Glutathione peroxidase 4 inhibits Wnt/β-catenin signaling and regulates dorsal organizer formation in zebrafish embryos

Xiaozhi Rong1,2,3,4, Yumei Zhou1, Yunzhang Liu1, Beibei Zhao1, Bo Wang1, Caixia Wang1, Xiaoxia Gong1, Peipei Tang1, Ling Lu1, Yun Li1, Chengtian Zhao2,3, and Jianfeng Zhou1,4*

1Key Laboratory of Marine Drugs (Ocean University of China), Chinese Ministry of Education, and School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao 266003, China; 2Institute of Evolution and Marine Biodiversity and College of Marine Biology, Ocean University of China, 5 Yushan Road, Qingdao 266003, China. 3Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266003, China. 4Laboratory for Marine Drugs and Biological Products, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266003, China.

*To whom correspondence should be addressed: Jianfeng Zhou, School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao 266003, China. Tel.: 86-532-82032957; Fax: 86-532-82032957; E-mail address: jfzhou@ouc.edu.cn.

Keywords: Glutathione peroxidase 4, Wnt/β-catenin signaling, Tcf/Lef, zebrafish, dorsal organizer

Summary statement

Selenoprotein glutathione peroxidase 4 inhibits Wnt/β-catenin signaling independently selenocysteine activation by preventing transcription factor Tcf/Lefs binding to target promoters and regulates dorsal organizer formation in zebrafish embryos.
Abstract

The Wnt/β-catenin signaling pathway plays pivotal roles in axis formation during embryogenesis and in adult tissue homeostasis. Glutathione peroxidase 4 (GPx4) is a selenoenzyme and participates in the reduction of peroxides. Its synthesis depends on the availability of the element selenium. However, the roles of GPx4 in vertebrate embryonic development and underlying mechanisms are largely unknown. Here, we show that maternal loss of zebrafish gpx4b promotes embryonic dorsal organizer formation, whereas overexpression of GPx4b inhibits the development of the dorsal organizer. Depletion of GPx4/GPx4b increases, while GPx4/GPx4b overexpression decreases, Wnt/β-catenin signaling in vivo and in vitro. Functional and epistatic studies showed that GPx4 functions at the Tcf/Lef level, independently of selenocysteine activation. Mechanistically, GPx4 interacts with Tcf/Lefs and inhibits Wnt activity by preventing the binding of Tcf/Lefs to the promoters of Wnt target genes, resulting in inhibitory action in the presence of Wnt/β-catenin signaling. Our findings unravel GPx4 as a suppressor of Wnt/β-catenin signals, suggesting a possible relationship between the Wnt/β-catenin pathway and selenium via the association of Tcf/Lef family proteins with GPx4.
**Introduction**

Animal development and adult tissue homeostasis are controlled by a wide range of molecules. Among these, the Wnt/β-catenin pathway, which is evolutionarily conserved among vertebrates, plays key roles (Clevers and Nusse, 2012; MacDonald et al., 2009). Misregulation of Wnt/β-catenin signaling has been implicated in birth defects, tumorigenesis, and other diseases (Anastas and Moon, 2013; Clevers and Nusse, 2012; MacDonald et al., 2009). In the presence of Wnt ligands, the Wnt signal inhibits the degradation of β-catenin (Clevers and Nusse, 2012; MacDonald et al., 2009; Stamos and Weis, 2013). Stabilized β-catenin translocates to the nucleus where it binds to transcription factors of the Tcf/Lef family, leading to the transcriptional activation of Wnt target genes (Cadigan and Waterman, 2012; Clevers and Nusse, 2012; MacDonald et al., 2009). In the absence of Wnt ligands, β-catenin is phosphorylated and targeted by a destruction complex for proteasomal degradation, and Tcf/Lefs interact with Groucho transcriptional repressors, preventing gene transcription (Cadigan and Waterman, 2012; Clevers and Nusse, 2012; MacDonald et al., 2009). Thus, in the Wnt/β-catenin pathway, Tcf/Lefs are the key molecules converting transcriptional repressors into transcriptional activators of Wnt target genes upon stimulation of Wnt signals.

Wnt/β-catenin signaling is critically involved in the axial patterning in vertebrate embryogenesis (Hikasa and Sokol, 2013; Langdon and Mullins, 2011; Petersen and Reddien, 2009). In zebrafish embryos, maternal Wnt/β-catenin signaling promotes the dorsal organizer formation before gastrulation (Bellipanni et al., 2006; Schneider et al., 1996). In contrast, zygotic Wnt/β-catenin signaling promotes ventrolateral mesodermal development to limit the organizer after the onset of gastrulation, and whereafter promotes posterior neural development (Baker et al., 2010; Erter et al., 2001; Lekven et al., 2001; Ramel et al., 2005).

Glutathione peroxidase 4 (GPx4) is a monomeric selenoenzyme harboring a selenocysteine (Sec) in the catalytically active center (Brigelius-Flohe and Maiorino, 2013). Sec, the 21st amino acid, incorporates selenium in selenoproteins in response to the opal UGA codon (Hatfield et al., 2014). Therefore, the element selenium is essential for selenoproteins. Sec efficiently reduces hydroperoxides at the expense of glutathione or other low-molecular-weight thiol-containing compounds (Hatfield et al., 2014). Thus, GPx4 plays an important role in controlling the cellular redox status (Brigelius-Flohe and Maiorino, 2013). In addition, GPx4
has been found to control “ferroptosis”, a novel, nonapoptotic form of cell death involving lipid reactive oxygen species (ROS) (Dixon et al., 2012; Friedmann Angeli et al., 2014; Seiler et al., 2008; Yang et al., 2014). GPx4 is highly conserved in vertebrates from fish to mammals. Previous studies reported that Sec activation of GPx4 is critical for survival in mice, and depletion of GPx4 or genetically inactive Sec in mice leads to embryonic lethality around embryonic day 7.5 (Imai et al., 2003; Ingold et al., 2015; Yant et al., 2003). However, besides the reported cellular function and developmental roles in mice, the role of GPx4 in vertebrate embryogenesis and its underlying molecular mechanisms are still poorly understood.

In this study, we used the zebrafish model to investigate the role of GPx4 in early development by using both the CRISPR/Cas9 knockout system and the antisense morpholino (MO) knockdown technology. Unexpectedly, maternal loss of zebrafish gpx4b and knockdown of gpx4b led to dorsal organizer formation deficiency and GPx4 inhibited the Wnt/β-catenin signaling pathway. We found that GPx4 interacts with Tcf/Lefs and occupies the Wnt target gene promoters to suppress transcription by rendering the association between Tcf/Lefs and Wnt target gene promoters, resulting in reduced Wnt activity in the presence of Wnt signals. To our knowledge, this is the first report on the cellular function of GPx4 as a repressor of Wnt/β-catenin signaling and the critical role of maternally deposited GPx4b in regulating dorsal organizer formation during zebrafish embryogenesis. Importantly, our results also suggested that the Wnt/β-catenin pathway might be modulated by selenium, which, in the form of Sec, is indispensable for cellular synthesis of GPx4.
Results

Maternal loss of zebrafish *gpx4b* impairs dorsal organizer formation and depletion of GPx4b increases Wnt/β-catenin activity.

The zebrafish genome harbors two *gpx4* genes: *gpx4a* and *gpx4b*. Both of GPx4a and GPx4b contain a Sec site (Fig. S1A). RT-PCR and whole-mount *in situ* hybridization (WISH) with a *gpx4b* antisense probe indicated that the *gpx4b* transcript is maternally deposited and is ubiquitously expressed before 24 hours post fertilization (hpf) (Fig. S1B, C) (Mendieta-Serrano et al., 2015; Thisse et al., 2003). Unlike *gpx4b*, previous studies have indicated that *gpx4a* shows zygotic gene expression in the periderm covering the yolk cell only (Mendieta-Serrano et al., 2015; Thisse et al., 2003). Interestingly, recent work has shown that from the 128-cell to 512-cell stages, nuclear GPx4 expression increased gradually (Mendieta-Serrano et al., 2015). The ubiquitous and dynamic expression of GPx4 implies that GPx4 may play an important role in the regulation of embryonic development.

