## Supplementary data to:

**Glutathione peroxidase 4 and vitamin E cooperatively prevent hepatocellular degeneration**

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### Supplementary materials & methods

*Materials*

Selenoprotein R (SelR) antibodies were from the laboratory of one of us (VNG). Txnrd1 (Catalog No. ab124954), Gpx1 (Catalog No. ab22604) and Gpx4 (Catalog No. ab125066) antibodies were purchased from Abcam, SepW (Catalog No. 600-401-A29) antibodies from Rockland, Gapdh (Catalog No. G9545) antibodies from Sigma-Aldrich, and anti-rabbit HRP conjugated secondary antibodies (Catalog No. 7074) from Cell Signaling Technology. Dulbecco's phosphate buffered saline (DPBS) and NuPAGE polyacrylamide gels were obtained from Life Technologies. Wizard Genomic DNA Purification Kit was purchased from Promega. β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), 5,5'-dithiobis (DTNB) and aurothioglucose hydrate were from Sigma-Aldrich. PVDF membrane, iScript cDNA synthesis kit and SYBR green were from BioRad. Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit was purchased from Cayman Chemical. TriPure isolation reagent and Complete Protease Inhibitor Cocktail Tablets were from Roche. BCA protein assay kit and SuperSignal West Dura Extended Duration Substrate were from Thermo Scientific.

*Generation of Hepatocyte-specific Knockout Mice*

*Gpx4*$^{fl/fl}$ mice in a C57BL/6 background were obtained as described [1] and crossed with mice carrying the *Alb-Cre* transgene (C57BL/6) [2] to obtain hepatocyte-specific *Gpx4* knockout mice. Hepatocyte-specific *Trsp*$^{fl/fl}$ knockout mice and *Txnrd1*$^{fl/fl}$ conditional knockout mice were generated as described [3, 4]. Male F2 generation offspring with genotypes *Alb-Cre; Gpx4*$^{fl/+}$, *Alb-Cre; Trsp*$^{fl/+}$ or *Alb-Cre; Txnrd1*$^{fl/+}$ were mated with female *Gpx4*$^{fl/fl}$, *Trsp*$^{fl/fl}$ or *Txnrd1*$^{fl/fl}$ mice, respectively, and the resulting *Alb-Cre; Gpx4*$^{fl/fl}$, *Alb-Cre; Trsp*$^{fl/fl}$ and *Alb-Cre; Txnrd1*$^{fl/fl}$ knockout littermates were compared to the corresponding littermates, which were used as controls. Unless otherwise noted, littermates with the genotype *Gpx4*$^{fl/fl}$ served as controls for *Alb-Cre; Gpx4*$^{fl/fl}$ mice, littermates with the genotype *Trsp*$^{fl/fl}$ served as controls for *Alb-Cre; Trsp*$^{fl/fl}$ mice and littermates with the genotype *Txnrd1*$^{fl/fl}$ served as controls for *Alb-Cre; Txnrd1*$^{fl/fl}$ mice. To obtain combination knockout mice lacking liver *Trsp*, *Gpx4* or *Txnrd1*, *Alb-Cre; Trsp*$^{fl/fl}$ mice were mated with either *Alb-Cre; Gpx4*$^{fl/fl}$ or *Alb-Cre; Txnrd1*$^{fl/fl}$ mice.

*Mice, genotyping and diets*

Mice were maintained under standard conditions, with food and water given *ad libitum*. Genomic DNA from mouse tails was isolated using the Wizard Genomic DNA Purification Kit according to manufacturer's instructions and genotyped using previously described primer sets for *Gpx4* [1] and *Txnrd1* [5]. *Trsp* [6] and the *Alb-Cre* transgene [3] were amplified using primers as described. Vitamin E-deficient (TD.88163) and a vitamin E-enriched diet (TD.130835) containing vitamin E in the form of DL-α tocopheryl acetate (500 IU/kg) were

obtained from Harlan Laboratories. The standard rodent diet used in this study (NIH-31) contained 41 IU/kg of DL-α tocopheryl acetate. To determine the effect of vitamin E on *Alb-Cre; $Gpx4^{fl/fl}$* mice, breeding pairs were provided either a vitamin E-deficient diet or a vitamin E-enriched diet. Male and female mice obtained from breeders receiving a vitamin E-enriched diet were maintained on this diet for a minimum of 6 weeks. For experiments requiring dietary depletion of vitamin E, mice were given a vitamin E-deficient diet at 6 weeks of age (TD.88163). Diets were administered to both knockout and littermate control mice in the same manner. Mice were handled in accordance with the National Institutes of Health Institutional Guidelines (NCI, NIH, Bethesda, MD, USA), and all mouse experiments were approved by the Animal Ethics Committee at the National Institutes of Health.

