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Research paper

Glutathione peroxidase 4 and vitamin E cooperatively prevent hepatocellular degeneration

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ABSTRACT

The selenoenzyme glutathione peroxidase 4 (Gpx4) is an essential mammalian glutathione peroxidase, which protects cells against detrimental lipid peroxidation and governs a novel form of regulated necrotic cell death, called ferroptosis. To study the relevance of Gpx4 and of another vitally important selenoprotein, cytosolic thioredoxin reductase (Txnrd1), for liver function, mice with conditional deletion of *Gpx4* in hepatocytes were studied, along with those lacking *Txnrd1* and selenocysteine (Sec) tRNA (*Trsp*) in hepatocytes. Unlike *Txnrd1*- and *Trsp*-deficient mice, $Gpx4^{-/-}$ mice died shortly after birth and presented extensive hepatocyte degeneration. Similar to *Txnrd1*-deficient livers, $Gpx4^{-/-}$ livers manifested upregulation of nuclear factor (erythroid-derived)-like 2 (Nrf2) response genes. Remarkably, $Gpx4^{-/-}$ pups born from mothers fed a vitamin E-enriched diet survived, yet this protection was reversible as subsequent vitamin E deprivation caused death of *Gpx4*-deficient mice ~4 weeks thereafter. Abrogation of selenoprotein expression in $Gpx4^{-/-}$ mice did not result in viable mice, indicating that the combined deficiency aggravated the loss of Gpx4 in liver. By contrast, combined *Trsp*/*Txnrd1*-deficient mice were born, but had significantly shorter lifespans than either single knockout, suggesting that Txnrd1 plays an important role in supporting liver function of mice lacking *Trsp*. In sum our study demonstrates that the ferroptosis regulator Gpx4 is critical for hepatocyte survival and proper liver function, and that vitamin E can compensate for its loss by protecting cells against deleterious lipid peroxidation.

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1. Introduction

Glutathione peroxidase 4 (Gpx4) is unique among a family of eight glutathione peroxidases due to its broad substrate specificity, the fact that it functions as a phospholipid hydroperoxidase protecting cells from lipid peroxidation and serves a structural function in mature sperm cells [1]. Moreover, Gpx4 was recently described to control a novel, iron-dependent form of regulated non-apoptotic cell death, ferroptosis [2], which is characterized by generation of high levels of lipid hydroperoxides [3,4]. Ferroptotic cell death was found not only to be relevant for cell death of malignant cells, but emerged to play an important role in cells and tissue demise in kidney tubule cells [5], viral immunity [6], and motor neuron degeneration [7]. Among the proteins encoded by 25 selenoprotein genes in mammals, Gpx4 enzyme appears to be particularly critical for murine survival in that its knockout in mice is early embryonic lethal [8], and it can only be partially knocked down in cultured cells [9,10]. Furthermore, its targeted removal in neural and skin tissues virtually mimics the knockout of the gene encoding the selenocysteine (Sec)-specific tRNA, *Trsp* (see [11,12],

Keywords: Gpx4, glutathione peroxidase 4; Txnrd1, cytosolic thioredoxin reductase; Sec, selenocysteine; Trsp, Sec tRNA gene; Nrf2, nuclear factor (erythroid-derived)-like 2; Alb-Cre, Albumin-Cre; H&E, hematoxylin and eosin; qPCR, Quantitative PCR; GSH, glutathione; SEM, standard error of the mean; Gpx1, glutathione peroxidase 1; SelR, Selenoprotein R; SepW, Selenoprotein W; Gsta1, glutathione S-transferase alpha 1; Srxn1, sulfiredoxin 1; Cbr3, carbonyl reductase 3; Gclc, glutamate-cysteine ligase; Gsr, glutathione reductase; TBARS, thiobarbituric acid reactive substances

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respectively).

A remarkable interrelationship exists between Gpx4 and vitamin E, which is a lipid soluble antioxidant responsible for protecting membranes from oxidative damage [5,13]. Inducible inactivation of Gpx4 in cells has been shown to result in lipid peroxidation-mediated cell death, which could be prevented by vitamin E supplementation [10]. In addition, this relationship was also demonstrated by disrupting Gpx4 in mouse endothelium, which had no apparent effect on normal vascular homeostasis, unless the mice were restricted from dietary vitamin E [13]. Vitamin E removal from the diets of these animals resulted in endothelial cell death and subsequent thrombus formation in multiple organs and consequently early death of mice.

Knockout of either *Trsp* (*Trsp*$^{-/-}$) [14,15] or *Txnrd1* (*Txnrd1*$^{-/-}$) [16] was found to be embryonic lethal; however, the targeted knockout of either gene in liver was not embryonic lethal [17–19]. The incidence of hepatocarcinogenesis in hepatocyte-specific conditional knockout mice carrying the *Albumin-Cre (Alb-Cre)* transgene (genotype *Alb-Cre*; *Txnrd1*$^{fl/fl}$) was dramatically increased compared to control mice expressing normal levels of Txnrd1 (genotype *Alb-Cre*; *Txnrd1*$^{+/+}$), suggesting that this major redox regulator selenoprotein has a role in protecting hepatocytes from cancer [18].

Since mice carrying hepatocyte-specific knockout of *Trsp* survived for several months and their death appeared to be due to severe hepatocellular degeneration and necrosis [17], while mice carrying hepatocyte-specific knockout of *Txnrd1* exhibited no apparent phenotype [18], we interrogated whether the ferroptosis regulator *Gpx4* could be the critical selenoprotein responsible for the effect observed in the *Trsp* knockout mice. To this end, we generated a series of mice with hepatocyte-specific deletion of *Gpx4* alone or in combination with *Trsp*, and as controls hepatocyte-specific knockout of *Txnrd1* in combination with *Trsp*. Findings obtained by these in vivo studies strongly suggest that Gpx4 is a critical factor for hepatocyte survival which is highly sensitive to vitamin E status.

2. Materials and methods

Please refer to the Supplementary Materials and Methods for more detailed descriptions.

2.1. Materials

A detailed list of materials is given in Supplementary Information.

2.2. Generation of hepatocyte knockout mice, genotyping and diets

All hepatocyte-specific mouse lines were generated using mice carrying the *Alb-Cre* transgene (C57BL/6) [20]. Mouse lines obtained, mouse genotyping and diets used are described in detail in the Supplementary Information. Mice were maintained under standard conditions, with food and water given ad libitum, 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.

2.3. Tissue harvest and histopathological analysis

Mice were euthanized, their livers removed immediately, washed briefly in DPBS, snap frozen in liquid nitrogen and stored at −80 °C until analysis. Skeletal muscle, tongue, heart, brain, lung, kidney, spleen and bone marrow were also taken in the same manner for further analysis. Tissues used for pathological analysis were immediately placed in 10% neutral-buffered formalin and paraffin embedded. Tissue sections were routinely stained with hematoxylin and eosin (H&E) and examined by light microscopy by a board certified veterinary pathologist.

