Supplementary material to the paper

Functional characterization of *cis*- and *trans*- regulatory elements involved in expression of phospholipid hydroperoxide glutathione peroxidase

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1. Sequence of the potential promoter region of the joint ph/snGPx gene

The phGPx gene consists of 7 exons (E1 to E7), but E1a and E1b are used alternatively (30, 31). E1a contains two in-frame translational initiation sites (5'-ATG and 3'-ATG, Scheme 1) and both of them are functional under certain conditions. The sequence between the two start codons encodes for a signal peptide required for mitochondrial import (11). However, for the cytosolic phGPx this sequence contributes to the 5'-untranslated region of the mRNA and thus, it may contain regulatory sequences. In Fig. S1 the sequence of the 5'-flanking region (potential promoter region) starting at the 3'-ATG is given.

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-100  ccgcgtccctatcactgggcatgcgcagttgctgataacaacaaactcgt
 - 50  aagcccagccccgcccaagccgtcccttcattcaggcttcccattggctg
+  1  CAGGGGCCTCGCGTCTTAGCGCAGGCGCATCTGACCAATAAGAGACGTCA
+ 51  GTGGGCGTGCCCGAGGGCGGGCAAGCCATACTCGGCCTCGCGCGTCCATT
                                                  5'ATG
+101  GGTCGGCTGCGTGAGGGGAGGAGCCGCTGGCTCCGGCCGCCGAGATGAGC
+151  TGGGGTCGTCTGAGCCGCTTACTTAAGCCAGCACTGCTGTGCGGGGCTCT
                              3'ATG
+201  GGCTGCGCCTGGTCTGGCAGGCACCATG
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Figure S1. Sequence of the potential promoter region of the ph/snGPx gene. The 5'-flanking region of the ph/snGPx gene is given starting at the 3'-ATG. The sequence encoding for the mitochondrial insertion peptide is indicated in italic letters. The in-frame translational initiation sites are given in bold. The most 5'-transcriptional start site identified so far in mice was set +1 (33).

2. Potential binding sequences for transcription factors in the footprint regions

DNase protection assays (Fig. 2 of the main paper) indicated 4 distinct protein-binding sites in the putative sn/phGPx promoter. Footprints FP1, FP2 and FP3 were of similar size (20-30 bp), but FP-4 comprised 50 bp. This size suggested the existence of two or more protein binding regions.

Foot-	transcription	position	functionality

print	factors		(EMSA)
FP-1	SP1	-47	+
FP-2	Ikaros2 NF-Y (5'-CCAAT)	-11 -8	- +
FP-3	CREB AP2 SP1	+47 +52 +54	- - +
FP-4A	SMAD NF-Y (3'-CCAAT)	+97 +98	+ +
FP-4B	MZF1 SP1	+115 +117	- +

Table S1 Protein binding sites in the footprint region of the basic sn/phGPx promoter

The nucleotide sequences of the binding regions were determined and then we searched for potential consensus binding sites of know transcription factors using the Matinspector software package. The location of the binding sites and their in vitro functionality are given in Table S1. The functionality of the binding regions was tested by EMSA and cell transfection with reporter gene constructs. It should be stressed that he SMAD site present in FP-4A was characterized by a rather low core-value.

3. Mutations of the consensus binding sequences of transcription factors

footprint	sequences
FP-1	AGC CCA **GCC CCG CCC** AAG CCG TC
mutated FP-1	AGC CCA **TGC TAG CCC** AAG CCG TC
FP-2	ATT CAG GCT TCC C**AT TGG** CTG CAG GGG
mutated FP-2	ATT CAG GCT TCC C**TA CCG** CTG CAG GGG
FP-3	AGA GAC GTC AGT **GGG CGT G**CC CGA G
mutated FP-3	AGA GAC GTC AGT **GTA CGT G**CC CGA G
FP-4A+B	ATA CTC GGC CTC GCG CGT CC**A TTG G**TC GGC TGC GTG A**GG GGA GGA G**CC GCT
mutated FP-4A	ATA CTC GGC CTC GCG CGT CC**A TTC G**TC GGC TGC GTG A**GG GGA GGA G**CC GCT
mutated FP-4B	ATA CTC GGC CTC GCG CGT CC**A TTG G**TC GGC TGC GTG A**GT GGA GGA G**CC GCT

Table S2 Site directed mutagenesis of *cis*-regulatory elements identified in the footprint regions of the sn/phGPx promoter. Consensus sequences are indicated in bold. Mutated nucleotides are underlined.