To investigate the role of *gpx4b*, we generated two lines of *gpx4b* null mutants with non-overlapping 4-bp and 10-bp deletions in the third exon by using the CRISPR/Cas9 system (Fig. 1A-C) (Chang et al., 2013). The mutations cause a frame shift early in the protein-coding region leading to early termination of translation (Fig. 1B). Zygotic mutant fish were viable, morphologically normal, and fertile. However, both lines of maternal (M) mutants showed dorsalized phenotypes with reduced ventral tail fins at 26 hpf although the allele penetrance and expressivity in the 4-bp deletion line appeared to be higher (Fig. 1D). These dorsalized phenotypes are characteristic of weakly dorsalized phenotypes, reminiscent of *mini fin* (*mfn*) and *lost-a-fin* (*laf*) mutant embryos at this stage (Mullins et al., 1996). Unlike the M mutants, all observed embryos from the two lines of maternal-zygotic (MZ) mutants were morphologically normal at 24 hpf (Fig. 1D). As an independent and complementary approach, a translation-blocking *gpx4b* MO was used to inhibit translation of both maternal and zygotic mRNAs. The effectiveness of the MO was confirmed by blocking the translation of a *gpx4b* 5′-UTR-GFP reporter (Fig. S2A). The specificity of the MO was confirmed by the lack of additional phenotypes in the MZ mutant background. Additionally, as shown in Fig. S2B, we generated a GPx4b expression vector which contains the ORF and the whole 3′-UTR including the Sec insertion sequence element to allow insertion of Sec in response to the UGA codon
instead of termination of translation (Berry et al., 1991). The expression of GPx4b was confirmed prior to rescue experiments using in vitro transcribed gpx4b mRNA (Fig. S2B). Injection of the MO caused relatively stronger dorsalized phenotypes with reduced trunk, thinner and curved tail, and reduced yolk extension at 26 hpf, which resembled the previously reported somitabun (sbn) and swirl (swr) mutants at this stage (Fig. S2C, D) (Mullins et al., 1996). The dorsalized phenotype could be neutralized by coinjection of 50 pg gpx4b mRNA, suggesting that the MO specifically targeted gpx4b (Fig. S2D). Similarly, coinjection of 50 pg gpx4a mRNA rescued the dorsalizing effect caused by the gpx4b MO, suggesting that GPx4a and GPx4b have comparable ventralizing action (Fig. S2E). Recent studies reported that zebrafish can activate a compensation mechanism, resulting in mild phenotype, in genetic mutants but not in morphants (Hu et al., 2016; Rossi et al., 2015). To evaluate the discrepancy between the phenotypes observed in mutants and morphants, we carried out quantitative real-time reverse transcription PCR (qRT-PCR) analysis of gpx4a and gpx4b at different developmental stages. The transcript levels of gpx4b were not decreased in M and MZ mutants at 4 hpf but were significantly decreased in MZ mutants at 6 hpf and 9 hpf, indicating a gradually increasing gpx4b mRNA degradation rate (Fig. S2F). Meanwhile, the gpx4a mRNA levels were significantly elevated (Fig. S2F). In gpx4b-knockdown morphants, neither the expression of gpx4b was reduced nor gpx4a was upregulated as compared to cMO-injected embryos (Fig. S2F). Collectively, these data suggested that GPx4a probably functions as a compensating protein in gpx4b mutants.

In zebrafish, maternal and zygotic Wnt/β-catenin appears to have different effects on dorsoventral patterning formation (Hikasa and Sokol, 2013; Langdon and Mullins, 2011; Petersen and Reddien, 2009). The phenotypic differences between M and MZ mutants as well as morphants prompted us to investigate whether or not dorsal organizer formation was impaired and Wnt/β-catenin signaling abrogated. To characterize the consequences of loss of GPx4b on embryonic patterning, we examined the expression of a suite of region-specific marker genes at different developmental stages. In both the M and MZ mutant lines of gpx4b, expression of the organizer-specific markers chordin (chd) and goosecoid (gsc) was laterally and ventrally expanded at 4.3 hpf as indicated by WISH (Fig. 2A, B and Fig. S3A, B). Similarly, the gpx4b-knockdown embryos showed broad expansion of chd and gsc expression at 4.3 hpf.
(Fig. S3C, D). At shield stage (6 hpf), the expression domains of *chd* and *gsc* were also laterally and ventrally expanded in M mutants (Fig. S3E, G). Another, maternal mutant allele1 showed a higher ratio of embryos with expanded expression of organizer markers at 4.3 hpf and 6 hpf, which was consistent with the higher penetrance of allele1 at 26 hpf (Fig. 2A, B and Fig. S3A, B, E, and G). Conversely, the ventrally restricted markers *even-skipped-1 (eve1)*, *bmp4*, *bmp2b*, and *sizzled (szl)* exhibited reduced expression domains at 6 hpf (Fig. S3F, G). Similar dorsoventral patterning was observed in the *gpx4b*-knockdown embryos (Fig. S3H, I). Unlike M mutants and *gpx4b*-knockdown morphants, however, the expression domains of the dorsoventral marker genes returned to the control levels in MZ mutants in comparison with WT (Fig. S3E-G). Next, we investigate the role of GPx4b in anteroposterior neural patterning. The expression of the forebrain marker *six3b*, midbrain-hindbrain boundary marker *pax2a*, and hindbrain marker *krox20* was used to assess the anteroposterior neural patterning. As shown in Fig. S3J, the anteroposterior neural patterning was not affected in *gpx4b* MZ mutant embryos at 12.5 hpf. Taken together, these results indicated that absence of maternal GPx4b promotes dorsal development before the onset of gastrulation, while loss of both maternal and zygotic GPx4b have little effect on dorsoventral patterning and anteroposterior neural patterning in the zebrafish gastrula.

The effect of loss of *gpx4b* on Wnt/β-catenin signaling pathway were investigated next. As *chd* and *gsc* are main targets of maternal Wnt at 4.3 hpf, we speculated that GPx4b may act as an inhibitor of the Wnt/β-catenin signaling pathway. To test this hypothesis, we investigated the expression of direct Wnt target genes at different developmental stages. At 4.3 hpf, the expression of three targets of maternal β-catenin, *boz/dharma, chd*, and *squint (sqt)/ndr1*, was significantly upregulated in both M and MZ mutants as determined by qRT-PCR (Fig. 2C and Fig. S4A). Additionally, the expression areas of three direct target genes of zygotic Wnt, *cdx4*, *sp5l*, and *tbx6*, were broadly expanded in MZ mutants, and *cdx4* as well as *sp5l* in morphants at 80-90% epiboly stage (9 hpf) as accessed by WISH (Fig. 2D and Fig. S4B, C). Similarly, at the 75% epiboly stage (8 hpf), the expression levels of direct zygotic Wnt targets *sp5l*, *cdx4*, *ccnd1*, *axin2*, and *vent* were significantly increased in MZ mutants as indicated by qRT-PCR (Fig. 2E and Fig. S4D). We used a well-established Wnt reporter construct TOPFlash to examine the role of endogenous GPx4b further. Injection of MZ mutants with TOPFlash alone
or of WT embryos with \textit{gpx4b}-MO resulted in significantly increased Wnt reporter activity (Fig. 2F and Fig. S4E). Taken together, these results suggested that maternal loss of zebrafish \textit{gpx4b} promotes dorsal organizer formation and depletion of GPx4b increases Wnt/\(\beta\)-catenin activity.

To further substantiate the developmental role of GPx4b, we next performed a series of analysis on the progeny of the homozygous females crossed to heterozygous males. The individual embryos were then genotyped to determine whether phenotypes were correlated with genotype or not. As shown in Fig. S5A, B, the expression areas of \textit{gsc} and \textit{eve1} at 6 hpf are correlated with genotypes. Similar results were obtained in the expression area of \textit{cdx4} at 80\% epiboly and phenotypes at 26 hpf (Fig. S5C-F). Due to variable penetrance and expressivity between the two alleles, the allele2 homozygous females were crossed to allele1 heterozygous males. As shown Fig. S5G and H, the dorsalized phenotype was confirmed by genotyping. Collectively, these data strongly suggest that maternal loss of zebrafish \textit{gpx4b} promotes dorsal organizer formation and depletion of GPx4b increases Wnt/\(\beta\)-catenin activity.

\textbf{Overexpression of \textit{gpx4b} ventralizes zebrafish embryos and inhibits Wnt/\(\beta\)-catenin activity.}

Next, we investigated the effect of forced expression of \textit{gpx4b}. Injection of 600 pg \textit{gpx4b} mRNA resulted in a ventralized phenotype with reduced head and enlarged ventral tail fin at 24 hpf (Fig. 3A, B). We speculated that these phenotypes may have resulted from the inhibition of maternal Wnt/\(\beta\)-catenin action. Therefore, we further examined the effect of \textit{gpx4b} overexpression on dorsal development at 4.3 hpf. As shown in Fig. 3C, embryos injected with \textit{gpx4b} mRNA consistently showed markedly reduced \textit{chd} and \textit{gsc} expression, indicating the ventralizing effect of GPx4b before gastrulation. To determine whether Wnt signaling is affected by GPx4b overexpression \textit{in vivo}, we measured the Wnt reporter activity in \textit{gpx4b} mRNA-injected zebrafish embryos. Forced expression of \textit{gpx4b} mRNA inhibited endogenous Wnt signaling activity in zebrafish embryos (Fig. 3D). Similarly, transfection of GPx4b into human embryonic kidney (HEK) 293T cells decreased basal Wnt reporter activity (Fig. 3E). These data indicated that GPx4b negatively regulates Wnt signaling both \textit{in vivo} and \textit{in vitro}. 
Inhibitory effect of GPx4/GPx4b on Wnt/β-cattnin signaling is evolutionarily conserved between humans and zebrafish.