*Protein isolation and western blotting*

Tissues were washed twice with DPBS and then harvested in ice cold lysis buffer (50 mM Tris; pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Igepal and protease inhibitor). Protein concentrations were measured in the resulting cell extracts using a BCA protein assay kit, and 40 µg of total protein were electrophoresed on NuPAGE polyacrylamide gels, transferred on to PVDF membranes and incubated overnight at 4°C in Tris-buffered saline (TBS-T) containing 0.1% Tween 20 and 5% milk with antibodies for either Gpx1, Gpx4, Txnrd1, SelR, SepW or Gapdh. Membranes were washed with TBS-T and incubated in secondary antibody for 1 h. Immunolabeling was detected using SuperSignal West Dura Extended Duration Substrate and exposed to x-ray film.

*Thioredoxin reductase activity assay*

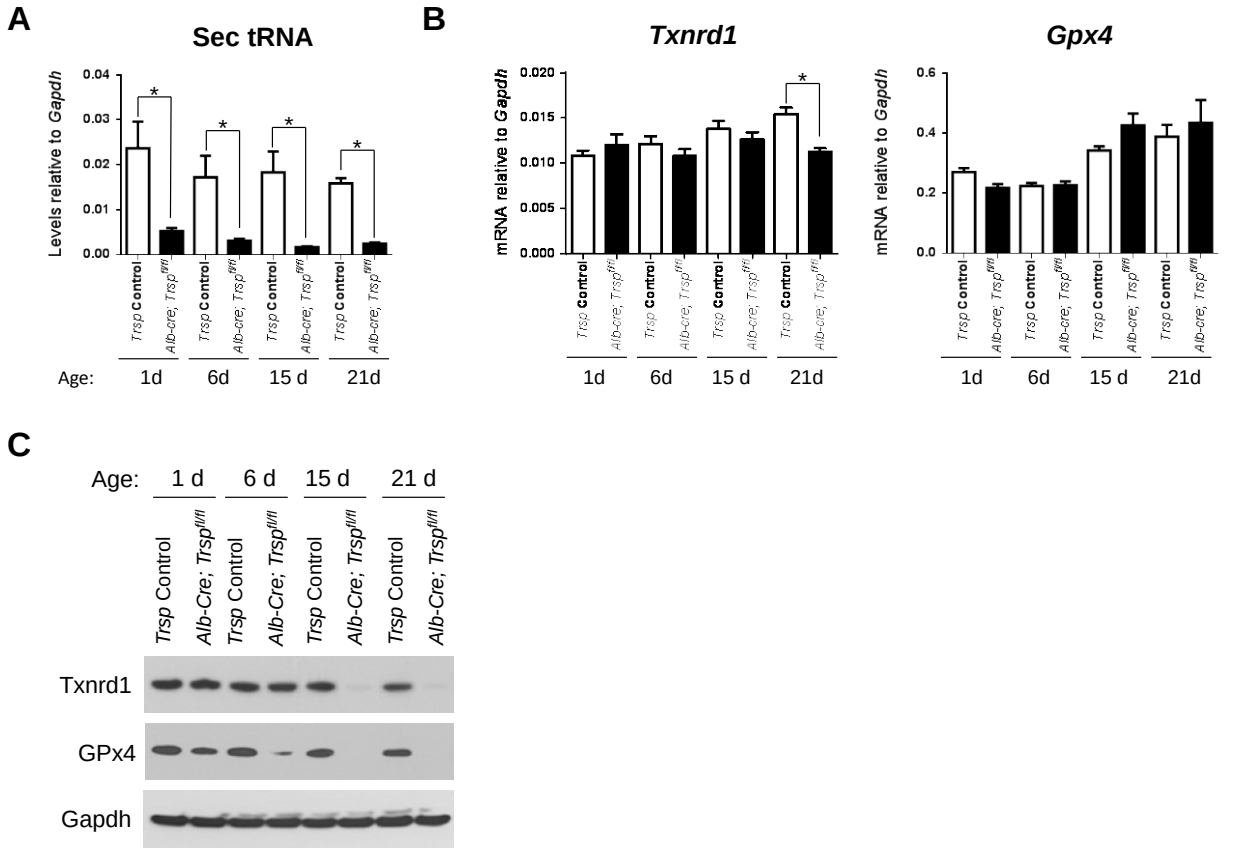
Txnrd1 activity was determined spectrophotometrically based on the method of Holmgren [7]. Briefly, Txnrd1 activity was determined as the difference between total Txnrd1 activity and the time-dependent increase in absorbance at 412 nm in the presence of aurothioglucose, a Txnrd1 inhibitor. Activity is expressed in μmol 5-thio-2-nitrobenzoic acid (TNB) formed/min/mg protein.

