

## Thioredoxin reductase 1 protects against chemically induced hepatocarcinogenesis via control of cellular redox homeostasis

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**Thioredoxin reductase 1 (TR1) controls the redox state of protein thiols in mammalian cells and has been shown to have roles in both preventing and promoting cancer. To define the role of this selenoenzyme in hepatocellular carcinoma development, we examined tumor incidence in the liver of mice with tissue-specific knockout of mouse TR1 subjected to the liver carcinogen, diethylnitrosamine (DEN). TR1-deficient livers manifested ~90% tumor incidence compared with ~16% in control livers. The TR1-dependent effect was observed independent of sex, and, in control mice, tumorigenesis did not affect the expression of TR1. On the other hand, we observed upregulation of another selenoenzyme, glutathione peroxidase 2 (GPx2), and components of the glutathione (GSH) system, including those that generate reduced GSH. Overall, this study shows that TR1 protects against chemically induced hepatocarcinogenesis via the control of the cellular redox state, whereas its role in promoting this type of cancer is minimal.**

### Introduction

Cytosolic thioredoxin reductase 1 (TR1, Txnrd1) is one of the major redox regulators in mammalian cells (1,2), whose primary function in normal cells is to keep thioredoxin (Trx) in the reduced state (3,4). It is an essential selenium-containing protein (selenoprotein; 5,6) with roles in DNA repair, angiogenesis, proliferation and transcription (7–10). By reducing Trx, TR1 serves as a redox regulator of cell signaling and contributes to the antioxidant defense of cells (1,2,7–10). Since one of the major characteristics of cancer cells is that they suffer from oxidative stress (e.g. see refs 11–13 and references therein) and TR1 acts in protecting normal cells from oxidative burden, TR1 has been proposed to have a role in cancer prevention (13–18). Several lines of evidence support this idea. For example, TR1 participates in activating the p53 tumor suppressor and promoting other tumor suppressor activities (14) and its downregulation by specific carcinogenic electrophilic compounds (15) results in altering some of the cell's malignancy phenotypes.

However, other studies suggested that TR1 promotes cancer development. For example, it is overexpressed in many cancer cell lines and cancers [(13) and see (17,18) for reviews]. TR1 has been targeted by a number of potent inhibitors and anticancer drugs that reduce its activity, which in turn reverses cell morphology and other cancer-like

**Abbreviations:** AOX1, aldehyde oxygenase 1; CAT, catalase; CBR3, carbonyl reductase 3; DEN, diethylnitrosamine; GCLC, glutamate–cysteine ligase; GPx1, glutathione peroxidase 1; GPx2, glutathione peroxidase 2; GPx4, glutathione peroxidase 4; GSR, glutathione reductase; GST- $\alpha$ 1, glutathione-S-transferase-alpha 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; GSSG, glutathione disulfide; mRNA, messenger RNA; MT1, metallothionein 1; PRDX1, peroxiredoxin 1; PB, phenobarbital; SRXN1, sulfiredoxin 1; SOD1, superoxide dismutase 1; TRX1, thioredoxin 1; TR1, thioredoxin reductase 1; Trx, Thioredoxin.

characteristics. These observations have led to the proposal that TR1 is a prime target for cancer therapy [(13) and see (17,18) for reviews].

Several studies suggested a link between the Trx and glutathione (GSH) systems with special emphasis on the interrelationships between these two redox regulatory systems in tumors and cancer cells that depend on these antioxidant systems due to the fact that malignant cells suffer from oxidative stress (11,22). The downregulation of one of these systems in cancer cells often results in an upregulation of the other system. In addition, the simultaneous inhibition of the Trx and GSH systems is being examined as an avenue for cancer therapy by promoting oxidative stress [(11,19,20) and references therein].

The above *in vitro* and *in vivo* studies suggest two opposing ways on how TR1-deficient hepatocytes may respond to a liver carcinogen. That is, TR1 has a major role in maintaining redox homeostasis and, for example, decreasing the rate of mutations that lead to cancer; but tumor growth also depends on this redox control, especially due to general vulnerabilities of tumors to oxidative stress (11,19–22). A TR1-deficient system may, therefore, lead to either increased resistance (23) or greater susceptibility to tumor growth [see references in (17,18)]. To shed light on which of these two TR1 roles prevail in chemically induced hepatocarcinogenesis, we examined whether mice lacking TR1 in liver were more or less susceptible to tumor development than control mice. Herein, TR1-deficient mice were found to develop a very high tumor incidence, even though the loss of this selenoenzyme was accompanied by an increase in another selenoenzyme, glutathione peroxidase 2 (GPx2), and elevated levels of components of the GSH system.