Zebrafish GPx4b shows a high degree of sequence identity with its human homolog GPx4 (Fig. S1A). This prompted us to explore whether the inhibitory effect of GPx4 on Wnt/β-catenin signaling is conserved in humans. When human GPx4 was overexpressed in zebrafish embryos, it exhibited a similar ventralizing action in embryos as zebrafish GPx4b did (Fig. 4A, B). We next took a loss-of-function approach to examine whether GPx4 inhibits Wnt/β-catenin signaling in HEK293T cells. To this purpose, a cell line stably expressing GPx4 shRNA was established. The knockdown efficiency was determined by Western blot analysis (Fig. 4C). GPx4 knockdown significantly increased endogenous Wnt reporter activity (Fig. 4D). In addition, it resulted in significantly upregulated expression of the direct Wnt target genes such as MYC, CCND1, AXIN2, DKK1, LEF1, CDK2, and CDK5 as indicated by qRT-PCR (Fig. 4E). Collectively, these data suggested that both human GPx4 and zebrafish GPx4b inhibit Wnt/β-catenin signaling.

GPx4/GPx4b inhibits Wnt/β-catenin signaling at the transcriptional level.

We then investigated the genetic interaction between GPx4/GPx4b and Wnt/β-catenin signals in zebrafish embryos and HEK293T cells. Wnt/β-catenin signals consisted of multiple steps including Wnt ligands, β-catenin, and Tcf/Lefs. Injection of mRNA for Wnt3a, constitutively active β-catenin (β-CatΔN), or constitutively active Tcf3 (VP16-Tcf3ΔN, β-catenin-independent VP16-Tcf3 fusion protein that lacks the β-catenin-binding site) in zebrafish embryos resulted in obviously dorsalized phenotype at 12.5 hpf (Fig. 5A, B). Co-injection of gpx4b mRNA rescued the dorsalization induced by Wnt3a, β-CatΔN, and VP16-Tcf3ΔN in zebrafish embryos (Fig. 5A, B). Similarly, GPx4b inhibited Wnt reporter activity induced by Wnt3a, β-CatΔN, and VP16-Tcf3ΔN in zebrafish embryos (Fig. 5C). Conversely, loss of maternal gpx4b synergistically enhanced β-catΔN- and vp16-tcf3ΔN-induced expression of boz, chd, and sqrt at 4.3 hpf as indicated by qRT-PCR analysis (Fig. 5D). Likewise, in HEK293T cells, GPx4 knockdown synergistically enhanced Wnt3a- and VP16-Tcf3ΔN-induced Wnt reporter activity (Fig. 5E, F). Collectively, these data implied that GPx4/GPx4b inhibits Wnt/β-catenin signaling at the level of Tcf/Lef.
The GPx4/GPx4b Sec active site residue is dispensable for Wnt signaling inhibition.

The Sec forms the active center of the GPx4 selenoenzyme for hydroperoxide reduction. To test whether or not it is required for Wnt signaling inhibition, we generated Sec-to-Cys mutants (GPx4\textsuperscript{U73C} and GPx4b\textsuperscript{U67C}) and a deletion mutant GPx4b-C, lacking both the N-terminal and the Sec site. Like wild-type GPx4b, GPx4b\textsuperscript{U67C} inhibited endogenous Wnt reporter activity in HEK293T cells (Fig. 6A). Additionally, consistent with wild-type GPx4/GPx4b, both GPx4\textsuperscript{U67C} and GPx4\textsuperscript{U73C} ventralized zebrafish embryos (Fig. 6B, C). Similarly, GPx4b-C inhibited endogenous Wnt reporter activity in HEK293T cells (Fig. 6D). In addition, GPx4b-C was sufficient to inhibit the Wnt3a- and β-CatΔN-induced Wnt reporter activity in HEK293T cells as well as to neutralize their dorsalizing activity in zebrafish embryos (Fig. 6E, F). Taken together, these results indicated that the Sec residue is dispensable for Wnt signaling inhibition.

GPx4 interacts with Tcf/Lefs and occupies Wnt target gene promoters, and depletion of GPx4 enhances Tcf/Lefs binding to target promoters.

The above genetic interaction analysis suggested that GPx4/GPx4b inhibits Wnt/β-catenin signaling at the level of Tcf/Lef. In addition, a recent study has shown clearly that zebrafish GPx4 preferentially accumulates in the nucleus from the 128- to 512-cell stages (Mendieta-Serrano et al., 2015). In cultured HeLa cells, GPx4 is distributed in the cytosol and nucleus (Fig. 7A). These findings suggested that GPx4/GPx4b and Tcf/Lefs may interact with each other at some stages during embryogenesis as well as in some cell types. To address this possibility, we first tested whether GPx4 and Tcf/Lefs interact with each other at endogenous levels. Co-immunoprecipitation (co-IP) assay indicated that endogenous GPx4 specifically retrieved endogenous TCF3 and 4 in HEK293T cells (Fig. 7B). Accordingly, endogenous GPx4 was detectable in Myc-tagged immunoprecipitates of 4 Tcf/Lef family members in reciprocal co-IP assays (Fig. 7B). Moreover, when Myc-tagged Tcf3 and Flag-tagged zebrafish GPx4b were co-expressed in HEK293T cells, they were observed in the same complex (Fig. 7C). Next, a bimolecular fluorescence complementation (BiFC) assay, which was recently established to directly visualize the β-catenin–Tcf interaction in living cells (Ding et al., 2014), was used to assess the interaction of GPx4b and Tcf in HeLa cells. The BiFC signals indicated that GPx4b and GPx4b-C interact with both Tcf3 and Tcf3ΔN in the nucleus, whereas they interact with Tcf3ΔNLS (lacking the nuclear localization signal) in the cytoplasm (Fig. 7D and Fig. S6A).
These results indicated that GPx4b and GPx4b-C specifically and directly interact with Tcf3 in the nucleus and that the binding does not require the activation of Wnt signals. Moreover, BiFC signals derived from GPx4b–β-catenin were barely detected, suggesting that they do not directly interact in living cells (Fig. S6B). Since GPx4/GPx4b overexpression inhibits VP16-Tcf3ΔN action while GPx4/GPx4b depletion synergistically enhances VP16-Tcf3ΔN action, we postulated that GPx4/GPx4b inhibits VP16-Tcf3ΔN action by preventing Tcf3 binding to target gene promoters. To test this hypothesis, the mutant VP16-Tcf3(295-441) was used. VP16-Tcf3(295-441) lacks both activator β-catenin and repressor Groucho binding domains and contains only the high-mobility group DNA-binding domain, which is sufficient to induce Wnt activity (Lu et al., 2015). Co-injection of GPx4 or gpx4b mRNA with VP16-Tcf3(295-441) reduced VP16-Tcf3(295-441)-induced dorsalizing activity in zebrafish embryos (Fig. 7E, F). Accordingly, co-injection with gpx4b mRNA inhibited VP16-Tcf3(295-441)-induced Wnt reporter activity (Fig. 7G). As GPx4/GPx4b forms a complex with Tcf/Lefs, it is possible that GPx4/GPx4b occupies a Wnt-regulated promoter. To examine this possibility, we carried out chromatin immunoprecipitation (ChIP)-PCR experiments using HEK293T cells to determine whether or not GPx4 occupies the promoters of Wnt target genes. ChIP assays showed that endogenous GPx4 associated with the promoters of Wnt target genes such as AXIN2, CCND1, DKK1, and LEF1 but not with those of α-Satellite and GAPDH (Fig. 7H). Since GPx4/GPx4b inhibited VP16-Tcf3(295-441)-induced Wnt activity and associated with the promoters of Wnt target genes, we speculated that GPx4/GPx4b may associate with Tcf/Lefs at target promoters and act by rendering the association between Tcf/Lefs with the promoters of Wnt target genes. To test this hypothesis, we performed ChIP-qPCR experiments using GPx4-depleted HEK293T cells to determine the binding between Tcf/Lefs with the promoter sequences of Wnt target genes. As shown in Fig. 7I, GPx4 knockdown in HEK293T cells strengthened the association of TCF3 and 4, TCF4 alone, and LEF1 with the promoters of AXIN2 and DKK1. Taken together, these data indicated that GPx4 is associated with Tcf/Lefs at the promoters of the Wnt target genes and prevents Tcf/Lefs binding to their target gene promoters.
Discussion

In this study, we report that depletion of maternal GPx4b by CRISPR/Cas9-mediated knockout or MO-mediated knockdown promoted dorsal organizer formation in zebrafish embryos. Conversely, gain-of-function of gpx4b in zebrafish embryos resulted in ventralized embryos. A variety of data showed that GPx4/GPx4b participates in Wnt/β-catenin signaling and that maternal GPx4b affects embryonic dorsal organizer formation through regulation of this pathway. We observed upregulated expression of Wnt target genes and increased Wnt reporter activity after GPx4/GPx4b depletion in vivo and in vitro. Additionally, the expression areas of both maternal and zygotic Wnt target genes were largely expanded after GPx4b depletion in zebrafish. Forced expression of GPx4/gpx4b impeded the development of the dorsal organizer and inhibited Wnt reporter activity. Genetic interaction analysis between GPx4/GPx4b and an array of Wnt activators indicated that GPx4/GPx4b acts at the level of Tcf/Lef. Importantly, we also showed that GPx4/GPx4b interacts with Tcf/Lef family members and occupies Wnt target gene promoters, thereby preventing the association between Tcf/Lefs and their target promoters. The interaction between GPx4 and Tcf/Lefs results in the repression of Wnt/β-catenin target genes in the presence of Wnt signals. Intriguingly, catalytically inactivated GPx4/GPx4b also ventralized embryos and was sufficient to inhibit the Wnt/β-catenin signal, suggesting that the Sec residue is dispensable for Wnt inhibition. These findings suggested that GPx4/GPx4b is a novel inhibitor of the Wnt/β-catenin pathway.

