 0      |

* indicates significant difference.
Suppl. FIG 3

| days after birth: | 0  | 5  | 10 | 21 |
|-------------------|----|----|----|----|
| **(-308/-274)Pss1 probe** |

A.

- **Brain**
- **Liver**

B.

- ![Brain mRNA Levels](chart)
- ![Liver mRNA Levels](chart)
Suppl. FIG 4