### Materials and methods

#### Mouse strains

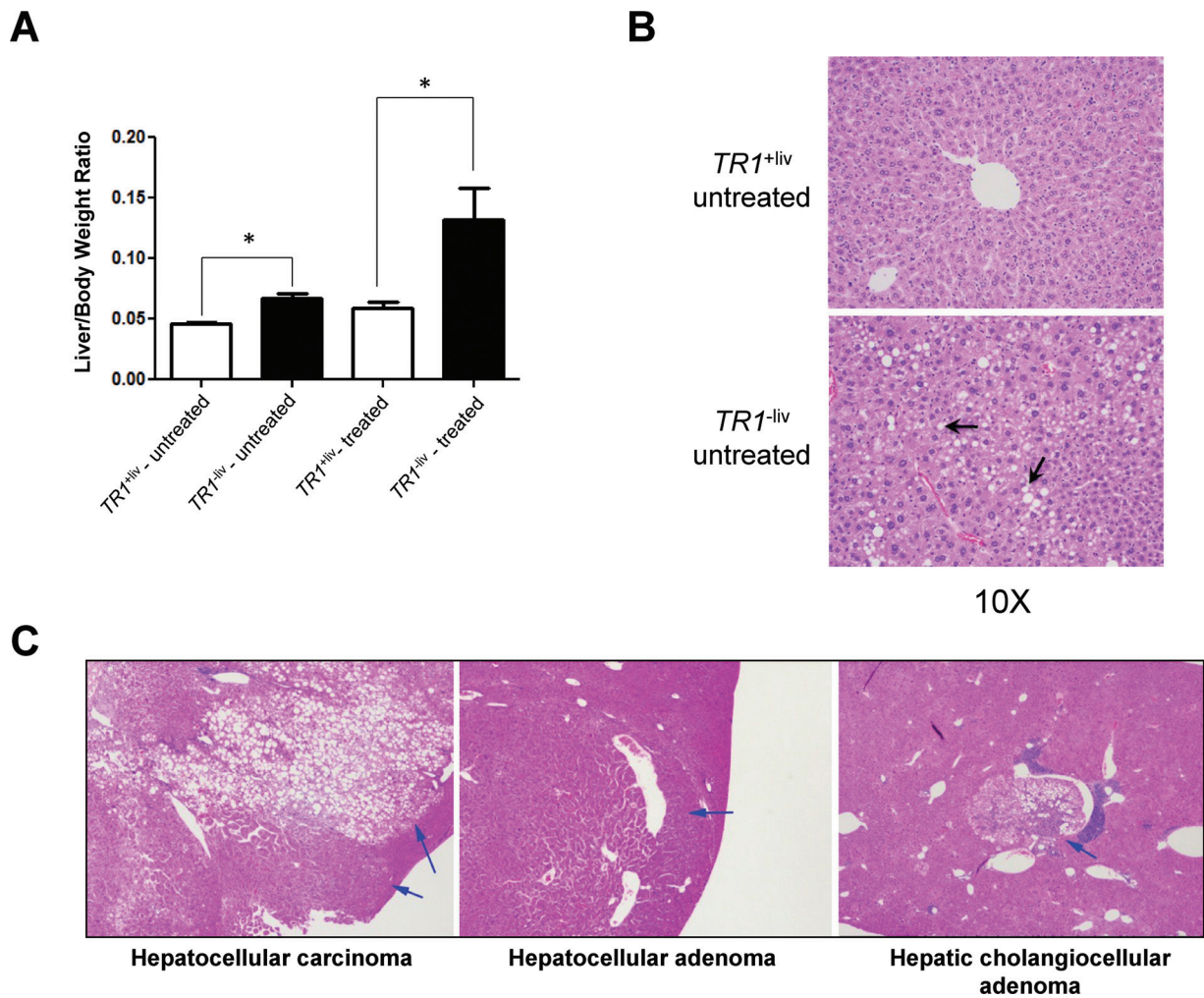
The TR1 conditional knockout mice in a C57BL/6 background (5) and the Alb-cre transgenic mice also in a C57BL/6 background (24) were crossed and the resulting heterozygous floxed male and female mice were then crossed to obtain the desired experimental genotypes, which are shown in Supplementary Figure 1, available at *Carcinogenesis* Online. All mice used in this study were therefore C57BL/6. Genotyping was performed as described (5,24). Homozygous floxed mice carrying the Alb-cre transgene were designated as *TR1*<sup>-liv</sup> and mice wild type for TR1 and homozygous for the Alb-cre transgene were designated as *TR1*<sup>+liv</sup>. Both males and females were used in all experiments. Mice were handled and humanely killed in accordance with the National Institutes of Health Institutional Guidelines under the expert direction of Dr John Dennis (NCI, NIH, Bethesda, MD, USA) and all mouse experiments were approved by the Animal Ethics Committee at the National Institutes of Health.

#### Experimental design

Four-week-old *TR1*<sup>+liv</sup> ( $n = 19$ ; 10 males, 9 females) and *TR1*<sup>-liv</sup> ( $n = 18$ ; 8 males, 10 females) mice were injected intraperitoneally with 5 mg/kg diethylnitrosamine (DEN; Sigma, St. Louis, MO) in phosphate-buffered saline weekly for 2 weeks (i.e. a total of two DEN injections) to initiate tumors and 2 weeks following DEN injections, the mice received 0.01% phenobarbital (PB; Sigma) in the drinking water to promote tumor formation until their sacrifice at 13 months of age (i.e. animals were killed 12 months after the initial DEN injection). Control *TR1*<sup>+liv</sup> ( $n=4$ ; 1 male, 3 females) and *TR1*<sup>-liv</sup> ( $n = 6$ ; 3 males, 3 females) mice did not receive DEN/PB treatment and were killed at 13 months of age.

#### Liver harvest and tumor analysis

Mice were killed, weighed and their livers removed immediately and weighed. The presence of liver tumors was evaluated and recorded. When tumors were present, fractions of tumors of sufficient sizes and nearby macroscopically normal tissue from the same liver were snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for biochemical analyses. The remaining samples were placed in 10% neutral-buffered formalin and paraffin embedded. Tissue sections were stained routinely with hematoxylin and eosin and the presence of hepatic foci,



**Fig. 1.** Hepatocarcinogenesis in  $TR1^{+liv}$  and  $TR1^{-liv}$  mice. (A) Liver and body weight ratios in  $TR1^{+liv}$  and  $TR1^{-liv}$  mice ( $n = 4$  and  $6$  for  $TR1^{+liv}$  and  $TR1^{-liv}$  untreated and  $19$  and  $18$  for  $TR1^{+liv}$  and  $TR1^{-liv}$  DEN/PB-treated mice, respectively; \*Denotes statistical difference,  $P \leq 0.05$ ). (B) Lipidosis revealed by liver pathology analysis. Livers from untreated  $TR1^{+liv}$  and  $TR1^{-liv}$  mice were sectioned, stained with hematoxylin and eosin and analyzed. Representative examples of lipidosis are shown. Arrows point to the characteristic clear staining cytoplasmic vacuoles. (C) Carcinogenesis in  $TR1^{+liv}$  and  $TR1^{-liv}$  mice. Livers from  $TR1^{-liv}$  mice were sectioned and stained with hematoxylin and eosin and analyzed. Representative examples of a hepatocellular carcinoma, hepatocellular adenoma and hepatic cholangiocellular adenoma are shown. Arrows designate hepatocellular carcinoma characterized by invasiveness, varied growth patterns and cellular pleomorphism; hepatocellular adenoma is circumscribed, noninvasive and composed of well-differentiated hepatocytes; and hepatic cholangiocellular adenoma is a circumscribed tumor with areas of hepatic and biliary differentiation. The pathology of the hepatocellular carcinoma and adenoma tumors from control mice was indistinguishable from the corresponding tumors in  $TR1^{-liv}$  mice.

cellular alteration, adenomas and carcinomas was analyzed qualitatively in a blind manner. Neoplastic lesions were classified according to the *International Classification of Rodent Tumors: The Mouse* (25). Tumors were not observed in other organs during necropsy and other tissues were not further examined.