An interesting observation made in this study is that maternal GPx4b regulates dorsal organizer formation in zebrafish embryos. Previous studies have reported that genetic ablation of GPx4 or targeted mutation of the active-site Sec in mice leads to embryonic lethality around E7.5, implying that the survival function of GPx4 is conferred by its peroxidase activity mediated through Sec (Imai et al., 2003; Ingold et al., 2015; Yant et al., 2003). In contrast, zygotic gpx4b-mutant fish are viable, morphologically normal, and fertile. This is not surprising as the zebrafish genome harbors two GPx4 genes which both have a Sec site. Another, GPx4a can rescue gpx4b morphants. Thus, GPx4a may partially compensate the genetic loss of function of GPx4b. In addition, we cannot exclude the possibility that the presence of maternal GPx4 protein may also have contributed to the rescue of the early developmental defect in zygotic gpx4b mutant embryos (Mendieta-Serrano et al., 2015). Intriguingly, M and MZ
mutants and morphants all exhibited an enlarged dorsal organizer at 4.3 hpf, and yet, they showed distinct phenotypes at 24 or 26 hpf. M mutants showed a weakly dorsalized phenotype, while dorsalization was relatively stronger in morphants. Our results indicate that genetic loss of maternal GPx4b but not gpx4b knockdown may induce the activation of a compensatory network in zebrafish embryos. The evidences include: 1) both M mutants and morphants displayed robust elevation of Wnt signaling although the strength of the dorsalized phenotype between them is largely different; 2) the transcript level of gpx4a was significantly upregulated in M mutant embryos but not in gpx4b-knockdown morphants at 4 hpf; 3) GPx4a and GPx4b had comparable ventralizing action. Similarly, Rossi et al., (2015) recently observed different phenotypes in egfl7-- genetic mutants and Egfl7 morphants and demonstrated that compensation mechanisms could be activated to buffer against deleterious mutations in zebrafish (Rossi et al., 2015). On the other hand, variable compensation may also contribute to penetrance and expressivity between intra- and inter-genetic lines. Recently, similar incomplete penetrance and variable expressivity observation in common even in a high-throughput analysis of developmental phenotypes in mice supported this view (Dickinson et al., 2016). Additionally, genetic knockout and MO-based knockdown have different effects; genetic knockout of gpx4b caused complete loss of GPx4b protein while the injected MO might be gradually diluted throughout embryonic development, with insufficient MO remaining to block the translation of zygotic gpx4b mRNA. As mentioned earlier, maternal Wnt promotes dorsal development while zygotic Wnt limits dorsal development. GPx4b inhibits both maternal and zygotic Wnt/β-catenin activity. Thus, a possible explanation for the MZ mutant phenotype is that these two events likely canceled each other out on dorsoventral patterning. The similar dorsoventral patterning between MZ mutants and morphants before the onset of gastrulation and distinct dorsoventral patterning between them after the onset of gastrulation support this view. This may underlie the different phenotypes observed in MZ mutants and morphants. In zebrafish, zygotic Wnt/β-catenin signaling is also involved in anteroposterior neuroectoderm patterning (Erter et al., 2001; Lekven et al., 2001). Although significantly increased zygotic Wnt activity was detected in the MZ gpx4b mutant embryos, no change in anteroposterior neural patterning was observed, indicating that there might be functional redundancy between GPx4 and other Wnt/β-catenin inhibitors. Similar dorsoventral patterning and anteroposterior neural patterning has
been recently observed in MZ mutant embryos of Ptk7, an inhibitor of Wnt/β-catenin (Hayes et al., 2013). In addition, the expression domains between gpx4b and ptk7 during gastrulation and segmentation stages were also overlapped. Similar phenotype in MZ mutant embryos suggest that other Wnt/β-catenin inhibitors may be functionally redundant with GPx4.

Another key finding in this study is that GPx4/GPx4b inhibits Wnt/β-catenin signaling at the level of Tcf/Lef and prevents the binding of Tcf/Lefs to promoter of Wnt target genes. We provided multiple lines of evidence to support the conclusion. First, epistasis experiments in zebrafish embryos suggested that inhibition of the Wnt pathway goes down to the inhibition of VP16-Tcf3ΔN. In addition, maternal loss of gpx4b in zebrafish embryos synergistically elevated expression of vp16-tcf3ΔN-induced maternal Wnt target genes. Likewise, depletion of GPx4 in cultured HEK293T cells synergistically augmented VP16-Tcf3ΔN-induced Wnt reporter activity. Second, co-IP assays confirmed that GPx4 and TCF3/4 are in a same complex under physiological conditions and that endogenous GPx4 interacts with 4 Myc-tagged Tcf/Lef family members. Importantly, BiFC signals derived from GPx4b and Tcf3, Tcf3ΔN, and Tcf3ΔNLS but not from GPx4b–β-catenin were detected when these proteins were co-expressed in HeLa cells, suggesting that GPx4b and Tcf3 do directly interact in living cells. Third, ChIP-PCR experiments showed that endogenous GPx4 occupies the promoters of AXIN2, CCND1, DKK1, and LEF1. Fourth, GPx4/GPx4b inhibited VP16-Tcf3(295-441)-induced activity. When GPx4 was depleted, the association of TCF3 and 4, TCF4 alone, and LEF1 with the promoters of AXIN2 and DKK1 was strengthened. These data strongly suggest that GPx4/GPx4b interacts with Tcf/Lefs, occupies the promoters of Wnt target genes, and prevents Tcf/Lefs to bind target genes promoter. It should be noted that Tcf3 has been shown to function primarily as a transcriptional repressor in various contexts (Dorsky et al., 2003; Kim et al., 2000; Wu et al., 2012; Yi et al., 2011). Our results indicate that GPx4 also binds to Tcf3 to inhibit its action. The physiological effect in the interaction between GPx4 and Tcf3 need further investigation.

We demonstrated that GPx4/GPx4b Sec activation is not required for Wnt signaling inhibition. The evidences include: 1) both Sec-to-Cys point mutants as well as N-terminal and Sec-site deletion mutants had similar Wnt inhibitory activity as wild-type GPx4/GPx4b in zebrafish embryos and cultured cells; 2) human and zebrafish Sec-to-Cys point mutants had
similar ventralizing action in zebrafish embryos; 3) N-terminal and Sec-site deletion mutant and Tcf3, Tcf3ΔN, and Tcf3ΔNLS produced BiFC signals when they were co-expressed in HeLa cells. It should be mentioned that, although inactivation of Sec was sufficient to inhibit Wnt activity, we cannot exclude that GPx4/GPx4b might partially affect Wnt signaling indirectly. For example, depletion of GPx4 leads to increased cellular ROS, which activates multiple signaling pathways including Wnt/β-catenin signaling (Funato et al., 2006; Funato et al., 2010; Kajla et al., 2012; Love et al., 2013; Rharass et al., 2014; Sandieson et al., 2014; Wen et al., 2012). Future studies are needed to elucidate such additional mechanisms. In addition, GPx4 might modulate other components of Wnt signaling: this needs to be investigated in future.

In summary, we characterized the phenotypes of gpx4b-deficient zebrafish embryos in detail. Our study revealed a novel function of selenoprotein GPx4 as an inhibitor of canonical Wnt signaling that prevents the association of Tcf/Lef5s with the promoters of Wnt target genes. The findings reported here will improve our understanding of the molecular mechanism of GPx4/GPx4b function at the cellular level and in embryonic development. Although the active-site Sec of GPx4 is dispensable for Wnt inhibition, selenium in the form of Sec is a prerequisite for GPx4 synthesis. Dietary selenium has been clearly shown to influence the translational efficiency of GPx4 in rats (Weiss Sachdev and Sunde, 2001). This raises interesting questions about the relations between dietary selenium, GPx4, Wnt/β-catenin signaling activity, and even disease, providing scope for future research.
Materials and Methods

Zebrafish strains.
The wild-type zebrafish (Danio rerio) strain Tübingen was used in this study. The gpx4b knockout mutant strain was constructed using the CRISPR/Cas9 system (Chang et al., 2013). Embryos obtained by natural cross were kept in embryo rearing solution at a standard 28.5°C as described previously (Rong et al., 2014). Embryos were strictly staged according to standard methods (Kimmel et al., 1995). All experimental protocols were approved by and conducted in accordance with the Ethical Committee of Experimental Animal Care, Ocean University of China.