*Lipid peroxidation assay*

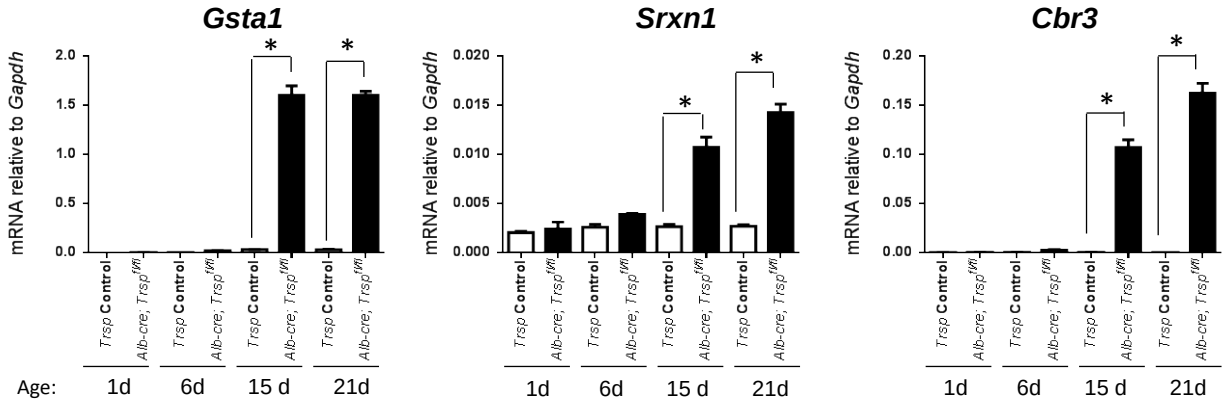
Lipid peroxidation, expressed in terms of malondialdehyde (MDA), was measured colorimetrically (540 nm) using the TBARS Assay Kit according to the manufacturer's instructions in plasma obtained from *Gpx4* control and *Alb-Cre; Gpx4*$^{fl/fl}$ mice, either maintained on a vitamin E-supplemented diet for 9 weeks or maintained on a vitamin E-supplemented for 6 weeks and then placed on a vitamin E-deficient diet for 3 additional weeks.