In order to test the functionality of the consensus binding sequences identified by the DNase protection assay the sequences were mutated so that their protein binding capacity was abolished. In separate experiments we found that for some transcription factors single point mutations of their consensus binding sequences were not sufficient to prevent the binding of the corresponding trans-

regulatory proteins. Thus, we were forced introduce step by step multiple mutations into the binding regions. The binding capacity of the mutated oligonucleotides was tested for the different constructs and for the final experiments only those oligonucleotides were used, which did not bind the corresponding *trans*-regulatory proteins any more. In Table S2 the mutations performed for the different consensus binding sequences are summarized.

4. Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed in order to find out whether or not binding of the identified transcription factors to the sn/phGPx promoter may actually occur inside the cells.

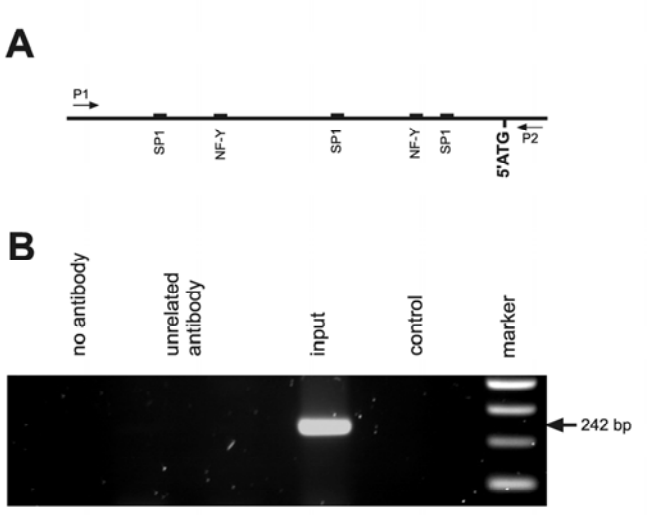

Figure S2. Chromatin immunoprecipitation with unrelated antibodies. Chromatin immunoprecipitation was carried out as described in Material and Methods. A) Localization of the relevant transcription factor binding sites in the promoter of the ph/snGPx gene. The arrows indicate the positions of the primers used for amplification of the immunoprecipitated DNA. B) Sheared DNA/protein complexes were precipitated with a polyclonal antibody raised against the 15-Lipoxygenase and the precipitated genomic DNA was amplified. Input – sheared DNA prior to immunoprecipitation. Control – PCR control.

From Fig. 9 (main paper) it can be seen that SP1 and NF-Y appear to interact with the sn/phGPx promoter in vivo. However, in chromatin immunoprecipitation there is always the danger of an unspecific precipitation of unrelated DNA fragments. In order to exclude that such unspecific co-precipitation may be responsible for the PCR signals a series of negative control experiments were carried out, in which an unrelated antibody (polyclonal anti-15-lipoxygenase antibody) was used for the precipitation procedure. The lack of any PCR signal in this lane (Fig. S2) indicated the specificity of the analytical procedure.

5. EMSA experiments using oligonucleotides containing mutated footprint sequences

To test the specificity of the electrophoretic mobility shift signals negative controls were performed in which we used oligonucleotides as unlabelled competitors that contained mutated consensus binding sequences for the various transcription factors (see Table S2). As indicated in Fig. S3 we did not observe any alterations in the shift patterns using these mutated competitors.

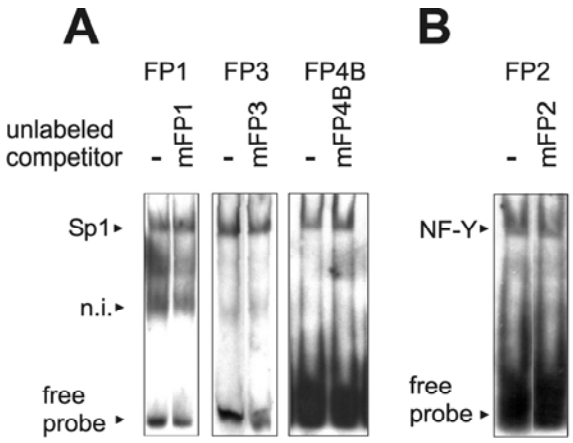

Figure S3. Competitive EMSA using mutated oligonucleotides as unlabeled competitors. The labeled oligonucleotide dimmers for FP1, FP2, FP3 and FP4B were incubated as described in Material and methods in the presence or absence of unlabeled competitors, in which the consensus sequences were mutated as indicated in Table S2. After 30 min of incubation at 4° C the assay sample was analyzed by PAGE. The arrows indicate the shift bands. The result for FP4A are shown in Fig. 6A in the main paper. A) SP1 sites, B) NF-Y site.