#### RNA analysis

Total RNA was isolated from liver tissues using Trizol (Invitrogen). Two-step qPCR was performed to determine the relative expression of genes using primer sequences shown in Supplementary Table 1, available at *Carcinogenesis Online*. For each sample,  $1 \mu\text{g}$  of total RNA was reverse transcribed using an iScript™ cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions and used for qPCR using DyNAmo™ SYBR Green qPCR Kit (Thermo Scientific). Reactions were carried out in triplicate and RNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

#### Western blot analysis

Protein extracts were prepared from liver tumor and surrounding control tissue by homogenizing the tissue in cold lysis buffer [50 mM Tris; pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1% Igepal and Complete Mini Protease Inhibitor (Roche)]. Total protein was electrophoresed on 10% NuPage polyacrylamide gels (Invitrogen), transferred onto polyvinylidene difluoride membranes and immunoblotted with antibodies against either glutathione

peroxidase 1 (GPx1), superoxide dismutase 1 (SOD1), thioredoxin 1 (TRX1), peroxiredoxin 1 (PRDX1; Abcam, Cambridge, UK; 1:1000 dilution); catalase (CAT), GPx2 (R&D Systems, Minneapolis, MN; 1:1000 dilution); glutathione peroxidase 4 (GPx4), TR1 (Epitomics, Cambridge, MA; 1:1000 dilution), glutathione reductase (GSR; Epitomics, Cambridge, MA; 1:1000 dilution); glutamate–cysteine ligase (GCLC; Thermo Scientific, Rockford, IL; 1:1000 dilution); glutathione-S-transferase-alpha 1 (GST- $\alpha$ 1; Detroit R&D, Detroit, MI; 1:2000 dilution) or carbonyl reductase 3 (CBR3; Proteintech Group, Inc., Chicago, IL, 1:1000 dilution). Following incubation of the primary antibody, membranes were washed with tris-buffered saline (20 mM Tris/HCl; pH 7.5, 150 mM NaCl) containing 0.1% Tween 20 and incubated in anti-rabbit (GPx1, GPx4, GSR, GCLC, TR1, TRX1, PRDX1, SOD1 and CBR3), anti-goat (GST- $\alpha$ 1, CAT) or anti-mouse (GPx2) horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific, Rockford, IL; 1:10 000). Following secondary antibody incubation, membranes were washed with tris-buffered saline, incubated in Supersignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL) and exposed to x-ray film.

#### GSH measurements

Total GSH [oxidized GSSG (glutathione disulfide) + reduced GSH] levels in liver were measured using a GSH assay kit according to the manufacturer's instructions (Sigma–Aldrich, St. Louis, MO).

**Table 1.** Liver tumor incidence and type in  $TR1^{+liv}$  and  $TR1^{-liv}$  mice with and without DEN/PB treatment

Group	Exposure	Total tumor incidence		Tumor type incidence (% total mice) and number of mice with tumors		
		Number of mice with tumor	Percent of mice with tumor	Hepatic carcinoma	Hepatic adenoma	Cholangiocellular adenoma
$TR1^{+liv}$ ( $n = 4$ ; 1 male, 3 females)	None	0	0	0	0	0
$TR1^{-liv}$ ( $n = 6$ ; 3 males, 3 females)	None	0	0	0	0	0
$TR1^{+liv}$ ( $n = 19$ ; 10 males, 9 females)	DEN/PB	3	15.8%	10.5% (1 male, 1 female)	5.3% (1 male)	0
$TR1^{-liv}$ ( $n = 18$ ; 8 males, 10 females)	DEN/PB	16	88.9%	66.7% (5 males, 7 females)	11.1% (1 male, 1 female)	11.1% (1 male, 1 female)

#### 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 ( $P \leq 0.05$ ) were considered significant.

## Results

### *TR1*-deficient livers are highly susceptible to chemically induced hepatocarcinogenesis

$TR1^{-liv}$  and the corresponding  $TR1^{+liv}$  mice were either treated or not treated with DEN/PB and killed 12 months following the initial exposure to the liver carcinogen. At necropsy, tumors were found only in livers of treated mice. Tumors were classified according to cell type as benign (adenoma) or malignant (carcinoma) by histological analysis and, in addition, the number of mice with each tumor type was recorded (Table 1). Almost 90% of the  $TR1^{-liv}$  mice (16 of 18) treated with DEN/PB developed liver tumors, whereas only about 16% of  $TR1^{+liv}$  mice (3 of 19) had liver tumors.

The types of tumors found in the  $TR1^{-liv}$  mice fell into three classes, hepatic carcinoma, hepatic adenoma and cholangiocellular adenoma, with carcinoma being by far the most prevalent. Two tumors in the  $TR1^{+liv}$  mice were carcinomas, and the only other tumor-bearing  $TR1^{+liv}$  mouse had an adenoma. Pathological analysis of the three tumor types found in  $TR1^{-liv}$  mice and their descriptions are given in Figure 1C. There was no preference with respect to sex as the number of tumors and tumor types were virtually identical in males and females.

### Elevated liver to body weight ratios in *TR1*-deficient mice

The liver/body ratios were much greater in  $TR1^{-liv}$ -treated mice due to the large tumor mass in livers of most of these animals compared with  $TR1^{+liv}$  mice (Figure 1A). Rollins et al. (26) examined the liver growth rates in  $TR1^{+liv}$  and  $TR1^{-liv}$  mice and found them to be similar, but examination of the liver/body ratios in the present study showed that these ratios were slightly but significantly higher ( $P \leq 0.05$ ) in  $TR1^{-liv}$  untreated mice compared with the corresponding untreated  $TR1^{+liv}$  mice. This observation prompted us to perform histological comparison of the livers (Figure 1B). Four of six untreated  $TR1^{-liv}$  mice had mid-zonal hepatic lipidosis compared with none of four  $TR1^{+liv}$  mice. The extent of lipidosis in the affected mice ranged from mild to severe with one mouse showing mild lipidosis, two moderate (see representative liver in Figure 1B, lower panel) and one having severe lipidosis.