*Quantification of GSH and GSSG*

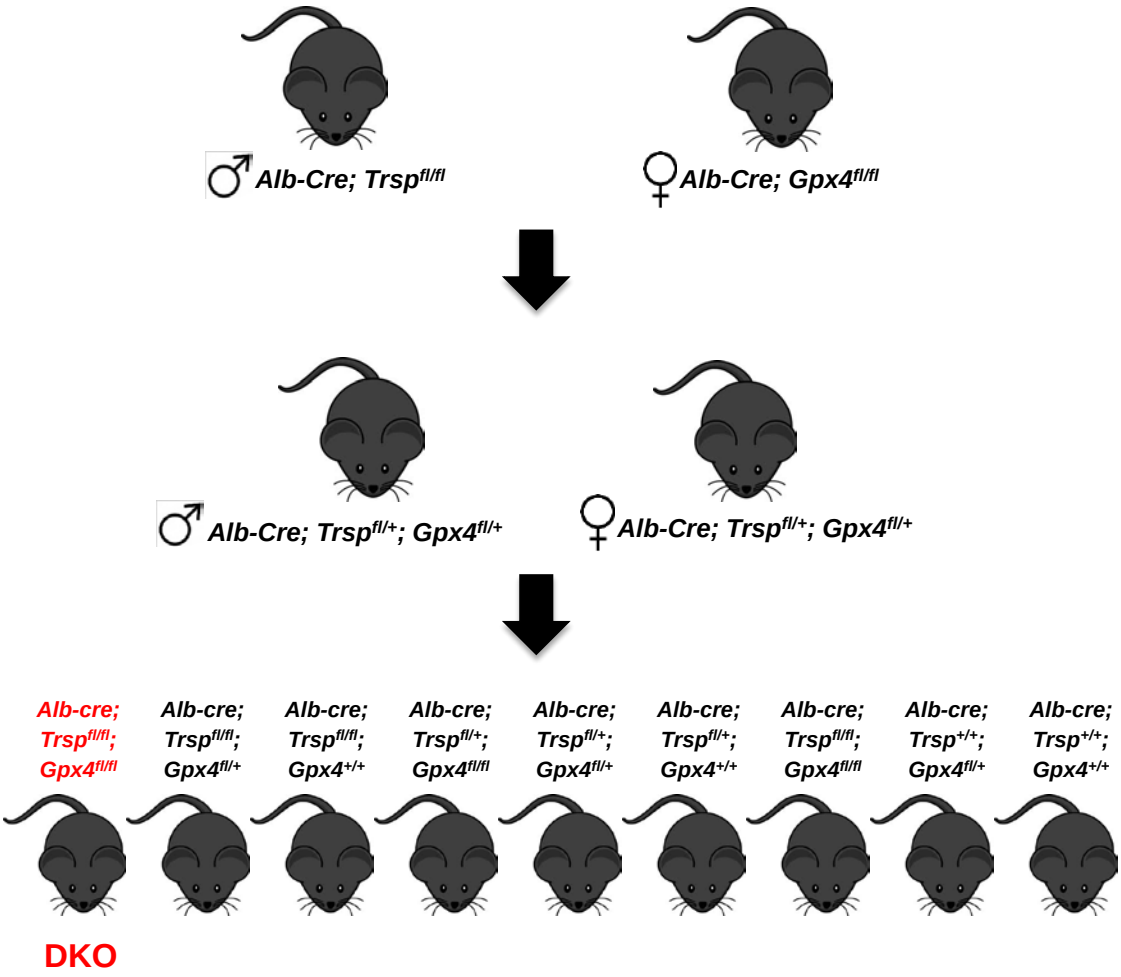
Total glutathione (GSH) levels in liver were quantified using a glutathione assay kit according to the manufacturer's instructions (Sigma-Aldrich).