Cell Culture and Luciferase Assays.
HEK293T cells were purchased from ATCC. Cell culture, transfection, and luciferase assay were performed as described previously (Feng et al., 2012). The in vivo luciferase assay was performed as reported previously (Rong et al., 2014).

Stable GPx4 Knockdown Cell Line Construction.
The stable GPx4 knockdown cell line was established by lentiviral delivery of shRNA in the HEK293T cell line. The interference sequence (GTGGATGAAGATCCAACCC) was designed and examined by Sigma.

Capped mRNA synthesis, Morpholino, and Microinjection.
Capped mRNA was synthesized using the mMESSAGE mMACHINE Kit. To knockdown gpx4b, a translation-blocking MO oligonucleotide targeting gpx4b was purchased and diluted as described previously (Rong et al., 2014). Diluted MO and/or mRNA were injected into one-cell stage zebrafish embryos. A GFP reporter plasmid containing the 5’-UTR and partial ORF (-27–114 bp) of zebrafish gpx4b was constructed and used to examine the efficiency of the MO.

Immunocytochemistry and BiFC.
For immunocytochemistry, HeLa cells grown on a coverslip were fixed with 4% paraformaldehyde for 20 min at room temperature, followed by 0.2% Triton X-100 treatment for 5 min and blocking with 20% BSA. The cells were then incubated with corresponding primary and secondary antibodies along with DAPI for visualization of the nuclei.

Plasmids for BiFC assays were a gift from Dr. Wei Wu (Ding et al., 2014). Zebrafish GPx4b^U67C and GPx4b-C were subcloned into the pCDNA3.1-Myc-VN vector and pCDNA3.1-
Flag-VC vector. HeLa cells were transfected with the constructs and Venus fluorescent protein was selected as the reporter for complementation.

Fluorescence images were acquired with a Leica TCS SP8 confocal microscope.

**Co-IP, Western Blotting, and ChIP Assay.**

Western blotting and Co-IP were performed mainly as described previously (Bai et al., 2014). ChIP assays were conducted using a ChIP assay kit (Millipore) according to the manufacturer’s protocol.

**Statistical Analysis.**

Data are presented as the means±s.e.m. Differences among groups were analyzed using GraphPad Prism version 5.01 (San Diego, CA, USA). Statistical significance was accepted at $P < 0.05$.

Detailed descriptions of experimental procedures, chemicals, reagents, antibodies, and primers are provided in the SI Text.
Author contributions

XR designed and performed the experiments, analyzed the data, and wrote the manuscript; YZ, YL, BZ, BW, CW, XG, and PT performed the experiments; LL, YL, and CZ provided analytical tools and reagents; and JZ supervised experimental design, data analysis, and wrote the manuscript.

The authors declare no conflict of interest.

Acknowledgements

We are grateful to Dr. Cunming Duan of the University of Michigan, Ann Arbor, and Dr. Shaojun Du of the University of Maryland, Baltimore, for critical suggestions. We thank Dr. Wei Wu of Tsinghua University and Dr. Ying Cao of Nanjing University for providing reagents. We thank Dr. Hiroyasu Kamei, Graduate School of Agriculture and Life Sciences, The University of Tokyo, for critical reading this manuscript. This work was supported by grants from Major Science Programs of China [2011CB943800 to J.Z. and Y.Li], NSFC-Shandong Joint Fund [U1406402 to J.Z.], and National Science Foundation of China [30972238 to J.Z., 31572261 to L.L., and 31601863 to X.R.], the Fundamental Research Funds for the Central Universities [201562028 to J.Z.], the Scientific and Technological Innovation Project Financially Supported by Qingdao National Laboratory for Marine Science and Technology [2015ASKJ02 to J.Z.], and program for Talents in Qingdao National Laboratory for Marine Science and Technology [C. Z.].
References


Funato, Y., Terabayashi, T., Sakamoto, R., Okuzaki, D., Ichise, H., Nojima, H., Yoshida, N. and Miki, H.


Fig. 1. Generation of the gpx4b mutant using the CRISPR/Cas9 system, and the gross phenotypes of maternal and maternal-zygotic loss of gpx4b in zebrafish embryos.

(A) Schematic representation of the gpx4b locus. Exons are shown as boxes (filled box, protein coding region; open box, UTR). Introns are shown as lines. The location of the Sec codon, TGA, is indicated with a red line. The green line indicates the location of the Cas9 target site. Black arrows show the position of genotyping primers. (B) The sequence of Cas9 target site and predicted protein sequences of wild-type (WT) and mutant gpx4b alleles (allele1 and allele2).

Upper panel: The sequence of the Cas9 target site is indicated with a black line and the two gpx4b null mutant alleles are shown. The black dashed line indicates a deletion. Red line, restriction enzyme BcI1301 recognition sequence. Lower panel: the protein sequences of WT and mutants are shown. (C) Genotyping result using restriction enzyme BcI1301 digestion assay. (D) Representative views of WT as well as maternal (M) and maternal-zygotic (MZ) mutants of gpx4b at the indicated stages. The arrow shows the reduced ventral tail fin. The frequency of embryos with the indicated patterns is shown in the bottom right corner of each panel. n = 4 females for allele1, n = 3 females for allele2. At least two clutches of embryos from each female were analyzed. Lateral views with anterior to the left. Scale bar = 200 µm.
Maternal loss of \textit{gpx4b} promotes dorsal organizer development and depletion of \textit{gpx4b} increases Wnt/\(\beta\)-catenin activity.

(A) Expression of dorsal organizer markers \textit{chd} and \textit{gsc} mRNA in each indicated group of embryos at 4.3 hpf as assessed by WISH. Upper panels are animal pole views with dorsal to the right. Arrows indicate the edges of the \textit{chd} and \textit{gsc} mRNA expression domains. Lower panels are dorsal views with animal pole up. The frequency of embryos with the indicated patterns is shown in the bottom right corner of each group.

(B) Quantification of the arc of marker expression shown in A.

(C) The expression levels of \textit{boz}, \textit{chd}, and \textit{sqt} mRNA in each indicated group of embryos at 4.3 hpf as analyzed by qRT-PCR.

(D) Expression of direct
zygotic Wnt markers cdx4, sp5l, and tbx6 in MZallele1 mutants and WT embryos at 80% or 90% epiboly stage (9 hpf). Asterisks indicate the edges of the indicated mRNA expression domains. Lateral views with dorsal to the right and animal pole up. (E) The mRNA expression levels of indicated zygotic Wnt direct target genes in WT and MZallele1 mutant embryos at 8 hpf analyzed by qRT-PCR. (F) MZallele1 mutants show increased endogenous Wnt signaling. Values are means±s.e.m. (n = 3). *P < 0.05; **P < 0.01. Unpaired t test, Two-tailed. Scale bar = 200 µm.
Fig. 3. Overexpression of gpx4b ventralizes zebrafish embryos and inhibits Wnt activity.
(A) Classification of phenotypes at 24 hpf caused by forced expression of 600 pg gpx4b mRNA.
(B) Percentage of embryos in each category as shown in A. Results are from three independent experiments and the total embryo numbers are given at the top. (C) Expression patterns of chd and gsc marker genes in embryos injected with 600 pg gfp or gpx4b mRNA at 4.3 hpf. The frequency of embryos with the indicated phenotypes is shown in the bottom right corner of each group. (D) Overexpression of GPx4b inhibits endogenous Wnt signaling in vivo. Embryos were injected with TOPFlash reporter DNA with 600 pg gfp or gpx4b mRNA. (E) GPx4b inhibits endogenous Wnt signaling in vitro. HEK293T cells were transfected with 600 ng GFP plasmid DNA or GPx4b plasmid DNA together with TOPFlash reporter DNA. Values are means ± s.e.m. (n = 3). *P < 0.05; **P < 0.01. Unpaired t test, Two-tailed. Scale bar = 200 µm.
Fig. 4. Wnt inhibitory activity is evolutionarily conserved between zebrafish GPx4b and human GPx4.

(A) The phenotypes of embryos injected with 600 pg gfp or GPx4 mRNA were scored and presented following the criteria described in Fig. 3A. Results are from three independent experiments and the total embryo numbers are given at the top.

(B) Human GPx4 alters the expression of chd and gsc in zebrafish embryos at 4.3 hpf. Embryos injected with 600 pg gfp or GPx4 mRNA were analyzed by WISH. The frequency of embryos with the indicated phenotypes is shown in the bottom right corner of each group.

(C) Effectiveness of GPx4 shRNA at the protein level in HEK293T cells. shCtrl, control shRNA. β-Tubulin, internal control.