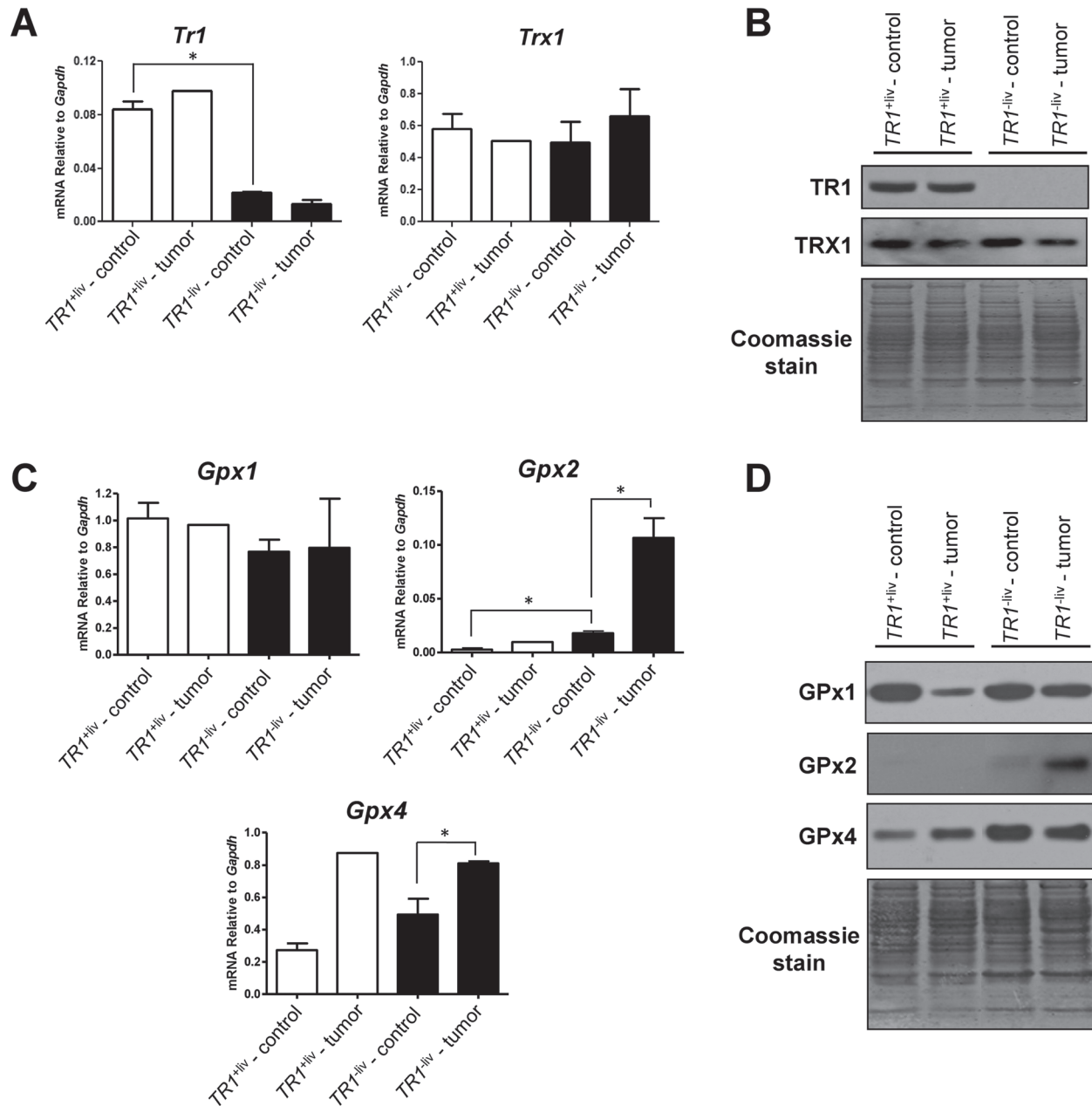
### Selenoenzyme expression is virtually unaffected by DEN exposure, but GPx2 is dramatically upregulated in carcinogen-induced *TR1*-deficient tumors

To assess the effects of DEN/PB treatment on the expression of TR1 and the cellular GPxs (GPx1, GPx2 and GPx4) in the livers of  $TR1^{+liv}$  and  $TR1^{-liv}$  mice, we compared the messenger RNA (mRNA) and protein levels of these selenoproteins in treated and untreated mice (Supplementary

Figure 2, available at *Carcinogenesis* Online). The *Tr1* and TR1 levels in the livers of these mice were similar and western blotting confirmed that, as expected, the corresponding  $TR1^{-liv}$  tissues did not express this selenoprotein (Supplementary Figure 2A and 2B, respectively, available at *Carcinogenesis* Online). The levels of *Gpx1* were similar in the livers of the two treated mouse lines compared with the *Gpx1* levels in the corresponding livers of untreated mice, which were lower but also in similar amounts (Supplementary Figure 2A, available at *Carcinogenesis* Online). However, the GPx1 levels appeared similar in both treated and untreated lines that showed some variations in levels in the various duplicate samples (Supplementary Figure 2B, available at *Carcinogenesis* Online). Treatment of these mice with DEN/PB caused an increase in the *Gpx1* level, but it is not clear why the higher levels did not result in more GPx1 expression. *Gpx2* was poorly expressed in livers of the two untreated mouse lines but slightly higher in the  $TR1^{-liv}$  mouse. Upon treatment, *Gpx2* levels significantly increased in both  $TR1^{+liv}$  and  $TR1^{-liv}$  livers ( $P \leq 0.05$ ), with the most dramatic increase occurring in  $TR1^{-liv}$  livers ( $P \leq 0.05$ ), albeit still being at a relatively low level. The amounts of GPx2 expressed in the livers of these mice were below detection of the corresponding antibodies. *Gpx4* levels were slightly, but not significantly, increased in livers of  $TR1^{-liv}$  mice compared with the controls, but GPx4 protein levels were higher in livers of untreated  $TR1^{-liv}$  mice compared with the corresponding  $TR1^{+liv}$  mice. Similarly, treated  $TR1^{-liv}$  mice expressed higher levels of GPx4 than the corresponding  $TR1^{+liv}$  mice. The enhanced expression of GPx4 in livers of  $TR1^{-liv}$  mice compared with the corresponding  $TR1^{+liv}$  mice most probably was due to the loss of TR1 that resulted in higher availability of selenium and Sec for synthesis of other hepatic selenoproteins. Since treatment of mice with DEN/PB did not have major consequences on the relative expression of these selenoenzymes, we examined only the livers of treated mice in subsequent studies.