2.4. RNA isolation and qPCR analysis

Total RNA was isolated from tissues or cells using TriPure isolation reagent following the manufacturer's instructions and reverse transcribed using an iScript cDNA synthesis kit. Quantitative PCR (qPCR) was performed in triplicate using iTaq Universal SYBR Green Supermix according to the manufacturer's instructions. Results were normalized to mouse *Gapdh*. Primers sequences used for analyses are shown in Supplementary Table 3.

2.5. 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 inhibitors). Western blot analysis was carried out as described in Supplementary Information.

2.6. Thioredoxin reductase activity and lipid peroxidation assays and quantification of GSH and GSSG

Txnrd1 activity was determined spectrophotometrically based on the method of Holmgren [21] and lipid peroxidation assessed as described in Supplementary Information. Total glutathione (GSH) levels in liver were quantified using a glutathione assay kit according to the manufacturer's instructions (Sigma-Aldrich).

2.7. Statistical analysis

Values in all figures are presented as the standard error of the mean (SEM). Student's *t*-test was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). *P*-values less than or equal to 0.05 ($P \leq 0.05$) are considered significant.

3. Results

Gpx4$^{fl/fl}$ females were crossed with *Alb-Cre; Gpx4*$^{fl/+}$ males and maintained on a standard rodent diet (NIH-31). The resulting pups were initially genotyped at three weeks of age (Table 1). No mice bearing the genotype *Alb-Cre; Gpx4*$^{fl/fl}$ were found, indicating that knockout of *Gpx4* in liver leads to early lethality. To determine if hepatocyte-specific *Gpx4*$^{-/-}$ pups die during embryogenesis, pups from the above matings were genotyped at 15.5 dpc. This analysis detected *Alb-Cre; Gpx4*$^{fl/fl}$ embryos. To characterize them, six *Alb-Cre; Gpx4*$^{fl/+}$ and six *Alb-Cre; Gpx4*$^{fl/fl}$ whole embryos were taken for histo-pathological analysis, but no differences were observed suggesting that the knockouts were dying at a later stage. Genotyping of one day old pups identified mice bearing the genotype *Alb-Cre; Gpx4*$^{fl/fl}$ according to the expected Mendelian ratio (Table 1). Pups were carefully monitored following birth, and all hepatocyte-specific *Gpx4*$^{-/-}$ pups were found to die by 48 h. To examine the cause of death, 1 day old *Alb-Cre; Gpx4*$^{fl/fl}$ knockout and *Gpx4*$^{fl/fl}$ control pups were euthanized and livers taken for analysis. In addition, *Trsp*$^{fl/fl}$ females were crossed with *Alb-Cre; Trsp*$^{fl/+}$ males and *Txnrd1*$^{fl/fl}$ females were crossed with *Alb-Cre; Txnrd1*$^{fl/+}$ males, and one day old pups from these litters were analyzed. H&E staining of livers from one day old *Alb-Cre; Gpx4*$^{fl/fl}$ pups revealed extensive hepatocyte degeneration and necrosis (Fig. 1). Neonatal *Alb-Cre; Gpx4*$^{fl/fl}$ mice had hepatic degeneration characterized by a “fading out” of hepatocytes, by pale staining

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

Age examined	Mean number of pups/litter	Number of mice/genotype			
		Alb-Cre; Gpx4$^{fl/+}$	***Gpx4***$^{fl/+}$	***Gpx4***$^{fl/fl}$	***Alb-Cre; Gpx4***$^{fl/fl}$
3 weeks	4.83	26	17	15	0
1 day	8.33	16	12	10	12

nuclei and cytoplasm and loss of cytoplasmic and nuclear membrane detail similar to autolysis, which are likely the result of membrane damage due to oxidative stress. In addition, globules that may be fragments of hepatocytes were present. Livers from knockout mice also lacked veins and sinusoids (see arrow pointing to vein, Fig. 1). Neither *Trsp*-deficient nor *Txnrd1*-deficient livers displayed hepatocyte degeneration or necrotic cell death (not shown).

mRNA levels of several liver selenoproteins, *glutathione peroxidase 1* (*Gpx1*), *Gpx2*, *Gpx4*, *Txnrd1*, *selenoprotein R* (*SelR*) and *selenoprotein W* (*SepW*) [17,22], were measured by qPCR in livers of one day old hepatocyte-specific *Gpx4*$^{-/-}$, *Txnrd1*$^{-/-}$, *Trsp*$^{-/-}$ mice and controls (Fig. 2A). *Gpx4* and *Txnrd1* were significantly reduced in the corresponding knockout mice as expected, but there also appeared to be low levels of these selenoprotein mRNAs retained in the respective knockout mice likely due at least in part to cell types other than hepatocytes [23]. *Gpx1* and *SepW* mRNA levels were significantly reduced in *Trsp* liver knockout mice which is not surprising since these mRNAs are subject to nonsense mediated decay when not properly translated [24,25]. *Gpx2* was significantly increased in *Txnrd1*-deficient mice, which was also expected since the nuclear factor (erythroid-derived)-like 2 (Nrf2) is known to upregulate oxidative stress-induced proteins like Gpx2 [26], including other proteins in the selenoprotein and glutathione families under certain cellular conditions (e.g., *Txnrd1*-deficiency; see [18,27] and below). *Txnrd1* mRNA was significantly enriched in liver of *Gpx4*$^{-/-}$ mice, which is in line with a previous study, where it was found to be up-regulated in skin epithelial-specific *Gpx4*$^{-/-}$ mice [12].

Expression of the above selenoproteins, with the exception of Gpx2, was examined by western blotting (Fig. 2B). Gpx1, SelR and SepW were all dramatically down-regulated, and Gpx4 partially reduced, in *Trsp*-deficient mice (see also [17]). Txnrd1 and Gpx4 was efficiently reduced in the respective hepatocyte-specific null mice and were found to be reciprocally regulated in the respective knockouts (Fig. 2B). To further examine the loss of Txnrd1 and Gpx4 in *Trsp*-deficient mice, we examined the levels of Sec tRNA and the mRNA of these two proteins in mice at 1, 6, 15 and 21 days of age by qPCR analysis (Supplementary Fig. 1A and B). Sec tRNA levels in *Alb-Cre; Trsp*$^{fl/fl}$ livers were ~20% of the levels found in control livers at 1 day of age and remained at approximately the same level throughout the time period examined (Supplementary Fig. 1A). The mRNA levels of *Txnrd1* and *Gpx4* were largely unchanged between control and *Trsp*$^{-/-}$ livers over this time period, with the exception of a slight decrease in *Txnrd1* levels in *Alb-Cre; Trsp*$^{fl/fl}$ livers at 21 days of age. The levels of Gpx4 and Txnrd1 were also examined by immunoblotting (Supplementary Fig. 1C). As mentioned above, both Gpx4 and Txnrd1 were detected in one day old *Trsp*-deficient mice. At 6 days of age, only a small amount of Gpx4 remained in *Trsp*$^{-/-}$ livers, while Txnrd1 was present in similar levels as control mice. By 15 days, both Gpx4 and Txnrd1 were virtually absent, indicating that Txnrd1 and Gpx4 remain