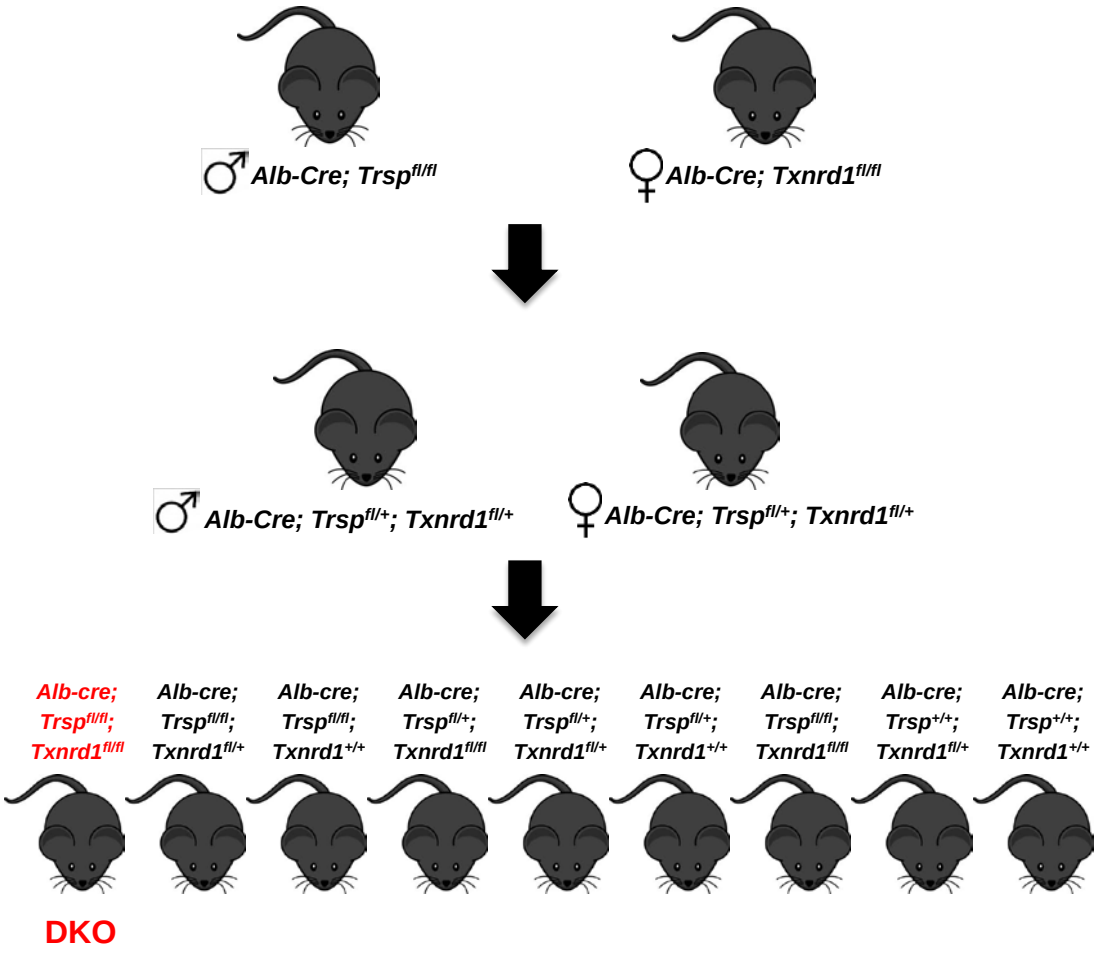

**Supplementary Fig. 1.** Expression levels of Sec tRNA, Txnrd1 and Gpx4 in livers of control and *Alb-Cre; Trsp$^{fl/fl}$ mice.* (A) Levels of Sec tRNA were analyzed by qPCR in control and knockout liver samples at 1, 6, 15 and 21 days post birth. Data are shown as relative levels normalized to *Gapdh* in all liver samples (n=3 for each genotype). *Denotes statistical difference ($P$ < 0.05). (B) mRNA levels of *Txnrd1 and Gpx4* were analyzed by qPCR in control and knockout liver samples at 1, 6, 15 and 21 days post birth. Data are shown as relative mRNA levels normalized to *Gapdh* in all liver samples (n=3 for each genotype). *Denotes statistical difference ($P$ < 0.05). (**C**) Protein levels of Txnrd1 and Gpx4 were analyzed by western blot in control and *Alb-Cre; Trsp$^{fl/fl}$* liver samples at 1, 6, 15 and 21 days post birth. Levels of Gapdh are shown in the bottom panel as a control for protein loading.




**Supplementary Fig. 2.** mRNA expression levels of Nrf2-regulated genes in livers of control and *Trsp*-deficient mice. mRNA levels of *Gsta1, Srxn1 and Cbr3* were analyzed by qPCR. Data are shown as relative mRNA levels normalized to *Gapdh* in all liver samples (n=3 for each genotype). *Denotes statistical difference ($P < 0.05$).




**Supplementary Fig. 3.** Breeding scheme for generation of combined *Trsp* and *Gpx4* knockout mice. Parental and expected offspring genotypes are shown. Double knockout (DKO) pups have the genotype *Alb-cre: Trsp*$^{fl/fl}$; *Gpx4*$^{fl/fl}$.




**Supplementary Fig. 4.** Breeding scheme for generation of combined *Trsp* and *Txnrd1* knockout mice. Parental and expected offspring genotypes are shown. Double knockout (DKO) pups have the genotype *Alb-cre: Trsp*$^{fl/fl}$; *Txnrd1*$^{fl/fl}$.