Henceforth, we designated nontumor-containing tissue that surrounded tumors in livers of DEN/PB-treated mice as control tissue. Analyses of mRNA and protein levels of TR1, Trx1, GPx1, GPx2 and GPx4 (Figure 2) in liver tumors and surrounding control tissue revealed that the levels of *Tr1* mRNA and protein were similar in  $TR1^{+liv}$  mice and the single tumor examined in the  $TR1^{+liv}$  mouse, and as expected, were substantially reduced in  $TR1^{-liv}$  tumor and  $TR1^{-liv}$  mice (Figure 2A and 2B). Substantial levels of *Trx1* were found in all liver samples from TR1-sufficient to knockout mice and the variations in amounts were not significantly different. TRX1 levels were slightly reduced in tumor tissues compared with control tissues in both  $TR1^{+liv}$  and  $TR1^{-liv}$  mice.

*Gpx1* mRNA levels were statistically unchanged in the four tissue samples (Figure 2C). However, the expression of GPx1 was reduced substantially in the tumor of the  $TR1^{+liv}$  mouse compared with control tissue and to the  $TR1^{-liv}$  tumor and its control tissue (Figure 2D). In addition, the GPx1 level appeared to be slightly lower in  $TR1^{-liv}$  tumor than its control tissue. *Gpx2*, on the other hand, was poorly expressed in  $TR1^{+liv}$  samples and in the control  $TR1^{-liv}$  sample, but it increased dramatically in the tumor  $TR1^{-liv}$  sample ( $P \leq 0.05$ ) suggesting that the large enhancement of GPx2 occurred in response to TR1 loss and to compensate for the reduced ability



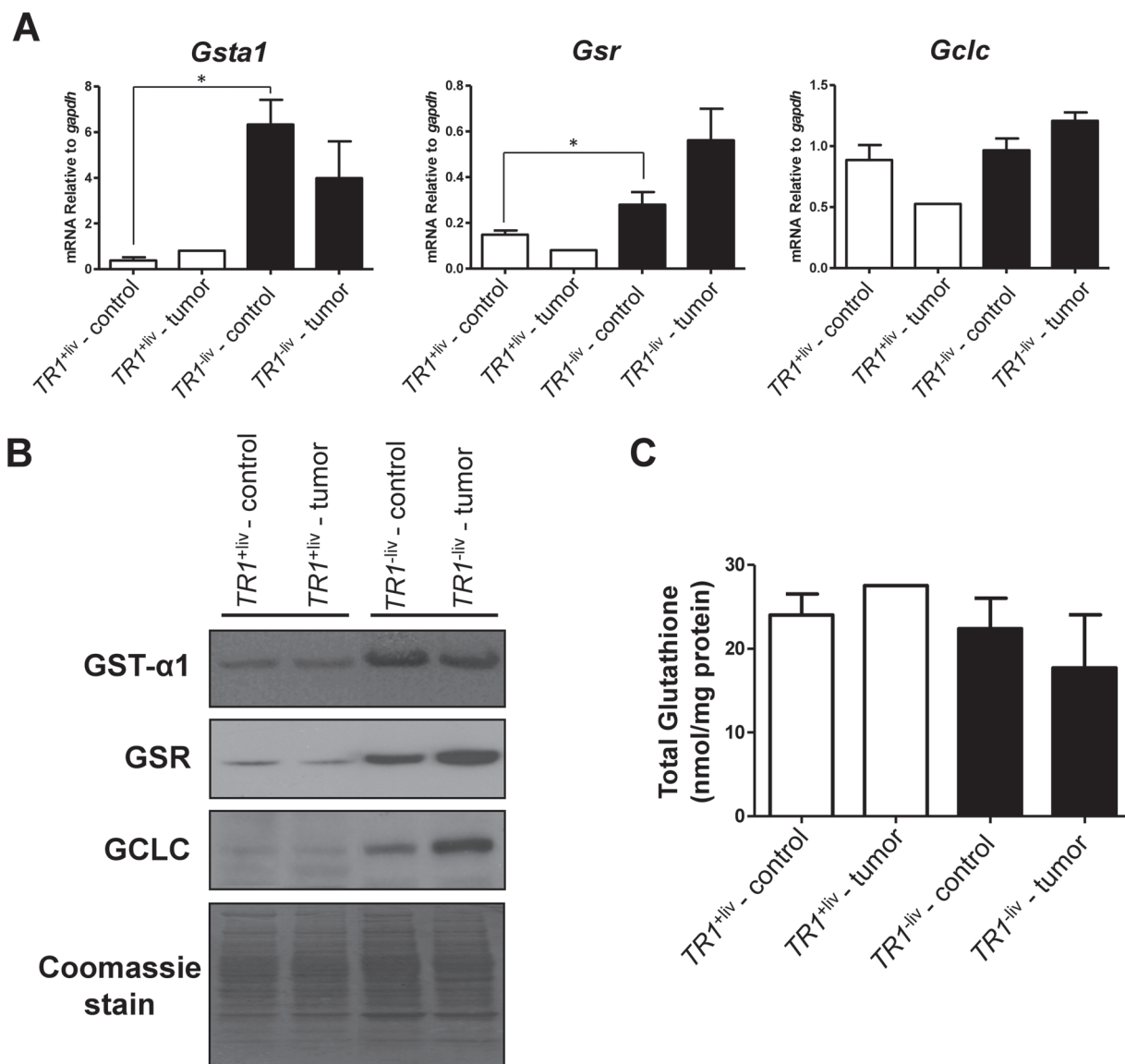
**Fig. 2.** Selenoprotein and Trx1 expression in  $TR1^{+liv}$  and  $TR1^{-liv}$  mice. (A) Gene expression in tumors and surrounding control tissue from livers of  $TR1^{+liv}$  and  $TR1^{-liv}$  mice. *Tr1* and *Trx1* mRNA levels were analyzed by real-time PCR. Data are shown as relative mRNA levels compared with *GAPDH* ( $n = 1$  for  $TR1^{+liv}$  tumor,  $n = 4$  for other samples; \*Denotes statistical difference,  $P \leq 0.05$ ). (B) Western blot analysis of TR1 and TRX1 in DEN/PB-treated  $TR1^{+liv}$  and  $TR1^{-liv}$  mice. A representative western blot is shown. Coomassie blue staining is shown in the bottom panel as control for protein loading. (C) mRNA levels of *GPx1*, *GPx2* and *GPx4*. Gene expression was analyzed by real-time PCR. Data are shown as relative mRNA levels compared with *GAPDH* ( $n = 1$  for  $TR1^{+liv}$  tumor,  $n = 4$  for other samples; \*Denotes statistical difference,  $P \leq 0.05$ ). (D) Western blot analysis of GPx1, GPx2 and GPx4 in livers of  $TR1^{+liv}$  and  $TR1^{-liv}$  mice. Protein extracts were prepared from tumors and surrounding control tissue from livers of  $TR1^{+liv}$  and  $TR1^{-liv}$  mice. Representative samples from a western blot are shown. Coomassie blue staining is shown in the bottom panel as control for protein loading.