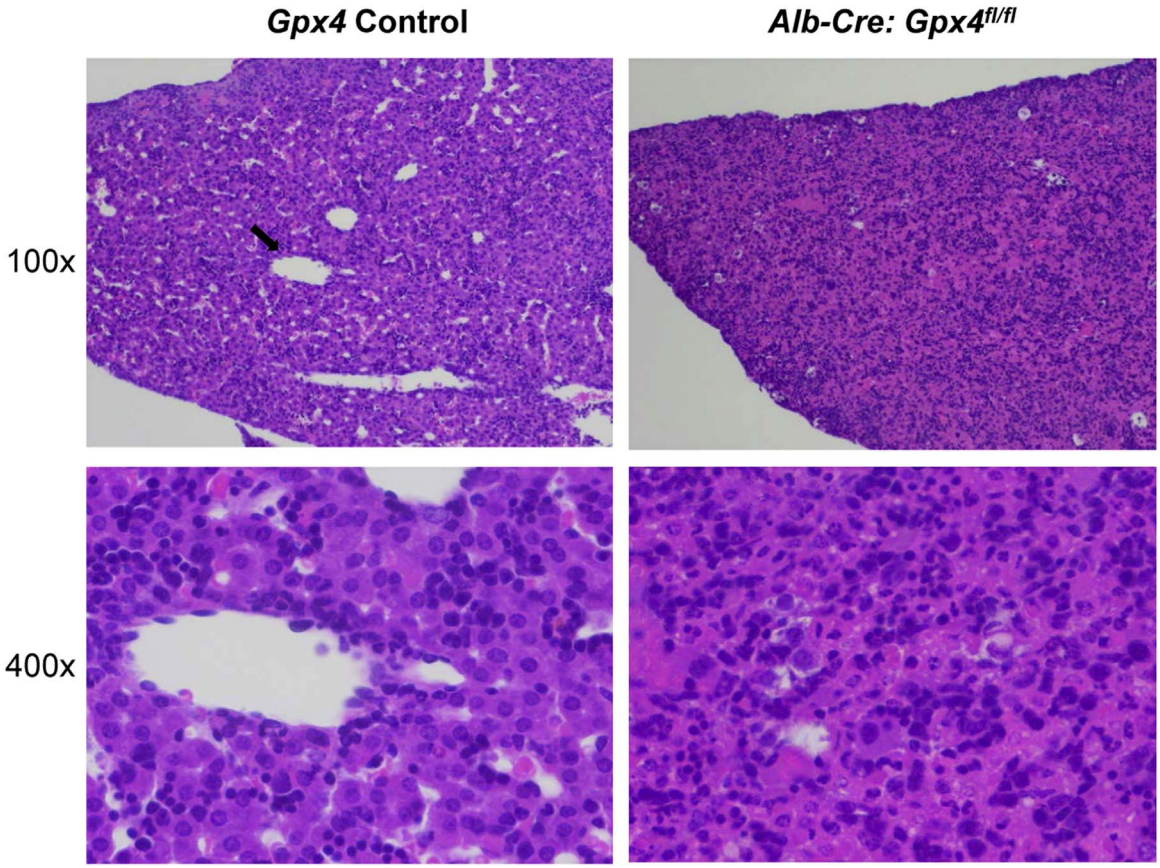

Fig. 1. Liver necrosis in *Alb-cre; Gpx4*$^{fl/fl}$ mice. H&E staining of *Gpx*4 control and *Alb-cre; Gpx4*$^{fl/fl}$ livers at 10 × and 40 × magnification from 1 day old pups revealed extensive hepatocyte degeneration and necrosis, lack of veins (arrow) and sinusoids in livers of *Gpx4*-deficient pups.