**Supplementary Table 1. Genotyping of pups of *Alb-Cre; Trs$^{fl/+}$; Gpx4$^{fl/+}$* female x *Alb-Cre; Trsp$^{fl/+}$; Gpx4$^{fl/+}$* male mice.**

| | | Number of mice/genotype | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Age examined** | **Mean number of pups/litter** | ***Alb-cre; Trsp$^{fl/fl}$; Gpx4$^{fl/fl}$*** | ***Alb-cre; Trsp$^{fl/fl}$; Gpx4$^{fl/+}$*** | ***Alb-cre; Trsp$^{fl/fl}$; Gpx4$^{+/+}$*** | ***Alb-cre; Trsp$^{fl/+}$; Gpx4$^{fl/fl}$*** | ***Alb-cre; Trsp$^{fl/+}$; Gpx4$^{fl/+}$*** | ***Alb-cre; Trsp$^{fl/+}$; Gpx4$^{+/+}$*** | ***Alb-cre; Trsp$^{+/+}$; Gpx4$^{fl/fl}$*** | ***Alb-cre; Trsp$^{+/+}$; Gpx4$^{fl/+}$*** | ***Alb-cre; Trsp$^{+/+}$; Gpx4$^{+/+}$*** |
| 1 day | 6.86 | 0 | 6 | 3 | 4 | 15 | 6 | 3 | 8 | 3 |

**Supplementary Table 2. Genotyping of pups of *Alb-Cre; Trsp$^{fl/+}$; Txnrd1$^{fl/+}$* female x *Alb-Cre; Trsp$^{fl/+}$; Txnrd1$^{fl/+}$* male mice.**

| | | Number of mice/genotype | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Age examined** | **Mean number of pups/litter** | ***Alb-cre; Trsp$^{fl/fl}$; Txnrd1$^{fl/fl}$*** | ***Alb-cre; Trsp$^{fl/fl}$; Txnrd1$^{fl/+}$*** | ***Alb-cre; Trsp$^{fl/fl}$; Txnrd1$^{+/+}$*** | ***Alb-cre; Trsp$^{fl/+}$; Txnrd1$^{fl/fl}$*** | ***Alb-cre; Trsp$^{fl/+}$; Txnrd1$^{fl/+}$*** | ***Alb-cre; Trsp$^{fl/+}$; Txnrd1$^{+/+}$*** | ***Alb-cre; Trsp$^{+/+}$; Txnrd1$^{fl/fl}$*** | ***Alb-cre; Trsp$^{+/+}$; Txnrd1$^{fl/+}$*** | ***Alb-cre; Trsp$^{+/+}$; Txnrd1$^{+/+}$*** |
| 1 day | 8.64 | 7 | 16 | 4 | 10 | 23 | 11 | 5 | 13 | 6 |

**Supplementary Table 3.** Primers used for qPCR analysis.

| Target | Forward Primer | Reverse Primer |
|---|---|---|
| *Cbr3* | GCGGGCATCGCCTTTAGAATGGA | TGCAGACTGCTGATGTTCACCAC |
| *Gclc* | CACCCCCGCTTCGGTACTCT | GACAGCAGTTGCCCATCCCG |
| *Gapdh* | TCTTGGGCTACACTGAGGAC | TGTTGCTGTAGCCGTATTCA |
| *Gpx1* | CAGGAGAATGGCAAGAATGA | GAAGGTAAAGAGCGGGTGAG |
| *Gpx2* | ATCAAACGGCTCCTCAAAGT | GGGACGATATTCAGGGAATG |
| *Gpx4* | GCAGGAGCCAGGAAGTAATC | GGCTGGACTTTCATCCATTT |
| *Gsr* | TCCGTGCCTGGTAGGAAGCC | GCAGCGATTGCAACTGGGGT |
| *Gsta1* | CGCAGACCAGAGCCATTCTC | TTGCCCAATCATTTCAGTCAGA |
| Sec tRNA | GCCCGGATGATCCTCAG | CGCCCGAAAGGTGGAATTGAA |
| *Sepr* | TCCAGTCACTCGAAGTACGC | CTTGCCACAGGACACCTTTA |
| *Sepw* | TAGAGGCAGGGTCCTGAAAG | AATCCATCTCTGGCCTGACT |
| *Srxn1* | GGTGGACACGATCCTGGCGG | GGTAGGCTGCATAGCGGTGGC |
| *Txnrd1* | CTACAGACCATTGCCTTGCT | ACCTCCTACCCACAAGATCC |

**Supplementary references**

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