to maintain redox homeostasis. *Gpx4* expression was enhanced about 3-fold in the  $TR1^{+liv}$  tumor sample over that observed in the surrounding control tissue and was also enriched in the  $TR1^{-liv}$  tumor sample over that observed in the control tissue ( $P \leq 0.05$ ). *Gpx4* was expressed higher in  $TR1^{-liv}$  tissues than the corresponding  $TR1^{+liv}$  samples.

#### Hepatic *TR1* deficiency is compensated for by elevated expression of enzymes involved in GSH metabolism

The expression of mRNAs and the corresponding proteins of three enzymes involved in GSH metabolism, GST- $\alpha 1$ , GSR and GCLC, was examined in  $TR1^{+liv}$  and  $TR1^{-liv}$  tumor and surrounding control

tissues (Figure 3A and 3B). The levels of *Gsta* and *Gsr* were low in both the  $TR1^{+liv}$  tumor and surrounding control samples but increased dramatically in the corresponding  $TR1^{-liv}$  liver samples ( $P \leq 0.05$ ), whereas those of *Gclc* appeared to be similar in these four samples; one exception involved *Gclc* expression that was about 2-fold lower in the  $TR1^{+liv}$  tumor sample compared with the corresponding control tissues (Figure 3A). The GST- $\alpha 1$ , GSR and GCLC levels were all dramatically increased in the  $TR1^{-liv}$  tumor and control samples compared with those in the corresponding  $TR1^{+liv}$  samples and the enrichment of GSR and GCLC was greater in the  $TR1^{-liv}$  tumor than  $TR1^{+liv}$  control samples (Figure 3B). The



**Fig. 3.** Expression of enzymes involved in GSH metabolism and GSH levels in *TR1<sup>+liv</sup>* and *TR1<sup>-liv</sup>* mice. (A) mRNA levels of *Gsta1*, *Gsr* and *Gclc*. Gene expression was analyzed by real-time PCR. Data are shown as relative mRNA levels compared with *GAPDH* ( $n = 1$  for *TR1<sup>+liv</sup>* tumor,  $n = 4$  for other samples; \*Denotes statistical difference,  $P \leq 0.05$ ). (B) Western blot analysis of GST- $\alpha$ 1, GSR and GCLC. Protein extracts were prepared from tumors and surrounding control tissue from livers of *TR1<sup>+liv</sup>* and *TR1<sup>-liv</sup>* mice. Representative samples from a western blot are shown. Coomassie blue staining is shown in the bottom panel as control for protein loading. (C) Total GSH levels were measured in livers from *TR1<sup>+liv</sup>* control ( $n = 3$ ), *TR1<sup>+liv</sup>* tumor ( $n = 1$ ), *TR1<sup>-liv</sup>* control ( $n = 3$ ) and *TR1<sup>-liv</sup>* tumor ( $n = 3$ ) tissues.

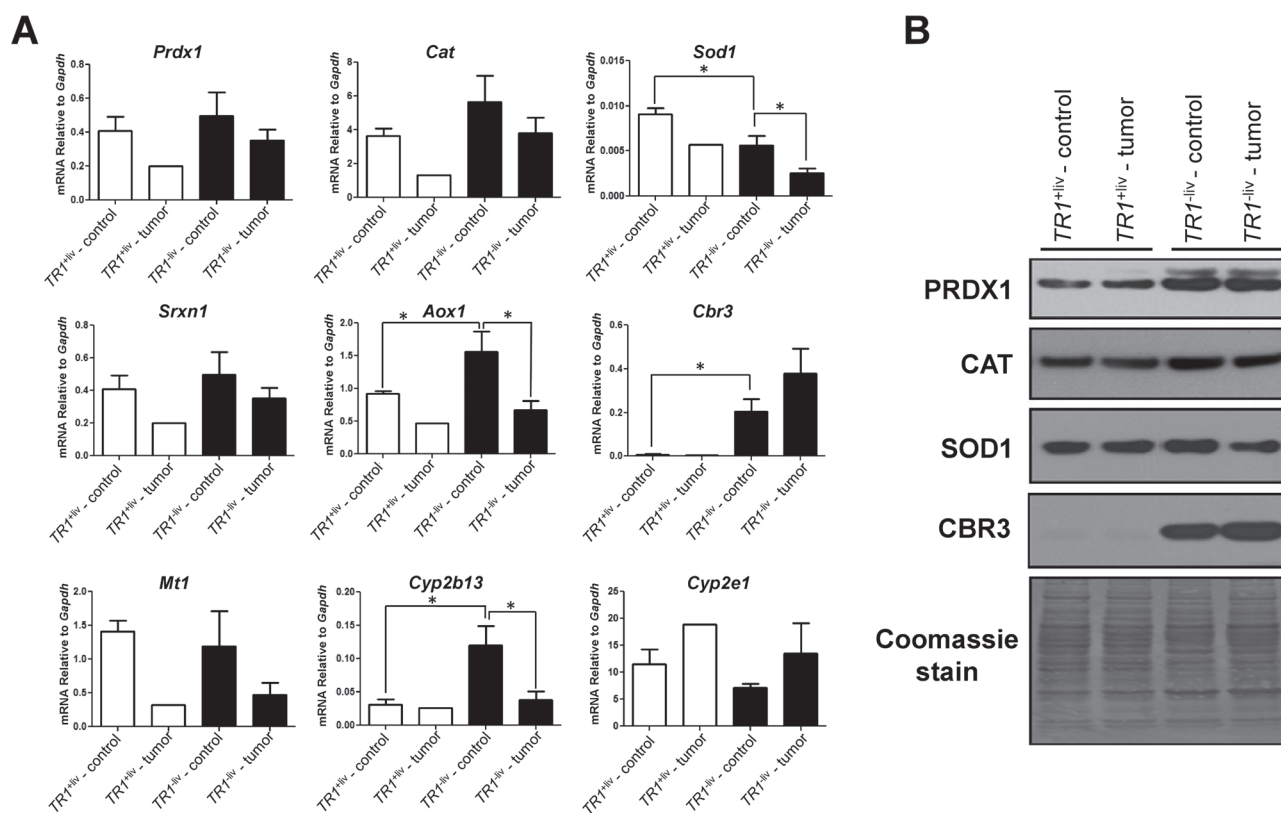
fact that GSR is involved in reducing GSSG to GSH and that GCLC is the first rate-limiting enzyme involved in GSH synthesis provides strong evidence that their elevated expression in *TR1<sup>-liv</sup>* tumors is to provide additional antioxidant support during TR1 deficiency.