(D) Knockdown of GPx4 enhances endogenous Wnt signaling in vitro. The stable control or GPx4 knockdown lines were transfected with TOPFlash reporter DNA. (E) Stable knockdown of GPx4 in HEK293T cells increased the expression of indicated Wnt direct target genes. Expression levels were analyzed by qRT-PCR. Values are means±s.e. (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001. Unpaired t test, Two-tailed. Scale bar = 200 µm.
Fig. 5. GPx4/GPx4b inhibits Wnt activity at Tcf level.

(A,B) GPx4b inhibits the action of indicated Wnt activators in vivo. Representative images of embryos injected with 680 pg gfp mRNA, mRNA of each indicated Wnt activator (20 pg wnt3a, 50 pg β-catΔN, and 80 pg vp16-Tcf3ΔN), and mRNA of each indicated Wnt activator plus 600 pg gpx4b mRNA at 12.5 hpf are shown in A. Quantitative results are shown in B. The frequency of embryos with the indicated phenotypes is shown in A.

(C) GPx4b inhibits Wnt activity induced by each indicated Wnt activator in vivo. Embryos were injected with TOPFlash reporter DNA with the indicated mRNAs (20 pg wnt3a, 50 pg β-catΔN, and 80 pg vp16-Tcf3ΔN).

(D)
Maternal loss of GPx4b synergistically elevated β-catΔN- and vp16-tcf3ΔN-induced expression of boz, chd, and sqrt at 4.3 hpf. One-cell stage WT and maternal mutant embryos were injected with gfp (40 pg), β-catΔN (25 pg), or vp16-tcf3ΔN (40 pg) mRNA, injected embryos were raised to 4.3 hpf and the expression levels of the indicated target genes were measured by qRT-PCR. (E, F) Knockdown of GPx4 synergistically enhances Wnt3a- and VP16-Tcf3ΔN-induced Wnt reporter activity. The indicated plasmid DNA (5 ng) was co-transfected with TOPFlash plasmid DNA into control or GPx4-knockdown HEK293T cells and the luciferase activity was measured. Results are from three independent experiments. Values are means±s.e.m. (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001. Unpaired t test, Two-tailed. Scale bar = 200 µm.
Fig. 6. Sec is dispensable for Wnt inhibition.

(A) GPx4b\(^{WT}\) and GPx4b\(^{U67C}\) inhibit endogenous Wnt activity. The indicated plasmid DNA (200 ng) was co-transfected with TOPFlash plasmid DNA into HEK293T cells. (B) Classification of ventralized phenotypes at 24 hpf caused by forced expression of 600 pg \(gpx4b^{U67C}\) or \(GPx4^{U73C}\) mRNA. (C) Percentages of embryos in each category as shown in B. Results are from three independent experiments and the total embryo numbers are given at the top. (D) GPx4b-C inhibits endogenous Wnt activity. The indicated plasmid DNA (600 ng) was co-transfected with TOPFlash plasmid DNA into HEK293T cells and the luciferase activity was measured. (E) GPx4b-C inhibits Wnt3a and β-Cat\(ΔN\) activity in vitro. The indicated plasmid DNA (20 ng Wnt3a and 50 ng β-Cat\(ΔN\)) was co-transfected with TOPFlash plasmid DNA into HEK293T cells and the luciferase activity was measured. (F) GPx4b-C inhibits Wnt3a and β-Cat\(ΔN\) action in vivo. Quantitative results are shown as in Fig. 5B. Embryos injected with 650 pg \(gfp\) mRNA, mRNA of each indicated Wnt activator (20 pg \(wnt3a\) and 50 pg β-\(catΔN\)) and mRNA of each indicated Wnt activator plus 600 pg \(gpx4b-c\) mRNA at 12.5 hpf. The total embryo numbers from three independent experiments are shown on top of each bar. Values are means±s.e.m. (n = 3). *\(P < 0.05\); ***\(P < 0.001\). Unpaired \(t\) test, Two-tailed. Scale bar = 200 µm.
Fig. 7. GPx4/GPx4b binds to and prevents Tcf/Lef binding to target promoters.

(A) Distribution of endogenous GPx4 protein in HeLa cells, visualized by immunofluorescence (green) with an anti-GPx4 antibody. Nuclei (blue) were stained with DAPI. Scale bar = 75 μm.

(B) Endogenous GPx4 interacts with Tcf/Lef members as indicated by co-IP. Left panel: GPx4 interacts with TCF3/4 in HEK293T cells. Right panel: 4 Myc-tagged Tcf/Lef members interact with endogenous GPx4 in HEK293T cells. The indicated proteins were immunoprecipitated or detected using appropriate antibodies. Vector, empty expressing plasmid with myc tag; WCL, whole-cell lysate; IP, immunoprecipitation; Input, positive control; IgG, negative control; arrow, IgG heavy chain.

(C) Exogenous GPx4b interacts with Tcf3. Proteins were extracted from lysates of cells that co-expressed tagged Tcf3 and GPx4b, immunoprecipitated, and subjected to Western blot analysis using the indicated antibodies. (D) GPx4b interacts with each indicated form of Tcf3 in living cells as indicated by BiFC assay. Flag-tagged GPx4b-VC expression vector was co-transfected with the indicated forms of Tcf3-VN. Expression of GPx4b detected by anti-Flag antibody immunostaining (red). NC, negative control. Scale bar = 50 μm. (E,F) Zebrafish GPx4b and human GPx4 inhibit VP16-Tcf3(295-441) action in vivo. One-cell stage
WT embryos were injected with indicated mRNAs (680 pg gfp mRNA alone or 80 pg vp16-tcf3(295-441) plus 600 pg gfp, gp4b, or GPx4 mRNA). Representative images of each group of injected embryos at 12.5 hpf are shown in E. Quantitative results of the indicated phenotypes are shown in F. The frequency of embryos with the indicated phenotypes is shown in E. Scale bar = 200 μm. (G) GPx4b inhibits VP16-Tcf3(295-441)-induced activity in vivo. Embryos were injected with TOPFlash reporter DNA with the indicated mRNAs (680 pg gfp mRNA alone or 80 pg vp16-tcf3(295-441) plus 600 pg gfp or gp4b mRNA). (H) Endogenous GPx4 is associated with the promoters of the indicated direct Wnt target genes in HEK293T cells as indicated by ChIP assay. a-Satellite and GAPDH promoters were used as negative controls. (I) Knockdown of GPx4 enhances the binding of TCF3/4, TCF4 alone, and LEF1 to the AXIN2 and DKK1 promoters in HEK293T cells. Values are means±s.e.m. (n = 3). *P < 0.05; ***P < 0.001. Unpaired t test, Two-tailed.
Supplemental Materials and Methods

Chemicals, Reagents, and Antibodies.

M-MLV Reverse Transcriptase and the Dual-Glo™ Luciferase Assay System were purchased from Promega (Madison, WI, USA). iQ SYBR Green Supermix was purchased from Bio-Rad (Hercules, CA, USA). mMESSAGE mMACHINE mRNA synthesis kit was purchased from Ambion (Austin, TX, USA). DIG-UTP and Anti-Digoxigenin-AP were purchased from Roche (Indianapolis, IN, USA). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), and T4 DNA ligase were purchased from Thermo (Waltham, MA, USA). OPTIMEM I reduced serum medium and Lipofectamine 2000 transfection agent were purchased from Invitrogen (Carlsbad, CA, USA). Morpholino oligonucleotides were purchased from Gene Tools, LLC (Philomath, OR, USA).

The following antibodies were used in this study: rabbit anti-GPx4 (1:200 for immunocytochemistry, 1:500 for Western blot, and 2 µg for co-IP and ChIP assay, Santa Cruz, sc-50497), mouse anti-TCF3+4 (1:1000 for Western blot, 2 µg for co-IP and ChIP assay, Abcam, ab12065), rabbit anti-TCF4 (2 µg for ChIP assay, Cell Signaling, #2569), mouse anti-LEF1 (4 µg for ChIP assay, Millipore, #17-604), mouse anti-c-Myc (1:1000, Santa Cruz, sc-40), murine anti-Flag (1:1000, Sigma, F1804), rabbit anti-β-tubulin (1:1000, Cell Signaling, #2146).

Primers and sequence information are listed in Table S1.

Molecular Cloning and Plasmids Construction.

The full-length cDNA of zebrafish gpx4a and gpx4b and human GPx4 with/without 3’-UTR were amplified and subcloned into the pCS2+ expression vector. Zebrafish GPx4b^{U76C} (full length with the 67th Sec mutated into Cys) and human GPx4^{U73C} (full length with the 73th Sec mutated into Cys) were generated by site-directed mutagenesis method. GPx4b-C (79-aa deletion in N-terminus) was also amplified and subcloned into the pCS2+ expression vector. The sequences of human GPx4 and mouse GPx4 (Fig. S1A) were retrieved from NCBI (www.ncbi.nlm.nih.gov). The amino acid sequence alignment was performed using ClustalW and GeneDoc (Free Software Foundation).