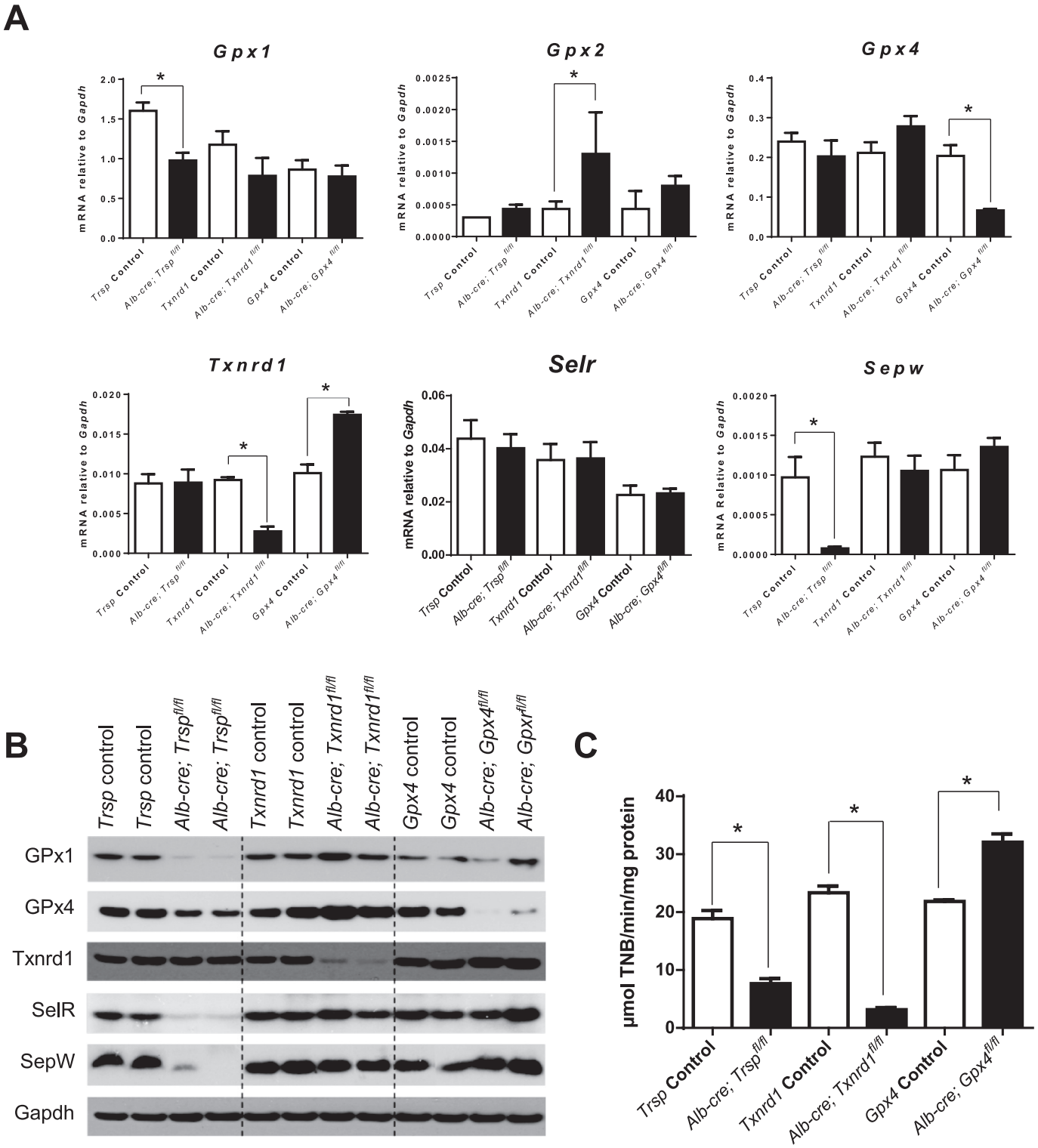

Fig. 2. Expression levels of selenoproteins in livers of 1 day old control, *Trsp*-, *Txnrd1*- and *Gpx4*-deficient mice. (A) mRNA levels of *Gpx1, Gpx2, Gpx4, Txnrd1, Selr, and Sepw* were analyzed by qPCR. Data are shown as relative mRNA levels normalized to *Gapdh* in all liver samples ($n=3$ for each genotype). *Denotes statistical difference ($P<0.05$). (B) Protein levels of Gpx1, Gpx4, Txnrd1, SelR and SepW were analyzed by immunoblotting in both control and knockout liver samples ($n=2$ each). Gapdh levels are used as a control for protein loading. (C) Txnrd1 activity in livers of control, *Trsp*-, *Txnrd1*- and *Gpx4*-deficient mice. Data are the mean ± SEM for three independent experiments and are expressed as µmol TNB/min/mg protein. *Denotes statistical difference ($P<0.05$).

present in *Trsp*-deficient livers of neonates longer than other selenoproteins examined. In addition, mRNA levels of the Nrf2-regulated enzymes, *glutathione S-transferase alpha 1* (*Gsta1*), *sulfiredoxin 1* (*Srxn1*) and *carbonyl reductase 3* (*Cbr3*), were measured by qPCR in mice at 1, 6, 15 and 21 days of age (Supplementary Fig. 2). mRNA levels of these genes were unaltered in *Trsp*-deficient mice at one and six days of age. However, at 15 and 21 days of age, the mRNA levels of all three genes increased dramatically, coinciding with the loss of both Txnrd1 and Gpx4. Txnrd1 activity was measured and its activity was significantly reduced in *Txnrd1*

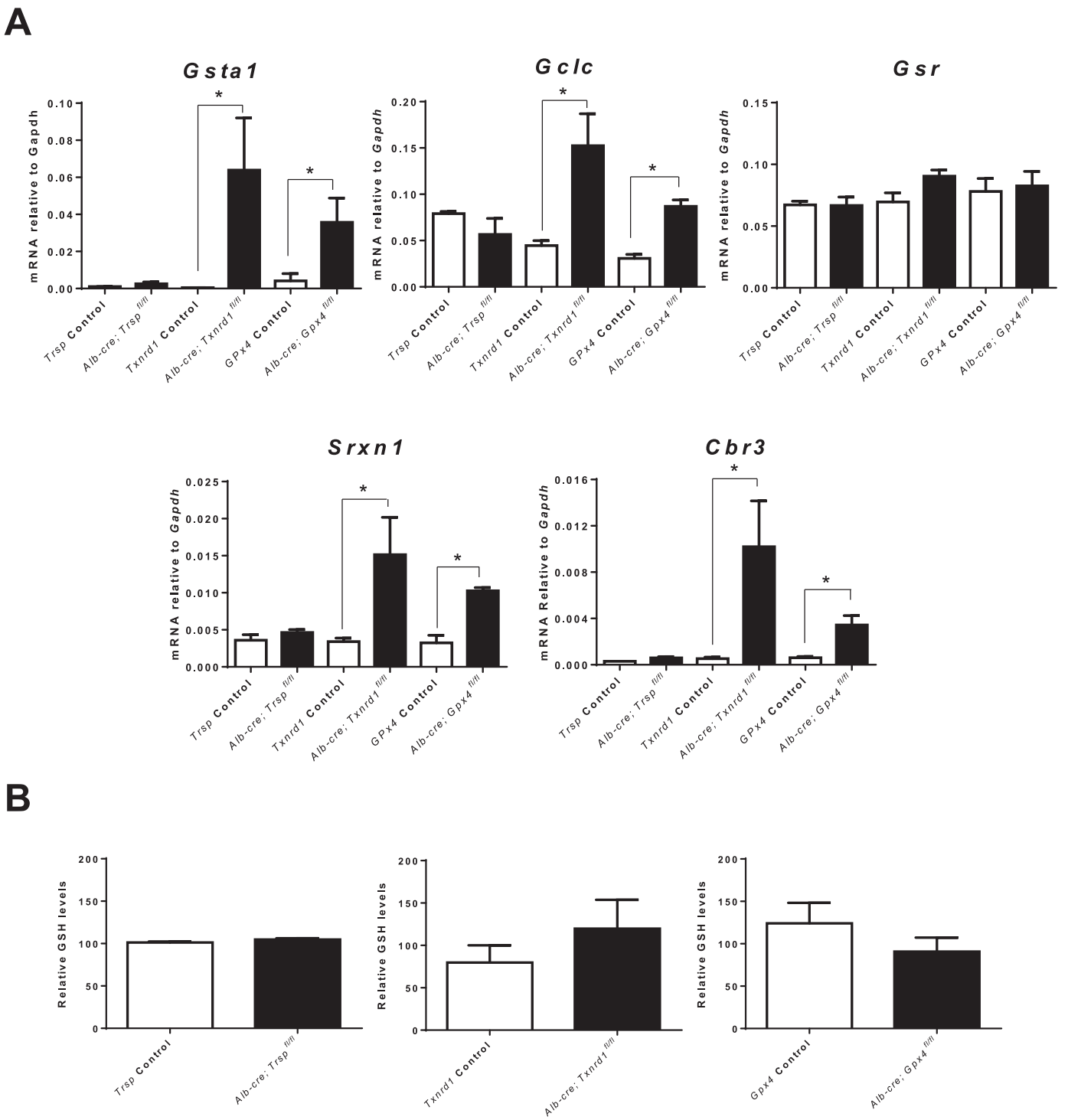

Fig. 3. mRNA expression levels of Nrf2-regulated genes in livers of 1 day old control, *Trsp*-, *Txnrd1*- and *Gpx4*-deficient mice. (A) mRNA levels of *Gsta1, Gclc, Gsr, Srxn1 and Cbr3* were analyzed by qPCR. Data are shown as relative mRNA levels normalized to *Gapdh* in all liver samples ($n=3$ for each genotype). *Denotes statistical difference ($P<0.05$). (B) Total GSH was measured spectrophotometrically and the levels calculated relative to the levels found in control mice. Total GSH concentrations are the mean ± S. D. for three independent experiments.

and *Trsp* liver knockout mice compared to the corresponding controls (Fig. 2C). Txnrd1 activity was significantly increased in *Gpx4*$^{-/-}$ liver as expected, reflecting the increase of *Txnrd1* mRNA and protein levels (Fig. 2A and B, respectively).