The levels of total GSH were slightly, but not significantly, lower in *TR1<sup>-liv</sup>* tumors than in control samples (Figure 3C).

#### *Other antioxidant systems probably do not provide additional antioxidant support to TR1-deficient liver samples*

We further examined mRNA expression of other antioxidant proteins, including PRDX1, CAT, SOD1, sulfiredoxin 1 (SRXN1), metallothionein 1 (MT1), CBR3, and three cytochrome P450 isozymes, CYP2E1, CYP2B13 and aldehyde oxygenase 1 (AOX1; Figure 4). The functions of these antioxidant proteins are summarized in Supplementary Table 2, available at *Carcinogenesis* Online. With the exception of *Cbr3* and *Cyp2e1*, antioxidant protein mRNAs appeared downregulated in the *TR1<sup>-liv</sup>* and *TR1<sup>+liv</sup>* tumor tissues

compared with the corresponding control tissues (Figure 4A). On the other hand, *Cbr3* was poorly expressed in *TR1<sup>+liv</sup>* tumor and control tissues but was dramatically upregulated in the *TR1<sup>-liv</sup>* tumor and control tissues ( $P \leq 0.05$ ). *Cyp2e1* was slightly upregulated in the tumor samples compared with the corresponding control tissues, but the differences were not statistically significant. *Cyp2b13* and *Aox1* were upregulated in *TR1<sup>-liv</sup>* control tissues compared with *TR1<sup>+liv</sup>* tissues, which was apparently due to the loss of TR1 expression, and both were downregulated in *TR1<sup>-liv</sup>* tumor compared with *TR1<sup>-liv</sup>* control tissues ( $P \leq 0.05$ ). *Sod1* expression was lower in *TR1<sup>-liv</sup>* control tissue when compared with *TR1<sup>+liv</sup>* control tissue and was also lower in the *TR1<sup>-liv</sup>* tumor samples compared with *TR1<sup>-liv</sup>* control tissues ( $P \leq 0.05$ ). We further examined the expression of PRDX1, CAT, SOD1 and CBR3 (Figure 4B). Although we found no significant change in mRNA levels, PRDX1 was upregulated in *TR1<sup>-liv</sup>* tumor and control tissues compared with the corresponding *TR1<sup>+liv</sup>* samples, whereas CAT and SOD1 expression



**Fig. 4.** Expression of oxidative stress-related genes. (A) Gene expression in tumors and surrounding control tissue from livers of *TR1*<sup>+liv</sup> and *TR1*<sup>-liv</sup> mice. mRNA levels of *Prdx1*, *Cat*, *Sod1*, *Srxn1*, *Aox1*, *Cbr3*, *Mt1*, *Cyp2b13* and *Cyp2e1* in tumors and surrounding control tissue from livers of *TR1*<sup>+liv</sup> and *TR1*<sup>-liv</sup> mice was analyzed by real-time PCR. Data are shown as relative mRNA level to *GAPDH* ( $n = 1$  for *TR1*<sup>+liv</sup> tumor,  $n = 4$  for other samples; \*Denotes statistical difference,  $P \leq 0.05$ ). (B) Western blot analysis of PRDX1, CAT, SOD1 and CBR3 in DEN/PB-treated *TR1*<sup>+liv</sup> and *TR1*<sup>-liv</sup> mice. Protein extracts were prepared from tumors and surrounding control tissue from livers of *TR1*<sup>+liv</sup> and *TR1*<sup>-liv</sup> mice. Representative samples from a western blot are shown. Coomassie blue staining is shown in the bottom panel.

was slightly higher in the *TR1*<sup>-liv</sup> control tissue compared with the three other tissues and SOD1 appeared to be slightly decreased in *TR1*<sup>-liv</sup> tumor samples compared with *TR1*<sup>-liv</sup> control tissues. CBR3 expression was dramatically upregulated in the *TR1*<sup>-liv</sup> tumor and control tissues and appeared to be slightly more upregulated in the *TR1*<sup>-liv</sup> tumor than the control tissue. With the possible exception of CBR3, the other antioxidant proteins examined did not appear to provide additional antioxidant support to *TR1*<sup>-liv</sup> tumor tissues.

## Discussion

The dramatically enhanced liver tumor incidence in DEN/PB-treated *TR1*<sup>-liv</sup> mice (~90% of the animals developed tumors) compared with DEN/PB-treated *TR1*<sup>+liv</sup> mice (~16% tumor incidence) clearly shows that the loss of this important selenoprotein in hepatocytes results in a considerable increase in tumor development. These data demonstrate the significance of TR1 in preventing chemically induced liver cancer in mice.

Since most cancer cells suffer from oxidative stress as a consequence of disrupted redox homeostasis, they rely on key redox regulators to perpetuate rapid growth and sustain their malignant status. Confronted with the deficiency in a major redox regulatory system, such cells would have to compensate by elevating functions of other antioxidant systems. Consistent with this possibility, the expression of GPx2 was dramatically enriched in tumors of *TR1*<sup>-liv</sup> mice compared with surrounding tissue and compared with the tumor in *TR1*<sup>+liv</sup> mice and its surrounding tissue. Although GPx2 is known as a gastrointestinal GSH peroxidase, it is also expressed in other tissues including human liver (27). Interestingly, its expression level is increased in malignant epithelial cells. It has an important role in cell proliferation,

is an antioxidant and has also been implicated in protection against carcinogenesis (27,28). Its large increase in tumors of *TR1*<sup>-liv</sup> mice observed in the present study suggests that GPx2 has a role in the development and/or maintenance of this cancer.