Cell Culture and Luciferase Assays.

HEK293T cells were maintained in DMEM supplied with 10% FBS. Cells were seeded into 12-well plates to reach 70%–80% confluence at the time of transfection. Plasmids were
transfected in duplicate with Lipofectamine 2000. Luciferase activities were measured 24 h after transfection using the Dual-Luciferase assay kit. Plasmids were co-transfected with 250 ng TOPFlash DNA and 50 ng Renilla DNA. Empty pCS2+ vector was used both as control and to adjust the DNA amount to 1.0–1.5 µg/well. The in vivo luciferase assay was performed as reported previously (Rong et al., 2014). In brief, one-cell stage embryos were injected with 100 pg TOPFlash DNA and 20 pg Renilla plasmid DNA, or MO and/or mRNA plus 100 pg TOPFlash DNA and 20 pg Renilla plasmid DNA, and raised to the shield stage. Two independent groups of embryos (each with >20 embryos) were lysed in passive lysis buffer. The luciferase reporter assay was performed using a Dual-Luciferase Assay Kit. The TOPFlash luciferase activity was normalized to the Renilla luciferase activity.

**Stable GPx4 Knockdown Cell Line Construction.**

The stable GPx4 knockdown cell line was established by lentiviral delivery of shRNA in the HEK293T cell line. The lentiviral pLKO.1-GFP+Puromycine vector was selected as the shuttle vector. shRNA constructs targeting human GPx4 and negative control shRNA were purchased from GeneChem (Shanghai, China). The shGPx4 plasmid and two packaging plasmids were co-transfected into HEK293T cells. Serum-free DMEM was added 24 h after transfection, and 48 h later, supernatant was collected and filtered through a 0.22 µm filter. Viral supernatant containing 8 µg/mL polybrene was added to 30–40% confluent HEK293T cells for infection. After 48 h of incubation, fresh DMEM was added for 24 h. The EGFP gene was engineered into the vector as a reporter gene for identification, and the puromycin resistance gene was utilized as a selectable marker for stably transformed mammalian cell lines. Fresh DMEM containing 0.5 µg/mL puromycin was added every day for at least one week. GPx4 knockdown efficiency was detected by measuring the endogenous GPx4 protein level.

**RT-PCR and Whole Mount in situ Hybridization.**

Total RNA was isolated from zebrafish embryos using RNAiso Plus and then reverse transcribed into first-strand cDNA using M-MLV with Oligo (dT)₁₈ as primer. RT-PCR was carried out using premix Taq DNA polymerase. qRT-PCR was performed in an iCycler iQ Multicolor real-time PCR detection system (Bio-Rad Laboratories). Samples from 3 independent experiments were used and each sample was measured in duplicate. The mRNA levels of the genes of interest were calculated using the 2⁻ΔΔCt method and normalized to β-actin
mRNA levels (Rong et al., 2014).

**Co-IP and Western Blotting.**

Western blotting and Co-IP were performed mainly as described previously (Bai et al., 2014). Briefly, HEK293T cells were transfected with pCDNA3.1-Myc-Tcf1, -Tcf3, -Tcf4, and -Lef1. Additionally, cells were co-transfected with pDNA3.1-Myc-Tcf3 and pCS2-Flag-GPx4b. HEK293T cells at 90% confluence or 48 h after transfection were lysed and the supernatant was incubated with appropriate antibody and protein A/G PLUS-Agarose (Santa Cruz) to enrich for target proteins. The bead-bound proteins were then eluted and subjected to immunoblotting.

**ChIP Assay.**

ChIP assays were conducted using a ChIP assay kit (Millipore) according to the manufacturer’s protocol. Briefly, HEK293T cells were fixed with fresh formaldehyde. Cells ($2 \times 10^7$) were used in each ChIP reaction. Chromatin in cell lysates was sheared to ~300–900 base pairs in length using a VCX 130 Sonicator (Sonics & Materials, Inc.; 18 $\times$ 10 s on, 17 $\times$ 10 s pulses, 30% amplitude. ChIP sample kept on ice water). Precipitated DNA samples were analyzed by semi-quantitative PCR or qPCR. Samples from 3 independent experiments were used and each sample was measured in duplicate. The semi-quantitative PCR figures shown are representative results. qPCR data were expressed as the percentage of input DNA.

**Supplemental reference**


Supplementary Figures

A

<table>
<thead>
<tr>
<th>human GPx4</th>
<th>mouse GPx4</th>
<th>zebrafish GPx4b</th>
<th>zebrafish GPx4a</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSLGRLCRLIKPPALLCSGAAPLCTMCASRDWRCAARMHEFSAKIDID</td>
<td>MSWGRSLCSRLKPPALLCSGAAPLCTMCASRDWRCAARMHEFSAKIDID</td>
<td>SFQRALLVGAOGSKSFIRANCAANDWQSAKSDKEFSAKIDID</td>
<td>SFITGSAVVVFSLVQ----TESAQLEDWOTAKSKIFETATID</td>
</tr>
<tr>
<td>GMYNLQGDYRPGFQIVTNVASQUGKTEVNYTQLVLHARYBECLKRLAF</td>
<td>GMYCLQGDYRPGFQIVTNVASQUGKTEVNYTQLVLHARYBECLKRLAF</td>
<td>GRDYSLEKYRGGVITINVASKUGTPVNYTQLAAHVTYAEKGLRLCF</td>
<td>GENEVSLEKYRGGVITINVASKUGTPVNYSQAEMHAKYBGRGLRLAF</td>
</tr>
<tr>
<td>PCNQPGKQEIPGSNIEIKEFACAGYNKMDFMSKIDVNGDAAHPLWKLKWMTQ</td>
<td>PCNQPGKQEIPGSNIEIKEFACAGYNKMDFMSKIDVNGDAAHPLWKLKWMTQ</td>
<td>PCNQPGKQEIPGSNIEKEFACAGYNKMDFMSKIDVNGDAAHPLWKLKWMTQ</td>
<td>PCNQPGKQEIPGSNIEKEFACAGYNKMDFMSKIDVNGDAAHPLWKLKWMTQ</td>
</tr>
<tr>
<td>PKGGLGNAIKMNFCTKFLKIDNKGCVKRYSDMDPLVIEKDLFHYF: 197</td>
<td>PKKRMGLNAIKMNFCTKFLKIDNKGCVKRYSDMDPLVIEKDLFHYF: 197</td>
<td>PKGGLGNAIKMNFCTKFLKIDNKGCVKRYSDMDPLVIEKDLFHYF: 197</td>
<td>PKGGLGNAIKMNFCTKFLKIDNKGCVKRYSDMDPLVIEKDLFHYF: 197</td>
</tr>
<tr>
<td>ENKGFNLQGDKMNFTKFLINREGCVKRYSDMDPSVEKDLFHYF: 186</td>
<td>ENKGFNLQGDKMNFTKFLINREGCVKRYSDMDPSVEKDLFHYF: 186</td>
<td>ENKGFNLQGDKMNFTKFLINREGCVKRYSDMDPSVEKDLFHYF: 186</td>
<td>ENKGFNLQGDKMNFTKFLINREGCVKRYSDMDPSVEKDLFHYF: 186</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>gpx4b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

Supplementary Fig. 1

1 cell | 4 cell | 2 hpf | 6 hpf | 12 hpf | 16 hpf | 24 hpf | 48 hpf, antisense | 48 hpf, sense
Fig. S1 Amino acid sequence alignment of GPx4 and spatiotemporal expression pattern of zebrafish gpx4b.

(A) Amino acid sequence alignment of human, mouse, and zebrafish GPx4. Identical amino acids are indicated in black and similar amino acids are indicated in grey. The Sec residue (U) is indicated with an asterisk and highlighted in red. The accession numbers are as follows: human GPx4 NP_002076.2, mouse GPx4 NP_032188.3, zebrafish GPx4b NP_001025241.2, zebrafish GPx4a NP_001007283.2. (B) RT-PCR analysis of zebrafish gpx4b mRNA at the indicated embryonic stages. Numbers indicate different developmental stages as hours post fertilization (hpf). β-actin was used as internal control. +, positive control; -, negative control. (C) Whole-mount in situ hybridization analysis of zebrafish gpx4b mRNA at the indicated stages. Panels are dorsal, top, or lateral views with animal pole up or anterior to the left. Scale bars = 200 µm.
Supplementary Fig. 2
Fig. S2 Knockdown of gpx4b results in dorsalized embryos and loss of gpx4b upregulates the expression of gpx4a.