Comparison of the Txnrd1 levels of *Trsp* in control and knockout liver, which are virtually the same amounts as examined by western blotting, (Fig. 2B) to Txnrd1 activity (Fig. 2C) suggests that the higher amounts of Txnrd1 likely are due to truncation and/or misreading or misinsertion of cysteine in place of Sec [28].

As mentioned above, two selenoproteins, Txnrd1 and Gpx2 are known Nrf2 targets [26]. Since Gpx4 appears to play an important role within the GSH system, the mRNA levels of Nrf2-regulated enzymes involved in the GSH system, *Gsta1, glutamate-cysteine ligase (Gclc), glutathione reductase (Gsr)*, as well as two other Nrf2-regulated enzymes involved in oxidative stress resistance, *Srxn1* and *Cbr3*, were measured by qPCR. Similar to *Txnrd1*-deficient

Table 2
Gpx4 knockout lethality is rescued by vitamin E.

Age examined	Mean number of pups/litter	Number of mice/genotype			
		Alb-Cre; Gpx4$^{fl/+}$	*Gpx4$^{fl/+}$*	*Gpx4$^{fl/fl}$*	*Alb-Cre; Gpx4$^{fl/fl}$*
3 weeks	5.73	20	13	12	18

Parents: *Gpx4$^{fl/fl}$* females × *Alb-Cre; Gpx4$^{fl/+}$* males.

livers, the mRNA levels of several enzymes induced by Nrf2 were upregulated in *Gpx4*-deficient livers, although to a lesser extent (Fig. 3A). Interestingly, no statistical differences were observed in *Trsp*-deficient livers compared to the respective controls. Total GSH levels were measured and remained statistically unchanged in all mice, but showed a slight increase in *Txnrd1*-deficient and decrease in *Gpx4*-deficient livers (Fig. 3B).

Gpx4$^{fl/fl}$ females were mated with *Alb-Cre; Gpx4$^{fl/+}$* males, which were maintained on diets with supplemented vitamin E (500 IU/kg, DL-α-tocopheryl acetate) through the gestation period, and the mothers were kept on these diets until the newborns were weaned and genotyped at 3 weeks of age. As shown in Table 2, *Alb-Cre; Gpx4$^{fl/fl}$* mice were born near the expected Mendelian ratios when given vitamin E-enriched diets, indicating that vitamin E compensated for *Gpx4*-deficiency in liver.

Gpx4$^{fl/fl}$ females were mated with *Alb-Cre; Gpx4$^{fl/+}$* males, maintained on diets with supplemented vitamin E (as above) through the gestation period and the mothers were kept on these diets until the newborns were weaned (Fig. 4A). The male and female offspring were kept on vitamin E-enriched diets for an additional three weeks, and then placed on diets lacking this vitamin and their survival monitored. None of the *Gpx4* control mice (*Alb-Cre; Gpx4$^{fl/+}$*, *Gpx4$^{fl/+}$* and *Gpx4$^{fl/fl}$*) died for the 78 days they were monitored on the vitamin E-deficient diet, while the *Alb-Cre; Gpx4$^{fl/fl}$* mice began dying at day 8 following vitamin E removal and continued to die over the next 43 days (Fig. 4B). *Alb-Cre; Gpx4$^{fl/fl}$* mice had an overall survival time of 69.5 days, or 27.5 days following vitamin E removal.

Livers and serum taken from control (*Gpx4$^{fl/fl}$*) and knockout mice following removal of vitamin E were examined for pathological changes (Fig. 5). The cause of morbidity in the moribund/dead mice was found to be extensive hepatocellular necrosis (Fig. 5A). Necrotic hepatocytes were centered on portal triads. Affected cells had swollen glassy cytoplasm and faded, pale-staining nuclei. Necrotic foci manifested variable hemorrhage and acute inflammation. Skeletal muscle, tongue, heart, brain, lung, kidney, spleen and bone marrow were also taken for further analysis. Degeneration of heart, tongue and skeletal muscle which is associated with severe vitamin E-deficiency in domestic species was not present. In order to estimate potential lipid peroxidation, we used the thiobarbituric acid reactive substances (TBARS) assay [29]. In mice maintained on a vitamin E-supplemented diet, TBARS plasma levels of *Alb-cre; Gpx4$^{fl/fl}$* were not higher than those of control mice (Fig. 5B). However, upon removal of vitamin E from the diet at six weeks of age and replacement with a vitamin E-deficient diet for 3 weeks, plasma levels of TBARS were elevated above controls ($P < 0.05$), suggesting an increase in lipid peroxidation.

To examine the effects of a combined *Trsp*-and *Gpx4*-deficiency in mouse liver, female *Alb-Cre: Trsp$^{fl/+}$; Gpx4$^{fl/+}$* mice were crossed with *Alb-Cre: Trsp$^{fl/+}$; Gpx4$^{fl/+}$* male mice (Supplementary Fig. 2). The resulting pups were genotyped at one day of age