The levels of enzymes involved in the GSH system, GST- $\alpha$ 1, GSR and GCLC, were also enhanced considerably in DEN/PB-treated *TR1*<sup>-liv</sup> mice compared with treated *TR1*<sup>+liv</sup> mice. GSR and GCLC were enriched in *TR1*<sup>-liv</sup> tumors compared with surrounding tissue. The increased expression of enzymes involved in generating GSH, GSR and GCLC is expected to provide tumors of *TR1*<sup>-liv</sup> mice with additional reducing equivalents necessary to counter increased oxidative stress in malignant *TR1*<sup>-liv</sup> tissue. Overall, our data suggest that the loss of an important redox regulator of protein thiols in liver led to an enrichment in several components of the GSH system including GPx2, most probably as a response to an increase in oxidized molecules normally reduced by the Trx system. However, this enrichment did not prevent tumor formation and may in turn be driving the malignancy.

The roles of TR1 and the GSH system (29–32) in preventing, promoting and/or sustaining cancer have been recognized for some time. However, the interrelationships and interdependence of these systems, particularly in cancer tissue, have received little attention until recently (11,19–22). An interesting finding in several recent studies, and in TR1-deficient hepatocytes as observed in our study, is the upregulation of the NF-E2-related factor 2 (Nrf2) stress-response pathway (33,34). This transcription factor has been shown to be upregulated in TR1-deficient hepatocytes (34), activating expression of many proteins involved in protective cellular effects (35,36), and is responsible for the upregulation of several antioxidant proteins involved in selenium and GSH metabolism, including TR1. As

previously reported and confirmed in this study, *TR1*<sup>-liv</sup> mice show an increase in Nrf2-regulated genes and proteins involved in phase I and phase II response, such as *Cbr3*, *Cyp2b13* and *Gsta* (34).

Upregulation of Nrf2 has been observed in many types of cancer and may play a role in promoting malignancy due to the subsequent upregulation of protective enzymes (37). However, Nrf2-deficient mice display an increased sensitivity to hepatic carcinogens, most probably due to the loss of detoxification enzymes regulated by Nrf2 (37). Clearly, despite the increase of many Nrf2-regulated enzymes in *TR1*<sup>-liv</sup> mice, tumor formation is elevated, implicating TR1 as a key Nrf2-regulated enzyme in preventing malignancy.

GPx4 expression was also upregulated in *TR1*<sup>-liv</sup> compared with *TR1*<sup>+liv</sup> tissues. However, the apparent slight downregulation of GPx4 in *TR1*<sup>-liv</sup> tumor tissues compared with the surrounding control tissues may be a reflection of the enhanced expression of specific selenoproteins (e.g. GPx2). This observation may also apply to GPx1 as the simultaneous upregulation in TR1 expression and downregulation of GPx1 in tumor tissues was initially reported several years ago (38). Such translational demands may also be responsible, along with the fact that SECIS-binding protein 2 is limiting in selenoprotein synthesis (39,40), for the disconnect observed in mRNA expression and corresponding protein synthesis.

Among other tested genes involved in antioxidant control that were examined in the present study, only *Cbr3* and *Cyp2e1* appeared to be upregulated in *TR1*<sup>-liv</sup> tumors compared with the surrounding tissue. *Cyp2e1* has been shown to be involved in the metabolic activation of DEN (41), possibly contributing to the increased tumorigenicity seen in *TR1*<sup>-liv</sup> mice. In addition, CBR3 was strongly upregulated in *TR1*<sup>-liv</sup> tumor and surrounding control tissue compared with the corresponding *TR1*<sup>+liv</sup> tissues. However, any possible role of this protein in liver cancer must await further examination. PRDX1 was upregulated in both *TR1*<sup>-liv</sup> tumor and nontumor tissue compared with the corresponding *TR1*<sup>+liv</sup> tissues suggesting that the enhanced expression of this protein is also needed to compensate for TR1 deficiency.

As discussed in the Introduction, overexpression of TR1 has been observed in many tumors and cancer cells, and this protein is a target for several drugs currently in use in cancer treatment, which has led to the proposal that this selenoenzyme is a prime target for chemotherapy. However, since the expression of this selenoenzyme was not enhanced in the TR1 sufficient liver tumor (compared with control tissues), it would seem that this selenoenzyme is not involved in promoting chemically induced hepatocarcinogenesis and thus its targeting may not help in liver cancer therapy. Instead, the evidence presented herein that TR1 deficiency results in a dramatic increase in chemical hepatocarcinomas suggests that its role is more of one in protecting hepatocytes against cancer initiation.

Clearly, cancers and cancerous tissues are different with regard to the role of TR1 and other selenoproteins in carcinogenesis (17,18). Selenium deficiency has also been shown to play a role in preventing some cancers (42,43), including mouse liver cancer driven by the *c-Myc* oncogene (43). In addition, differences may be seen when only one selenoprotein is compromised either by knockout, as in our study, or by targeted inactivation using specific inhibitors [see (16) for review] or when the entire selenoproteome is compromised, as in dietary studies and genetic manipulations involving essential factors (e.g. Sec tRNA) that affect selenoprotein expression as a whole. Further complications arise from compensatory changes in other systems regulating redox homeostasis. In our study, the loss of TR1 led to an increase in another selenoenzyme, GPx2 (Figure 2C), and other components of the GSH system (Figure 3), whereas the total loss of selenoprotein expression can no longer support such interplay. As investigators begin to elucidate the complex interactions between different redox regulators and redox regulatory systems (see Introduction), such studies will most certainly uncover the interrelationships involving selenium, individual selenoproteins, the entire selenoproteome and other antioxidant systems.

As we move into the era of personalized medicine, information on the specific links between tumors derived from mutations in certain genes and pathways, and their response to drugs, will play an

important role in clinical approaches. Here, we defined the role of one critical enzyme, previously implicated in both preventing and promoting cancer, in chemically induced hepatocarcinogenesis in mice. However, additional studies will be required to define other cancer types with regard to the roles of TR1, other selenoproteins and ultimately dietary selenium, in cancer. With this information, it may be possible, in the future, to precisely define conditions and genotypes that benefit from dietary selenium, as well as those for which dietary selenium will have adverse effects.

## Supplementary material

Supplementary Tables 1 and 2 and Figures 1 and 2 can be found at <http://carcin.oxfordjournals.org/>

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