(A) Effectiveness of the MO used. Fluorescence micrographs of zebrafish embryos at 12 hpf injected with the gpx4b-5’-UTR reporter plasmid DNA (50 pg) and control MO (cMO, 4 ng) or gpx4b-targeting MO (MO, 4 ng). (B) Validation of the GPx4b expression plasmid used. Top: Schematic representation of the constructed expression plasmid. A Flag tag was inserted just before the stop codon of the ORF. Bottom: Western blot showing the expression of Flag-tagged GPx4b. The Flag-tagged GPx4b expression vector was transfected into HEK293T cells, and lysates were analyzed by immunoblotting with an anti-Flag antibody. GFP expression vector was used as a negative control. (C) Classification of phenotypes caused by MO-mediated knockdown of gpx4b. Representative images of zebrafish embryos at 26 hpf injected with 6 ng control MO (cMO), 6 ng MO, or 6 ng MO plus 50 pg gpx4b mRNA (MO+gpx4b). Lateral views with anterior to the left. Scale bar = 200 µm. (D) Percentages of embryos in each category as shown in C. (E) The antagonizing effect of GPx4a to gpx4b knockdown in zebrafish embryos. One-cell stage embryos were injected with 6 ng gpx4b MO alone or 6 ng gpx4b MO plus 50 pg gpx4a mRNA. The phenotypes of injected embryos were scored and presented following the criteria shown in C. The results are from three independent experiments and the total embryo numbers are given at the top. (F) The transcript levels of gpx4a and gpx4b in gpx4b WT (WT and cMO), mutant (M and MZ), and morphant (MO) embryos at indicated stages. 6 ng cMO or gpx4b MO were injected at one-cell stage. Each group of embryos were raised and collected at indicated stage, the transcript levels of gpx4a and gpx4b were then measured by qRT-PCR. Values are means±s.e.m. (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. Unpaired t test, Two-tailed.
Fig. S3 Dorsoventral and anteroposterior patterning in *gpx4b* M and MZ mutants and morphants.

(A) Expression of dorsal organizer markers in M<sup>allele2</sup> and MZ<sup>allele2</sup> mutants compared to WT embryos at 4.3 hpf as revealed by WISH. Upper panels are animal pole views with dorsal to the right. Arrows indicate the edges of the *chd* and *gsc* mRNA expression domains. Lower panels are dorsal views with animal pole up. (B) Quantification of the arc of marker expression shown in A. (C) Effects of *gpx4b* knockdown on the expression of the indicated dorsal organizer marker genes at 4.3 hpf as revealed by WISH. (D) Quantification of the arc of marker expression shown in C. (E,F) Expression of the indicated dorsoventral markers in M and MZ mutants at 6 hpf as revealed by WISH. (G) Quantification of the arc of each marker expression shown in E and F. (H) Expression of the indicated dorsoventral markers in *gpx4b* morphants compared to cMO injected embryos at 6 hpf as revealed by WISH. (I) Quantification of the arc of marker expression shown in H. (J) Expression of the indicated anteroposterior neural markers in *gpx4b* mutants at 12.5 hpf. The frequency of embryos with the indicated pattern is shown in the bottom right corner of each group. Total embryo numbers are given at the top of each bar in B, D, G, and I. Asterisk and arrow in panels indicate the edges of the indicated mRNA expression domains. t, top view with dorsal to the right; d, dorsal views with animal pole up; l, lateral view with dorsal to the right. Values are means±s.e.m. (n = 3). ***P < 0.001. n.s., not significant. Unpaired t test, Two-tailed. Scale bar = 200 µm.
**Fig. S4 Wnt activity in gpx4b MZ<sup>allele2</sup> mutants and morphants.**

(A) The expression of *boz*, *chd*, and *sqt* mRNA in each indicated group of embryos at 4.3 hpf as analyzed by qRT-PCR. (B) Expression of direct zygotic Wnt markers *cdx4*, *sp5l*, and *tbx6* in MZ<sup>allele2</sup> mutants compared to WT embryos at 9 hpf as revealed by WISH. (C) Effects of *gpx4b* knockdown on the expression of *cdx4* and *sp5l* at 9 hpf as revealed by WISH. The frequency of embryos with the indicated patterns is shown in the bottom right corner of each panel. Asterisks indicate the edges of the indicated mRNA expression domains. 1, lateral views with dorsal to the right and animal pole up. (D) The mRNA expression levels of indicated zygotic Wnt direct target genes in WT and MZ<sup>allele2</sup> mutant embryos at 8 hpf analyzed by qRT-PCR. (E) Knockdown of *gpx4b* increased Wnt/β-catenin reporter activity. One-cell stage embryos were injected with TOPFlash reporter DNA together with cMO (6 ng), MO (6 ng), or 50 pg *wnt3a* mRNA. Values are means ±s.e.m. (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001. Unpaired t test, Two-tailed. Scale bar = 200 µm.
Supplementary Fig. 5
Fig. S5 Dorsoventral patterning and Wnt activity in \textit{gpx4b} mutants assessed by genotyping.
(A) Representative expression of \textit{gsc} and \textit{eve1} in progeny of allele1 homozygous female cross to heterozygous males at 6 hpf as revealed by WISH. Animal pole views with dorsal to the right. Arrows indicate the edges of the \textit{gsc} and \textit{eve1} mRNA expression domains. (B) Genotyping results of individual embryos with indicated expression domain as shown in A. (C) Representative expression of \textit{cdx4} in progeny of allele1 homozygous female cross to heterozygous male at 80\% epiboly as revealed by WISH. Asterisks indicate the edges of the \textit{cdx4} mRNA expression domains. (D) Genotyping results of individual embryos with indicated expression domain as shown in C. (E) Genotyping results of individual embryos with representative normal and dorsalized phenotypes (according to Figure 1D). (F) Quantification of genotyping results shown in E. (G) Genotyping results of individual embryos with representative normal and dorsalized phenotypes (according to Figure 1D). (H) Quantification of genotyping results shown in G. \(n = 5\) females for allele1, \(n = 3\) females for allele2. Scale bar = 200 \(\mu m\). M, marker; Ht, heterozygous mutant; Mt, homozygous mutant.
**Supplementary Fig. 6**

(A) **anti-Flag**

<table>
<thead>
<tr>
<th>Myc-VN-</th>
<th>NC</th>
<th>Tcf3</th>
<th>Tcf3ΔNLS</th>
<th>Tcf3ΔN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venus</td>
<td><img src="image" alt="Venus" /></td>
<td><img src="image" alt="Venus" /></td>
<td><img src="image" alt="Venus" /></td>
<td><img src="image" alt="Venus" /></td>
</tr>
<tr>
<td>Cy3</td>
<td><img src="image" alt="Cy3" /></td>
<td><img src="image" alt="Cy3" /></td>
<td><img src="image" alt="Cy3" /></td>
<td><img src="image" alt="Cy3" /></td>
</tr>
<tr>
<td>DAPI</td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
</tr>
<tr>
<td>merge</td>
<td><img src="image" alt="Merge" /></td>
<td><img src="image" alt="Merge" /></td>
<td><img src="image" alt="Merge" /></td>
<td><img src="image" alt="Merge" /></td>
</tr>
</tbody>
</table>

(B) **anti-Myc**

<table>
<thead>
<tr>
<th>Myc-VN-</th>
<th>Tcf3</th>
<th>Tcf3</th>
<th>Tcf3ΔNLS</th>
<th>Tcf3ΔN</th>
<th>GPx4b</th>
<th>GPx4b-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venus</td>
<td><img src="image" alt="Venus" /></td>
<td><img src="image" alt="Venus" /></td>
<td><img src="image" alt="Venus" /></td>
<td><img src="image" alt="Venus" /></td>
<td><img src="image" alt="Venus" /></td>
<td><img src="image" alt="Venus" /></td>
</tr>
<tr>
<td>Cy3</td>
<td><img src="image" alt="Cy3" /></td>
<td><img src="image" alt="Cy3" /></td>
<td><img src="image" alt="Cy3" /></td>
<td><img src="image" alt="Cy3" /></td>
<td><img src="image" alt="Cy3" /></td>
<td><img src="image" alt="Cy3" /></td>
</tr>
<tr>
<td>DAPI</td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
<td><img src="image" alt="DAPI" /></td>
</tr>
<tr>
<td>merge</td>
<td><img src="image" alt="Merge" /></td>
<td><img src="image" alt="Merge" /></td>
<td><img src="image" alt="Merge" /></td>
<td><img src="image" alt="Merge" /></td>
<td><img src="image" alt="Merge" /></td>
<td><img src="image" alt="Merge" /></td>
</tr>
</tbody>
</table>
Fig. S6 GPx4b-C interacts with Tcf3 whereas GPx4b does not bind with β-catenin in living cells as indicated by BiFC assay.

(A) Flag-tagged GPx4b-C-VC expression vector was co-transfected with each indicated Myc-tagged form of Tcf3-VN. Expression of GPx4b-C was detected by anti-Flag immunostaining (red). (B) Flag-tagged β-Cat-VC expression vector was co-transfected with each indicated Myc-tagged form Tcf3-VN, GPx4b-VN, and GPx4b-C-VN. Expression of each form of Tcf3-VN, GPx4b-VN, and GPx4b-C-VN was detected by anti-Myc immunostaining (red). Scale bar = 50 μm.

Table S1 Primers and sequences information.

Click here to Download Table S1