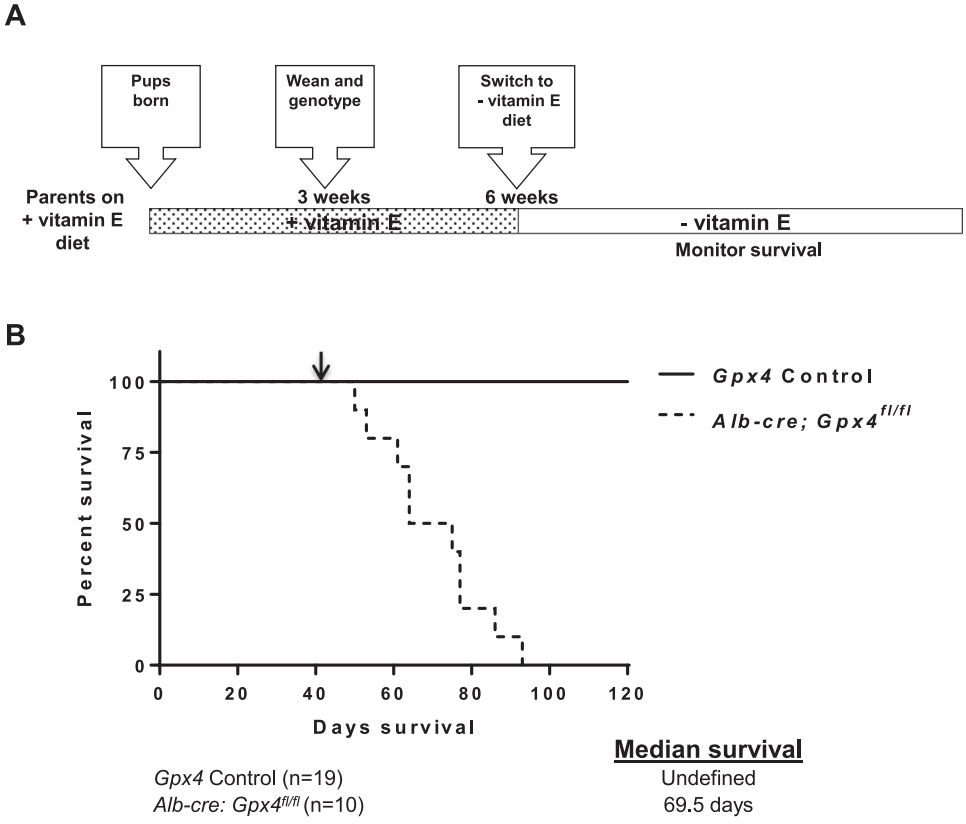

Fig. 4. Survival rate of *Gpx4* control and *Alb-cre; Gpx4$^{fl/fl}$* mice following removal of vitamin E from the diet. (A) Schematic representation of experimental design and feeding plan of mice maintained on vitamin E-deficient (−vitamin E) or vitamin E-supplemented (+vitamin E) diets. (B) Survival curve of control ($n=12$) and *Alb-cre; Gpx4$^{fl/fl}$* ($n=10$) mice on defined diets. Arrow notes the time when mice were placed on a vitamin E-deficient diet. A log-rank test was used for statistical analysis. *Denotes statistical difference ($P < 0.0001$).

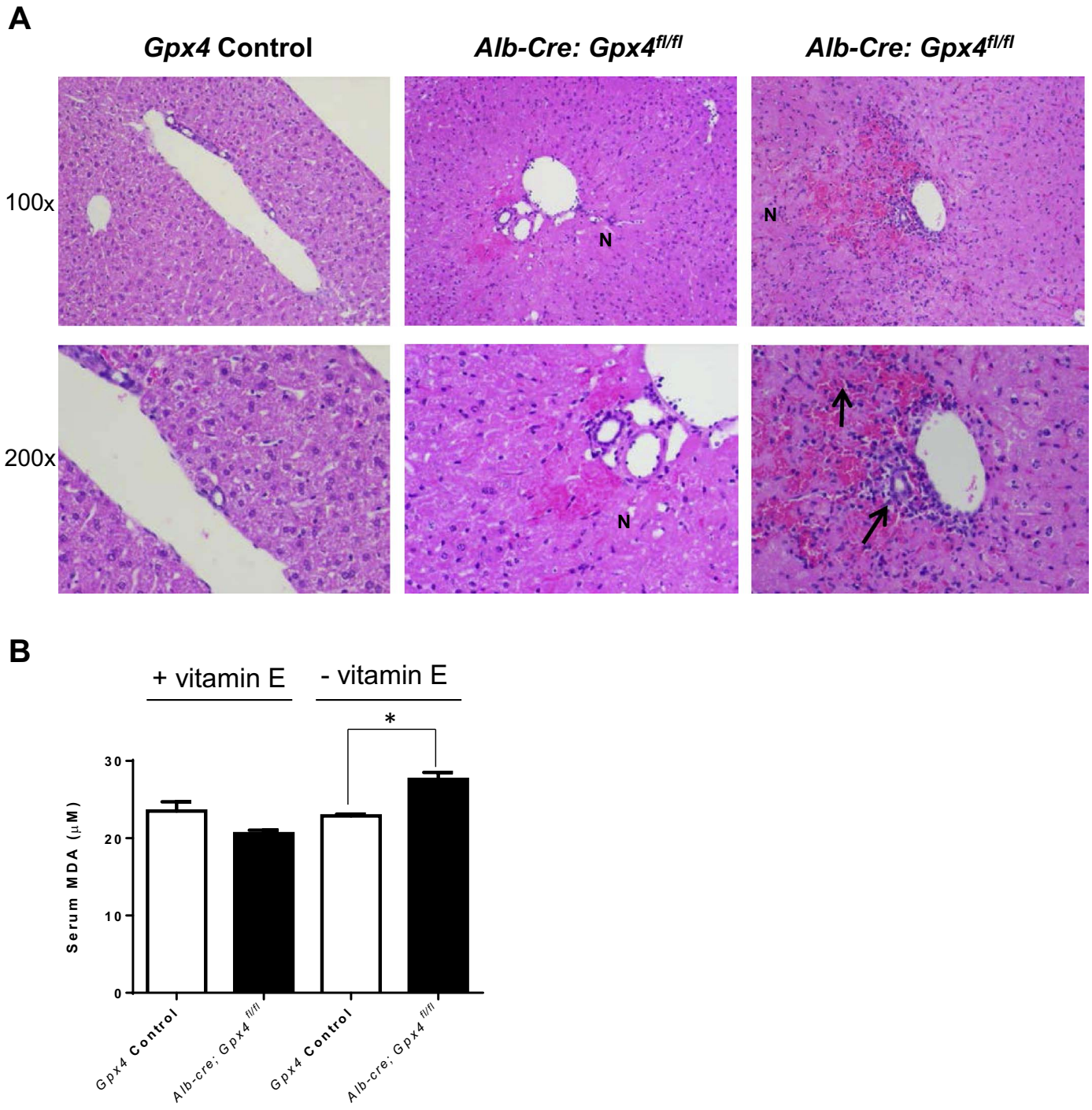

Fig. 5. Liver necrosis in *Alb-cre; Gpx4$^{fl/fl}$* mice following removal of vitamin E. (A) H&E staining of *Gpx4* control and two *Alb-cre; Gpx4$^{fl/fl}$* livers at 10× and 20× magnification from mice that have been maintained on a vitamin E-sufficient diet for 6 weeks, followed by removal of vitamin E for 3 weeks. N=areas of necrosis. Arrows point to areas of acute inflammation. (B) TBARS assay was used to assess plasma levels of malondialdehyde (MDA) in *Gpx4* control ($n=3$) and *Alb-cre; Gpx4$^{fl/fl}$* (n=3) mice maintained on a vitamin E-supplemented diet (vitamin E) for nine weeks and in *Gpx4* control ($n=3$) and *Alb-cre; Gpx4$^{fl/fl}$* ($n=3$) mice maintained on a vitamin E-supplemented diet for six weeks followed by removal of vitamin E (- vitamin E) for 3 weeks. *Denotes statistical difference ($P<0.05$).

(Supplementary Table 1). No pups with the genotype *Alb-cre: Trsp$^{fl/fl}$; Gpx4$^{fl/fl}$* were observed, indicating that the combined *Trsp* and *Gpx4* knockout in liver is embryonic lethal and, therefore, more severe than *Gpx4* knockout alone. Examination of lethality of these mice in embryogenesis was not further pursued.

Combined *Trsp*- and *Txnrd1*-deficient mice were generated by crossing female *Alb-Cre: Trsp$^{fl/+}$; Txnrd1$^{fl/+}$* with male *Alb-Cre: Trsp$^{fl/+}$; Txnrd1$^{fl/+}$* mice (Supplementary Fig. 3). As shown in Supplementary Table 2, these crosses generated offspring with the expected Mendelian ratios. Control (*Alb-cre: Trsp$^{fl/+}$; Txnrd1$^{fl/+}$*), *Alb-cre: Trsp$^{fl/fl}$; Txnrd1$^{fl/fl}$*, *Alb-cre: Trsp$^{fl/fl}$; Txnrd1$^{fl/+}$*, and *Alb-cre: Trsp$^{fl/fl}$; Txnrd1$^{+/+}$* male and female mice produced from these matings were maintained on standard chow diets and survival monitored for 140 days (Fig. 6). Median survival was 14.5 days for *Alb-cre: Trsp$^{fl/fl}$; Txnrd1$^{fl/fl}$* mice, 17 days for *Alb-cre: Trsp$^{fl/fl}$; Txnrd1$^{fl/+}$* mice and 61 days for *Alb-cre: Trsp$^{fl/fl}$; Txnrd1$^{+/+}$* mice. No control mice died during this period. Mice lacking only *Trsp* had a significantly shorter life span than control mice ($P<0.0001$).

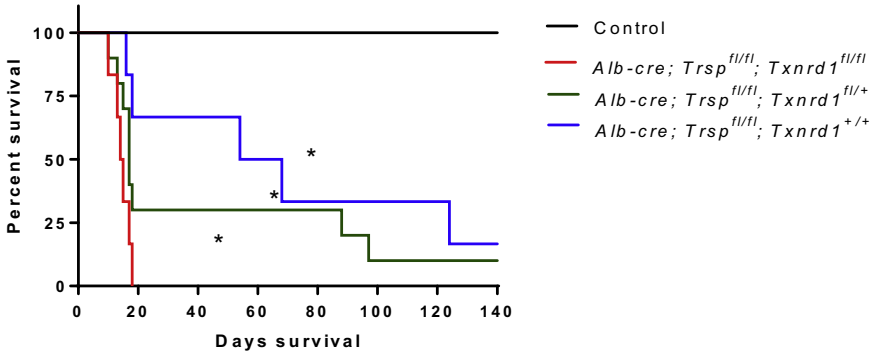

Fig. 6. Survival curve of combined *Trsp*- and *Txnrd1*-deficient mice. Survival curve of control ($n=10$), *Alb-cre: Trsp*$^{fl/fl}$; *Txnrd1*$^{fl/fl}$ (n=6), *Alb-cre: Trsp*$^{fl/fl}$; *Txnrd1*$^{fl/+}$ (n=6) and *Alb-cre: Trsp*$^{fl/fl}$; *Txnrd1*$^{+/+}$ mice ($n=10$) maintained on standard chow. A log-rank test was used for all statistical analyses. *Denotes statistical difference ($P<0.0001$) from control.

Mice lacking both *Trsp* and *Txnrd1* had a significantly shorter life span than control mice ($P<0.0001$) and mice only lacking *Trsp* ($P=0.0061$). Mice lacking *Trsp* and heterozygous for *Txnrd1* had a significantly shorter life span than control mice ($P<0.0001$), but did not have a significantly different life span than mice lacking only *Trsp* or mice lacking both *Trsp* and *Txnrd1*.

4. Discussion

Gpx4 is one of the most critical selenoproteins for murine survival and its knockout causes early embryonic lethality at E7.5 [30]. However, we found herein that tissue-specific loss of Gpx4 did not seem to impact hepatocyte development since the embryos at E15.5 appeared normal upon pathological examination. Neonatal *Alb-Cre; Gpx4*$^{fl/fl}$ mice died within 24–48 h after birth from severe liver degeneration. The hepatic degeneration also exhibited a "fading out" of hepatocytes and globules were present which appeared to be hepatocyte fragments, alterations that might be caused by oxidative membrane damage. In contrast, mice carrying knockouts in hepatocytes of either *Trsp* or *Txnrd1* (wherein their loss in early development, like *Gpx4*, is embryonic lethal [14,16]) did not appear to affect hepatocyte development or to provoke liver degeneration in neonates (Fig. 1). As evidenced by the fact that *Alb-Cre; Txnrd1*$^{fl/fl}$ mice survived, unless they were challenged [18], and *Alb-Cre; Trsp*$^{fl/fl}$ mice survived for several months without any apparent phenotype [17], provided further evidence that Gpx4 is apparently more critical to proper tissue function and survival than any other essential selenoprotein (e.g., see [11,12,18]). Interestingly, hepatocyte-specific *Trsp*$^{-/-}$ mice die about 3–4 months after birth from "severe liver degeneration and necrosis" [17], a time point when Gpx4 levels are likely reduced to a critically low amount resulting in this phenotype and death. It is interesting that the phenotype of hepatocyte-specific loss of Gpx4 is more severe than that of *Trsp*. It is possible, however, that a very low level of Gpx4 protein with a replacement of Sec to Cys under limiting selenium (or Sec) availability, as reported for Txnrd1 [31], may lead to a protein with residual Gpx4 activity able to partially compensate for the lack of *Trsp* in hepatocytes at least to some extent. Indeed, tetracycline-inducible, ectopic expression of a mutant Gpx4 with a Sec-Cys conversion (even in minute amounts) was able to rescue cell death in Tamoxifen-inducible *Gpx4*$^{-/-}$ fibroblasts [32]. We actually addressed a putative Cys insertion into Gpx4 in the absence of *Trsp* using immunoprecipitated Gpx4 followed by mass spectrometry in liver tissue from hepatocyte-specific Gpx4 knockout neonates multiple times (not shown). However, we failed to detect such a tryptic Gpx4 fragment as liver tissue from neonates might not be the ideal tissue to perform such a study because liver is the site of fetal hematopoiesis and thus has a substantial amount of hematopoietic cells still present at birth. Therefore, tissue heterogeneity may preclude such a challenging study.

If *Gpx4* is such an important gene in development, why do not the *AlbCre*; *Gpx4*$^{fl/fl}$ hepatocyte mice die before birth and in early embryogenesis? The reasons most certainly lie in large part in the relative activity of the *Alb-Cre* transgene and in the percent hepatocytes at different stages of embryonic liver development [33], the intracellular half-life of Gpx4, and the point at which the Gpx4 levels decline to critically low amounts that are severely detrimental to health. Weisend et al. [33] have carefully followed the transition of mostly hematopoietic to mostly hepatocytic cells in developing fetal and neonatal liver, along with the expression of the Cre recombinase. The Cre recombinase is expressed as early as E10.5 [34,35] and hepatocytes become more plentiful between E14.5 and P3 [33]. It is tempting to speculate from these observations that a critically low level of Gpx4 in *Alb-Cre*; *Gpx4*$^{fl/fl}$ pups was achieved within the first 24–48 h post-birth. Regarding the half-life of Gpx4 and the life-threatening levels of this selenoenzyme to survival, little is known, to our knowledge, about either, but these indeed are factors that require consideration. In addition, as placental vitamin E provision by the mother is greatly facilitated by the α-tocopherol transfer protein playing an essential role in the selective transfer of α-tocopherol across the placenta [36], one may hypothesize that knockout embryos additionally profit from a vitamin E-enriched environment.

The double knockdown of *Gpx4* and *Trsp* resulted in embryonic lethality demonstrating that loss of both genes confounded the loss of either single gene. Similarly, the double knockdown *Alb-Cre: Trsp*$^{fl/fl}$; *Txnrd1*$^{fl/fl}$ mice died earlier than mice with a loss of either individual gene. Interestingly, approximately 60% of *Alb-Cre: Trsp*$^{fl/fl}$; *Txnrd1*$^{fl/+}$ mice died in virtually the same period as *Alb-Cre: Trsp*$^{fl/fl}$; *Txnrd1*$^{fl/fl}$ mice, wherein their median survival was 17 days compared to 14.5 days in the former mice, while the median survival for *Alb-Cre; Trsp*$^{fl/fl}$ mice was 61 days. Clearly, the expression of both *Txnrd1* alleles in liver is critical for longer longevity of *Trsp*-deficient mice, whereas expression of both *Gpx4* alleles in this organ did not appear to be essential at this early age, as *Alb-Cre: Trsp*$^{fl/fl}$; *Gpx4*$^{fl/+}$ mice survived beyond weaning age.

The observation that the double knockout mice had greater impact on the animal's survival than the loss of any one of the three genes in liver raises a question as to why this is true when theoretically the removal of *Trsp* alone should prevent expression of all selenoproteins. Undoubtedly, there are residual amounts of all three components detectable at 6 days which are absent by 15 days. Since the liver *Trsp* knockout mice survive for several months, and apparently without selenoprotein expression, there appears to be a compensatory mechanism in total selenoprotein knockout mice that is absent in the *Gpx4* knockout alone. *Trsp*

knockout in skin resulted in hyperplastic epidermal development, abnormal hair follicle morphogenesis, progressive alopecia, a runt phenotype and premature death in the progeny [12]. *Gpx4* knockout alone in skin mimicked many of these same abnormalities, but the affected offspring survived the critical stage in early development and went on to live normal lives [12]. We speculate that the residual amounts of Sec tRNA$^{[Ser]Sec}$, Txnrd1 and Gpx4 observed in *Trsp* knockout livers are sufficient to carry the mice through a critical stage in early neonate liver development until the mice are less dependent on selenoproteins. Additionally, vitamin E might be sufficient at an advanced age to compensate for Gpx4 loss in hepatocyte-specific *Trsp* knockout mice once these mice have established their own liver metabolism. Data obtained here further stress the point that variable amounts of vitamin E found in commercially available diets may help to explain the differences in longevity seen in *Trsp* knockout mice [17,19].

Although Gpx4 is not under the regulation of Nrf2 [27], the knockout of *Gpx4* in liver increases the expression of Nrf2-induced enzymes such as Txnrd1 and Gpx2 and enzymes in the GSH system. mRNAs encoding enzymes in the GSH system, *Gsta1* and *Gclc*, and other enzymes involved in redox reactions, e.g., *Srxn1* and *Cbr3*, are not induced as much in *Gpx4*-deficient hepatocytes as in *Txnrd1*-deficient hepatocytes. However, since the expression of these enzymes are known to be under Nrf2 control, this transcription regulatory factor likely responds to lipid peroxidation by-products able to alkylate Keap-1 and thus stabilize Nrf2 [26].

As reported in earlier studies [6,13], vitamin E can compensate for Gpx4 loss in some tissues in vivo (i.e. endothelium and T cell function) and, as further observed herein, *Gpx4* hepatocyte knockout mice appear to survive normally. However, if vitamin E is removed from the diet of Gpx4-deficient animals after 6 weeks, they begin dying eight days later and continue to expire for the next 43 days with a medium survival of 27.5 days. Pathological analysis of the livers revealed that the mice die from severe liver degeneration. Yet, it remains to be shown at a molecular level, how vitamin E (and/or its metabolic products) protects hepatocytes in vivo when Gpx4 expression is abrogated. It is conceivable that vitamin E, as a lipid peroxidation chain breaking antioxidant, protects from detrimental lipid peroxidation in Gpx4 null hepatocytes. Alternatively, it is also possible that vitamin E may inhibit lipoxygenase activity, thus preventing uncontrolled oxidation of polyunsaturated fatty acids (PUFAs) in membranes [37]. In this respect, we previously provided evidence that cell death induced by Gpx4 disruption in murine fibroblasts could be rescued by inhibitors targeting 12/15-lipoxygenase (*Alox15*) but not other lipoxygenase isoforms [10]. Along the same line, *Alox15*$^{-/-}$ fibroblasts were more resistant to GSH depletion than wildtype cells, indicating that 12/15-lipoxygenase is indeed involved in cell death downstream of GSH depletion and Gpx4 inactivation. However, we have recently shown that cross-breeding *Alox15*$^{-/-}$ mice with tamoxifen-inducible whole body Gpx4 null mice does not rescue acute renal failure and early death of *Gpx4*$^{-/-}$ mice, and that cells isolated from *Alox15*/*Gpx4* double knockout mice die like *Gpx4*$^{-/-}$ cells [5]. Similarly, Borchert's group has recently demonstrated that cross-breeding mice expressing inactive Gpx4 die as early as systemic *Gpx4*$^{-/-}$ mice (E7.5) and that this early embryonic lethality cannot be rescued by Alox15-deficiency [38]. From these studies, one can conclude that there is either redundancy between different lipoxygenases in modulating cellular lipids or that there are mechanisms other than lipoxygenase activity contributing to lipid peroxidation and related cell death.

Our present study has provided conclusive, molecular evidence about the interrelationship between selenium - in form of Gpx4 - and vitamin E in the prevention of hepatocellular degeneration, a relationship that was postulated almost 50 years ago by Schwarz and Foltz [39]. In these early studies, it was determined that liver necrosis could be induced only when both vitamin E and "Factor 3", identified to be selenium, are concomitantly omitted from the diet. Our study, thus, has at least two important implications that might be considered in future studies, particularly when addressing pathological conditions that ultimately impinge on hepatocyte survival: (1) dietary vitamin E content should be carefully controlled in toxicological (e.g. drug-induced, alcohol) or pathological (e.g. ischemia/reperfusion) settings which will directly impinge on the glutathione/Gpx4 axis as this may lead to entirely different study outcomes in different laboratories; and (2) inhibition of ferroptotic cell death (e.g. through vitamin E and ferroptosis inhibitors) may harbor substantial therapeutic potential to be applied in clinical settings.

5. Conclusion

Data presented here show that Gpx4 and vitamin E are two important factors for hepatocyte and thus mouse survival. As many deleterious conditions are known to directly impair the glutathione/Gpx4 system, our data imply that ferroptosis inhibition may represent a viable therapeutic approach to ameliorate hepatocyte cell death. Our findings further imply that the vitamin E status in the mouse diet may substantially influence the outcome of preclinical models of liver disease, potentially leading to conflicting results among different laboratories.

Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2016.05.003.

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