Supplementary 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).

Molecular Cloning and Plasmid Construction.

The full-length cDNA of zebrafish *gpx4a* and *gpx4b* and human *GPX4* with/without 3'-UTR were amplified and cloned into the pCS2+ expression vector. Zebrafish Gpx4b^{U67C} (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. The shRNA constructs targeting human *GPX4* and the 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 and HEK293T cells 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 $2^{-\Delta\Delta Ct}$ method and normalized to *β-actin* mRNA levels (Rong et al., 2014).

Plasmids containing a *gpx4b* ORF and 3'-UTR were used to generate sense and antisense riboprobes using DIG RNA labeling mix following standard procedures. The specificity of the riboprobes was verified using dot-blot assay. *In situ* hybridization was performed as described previously (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.

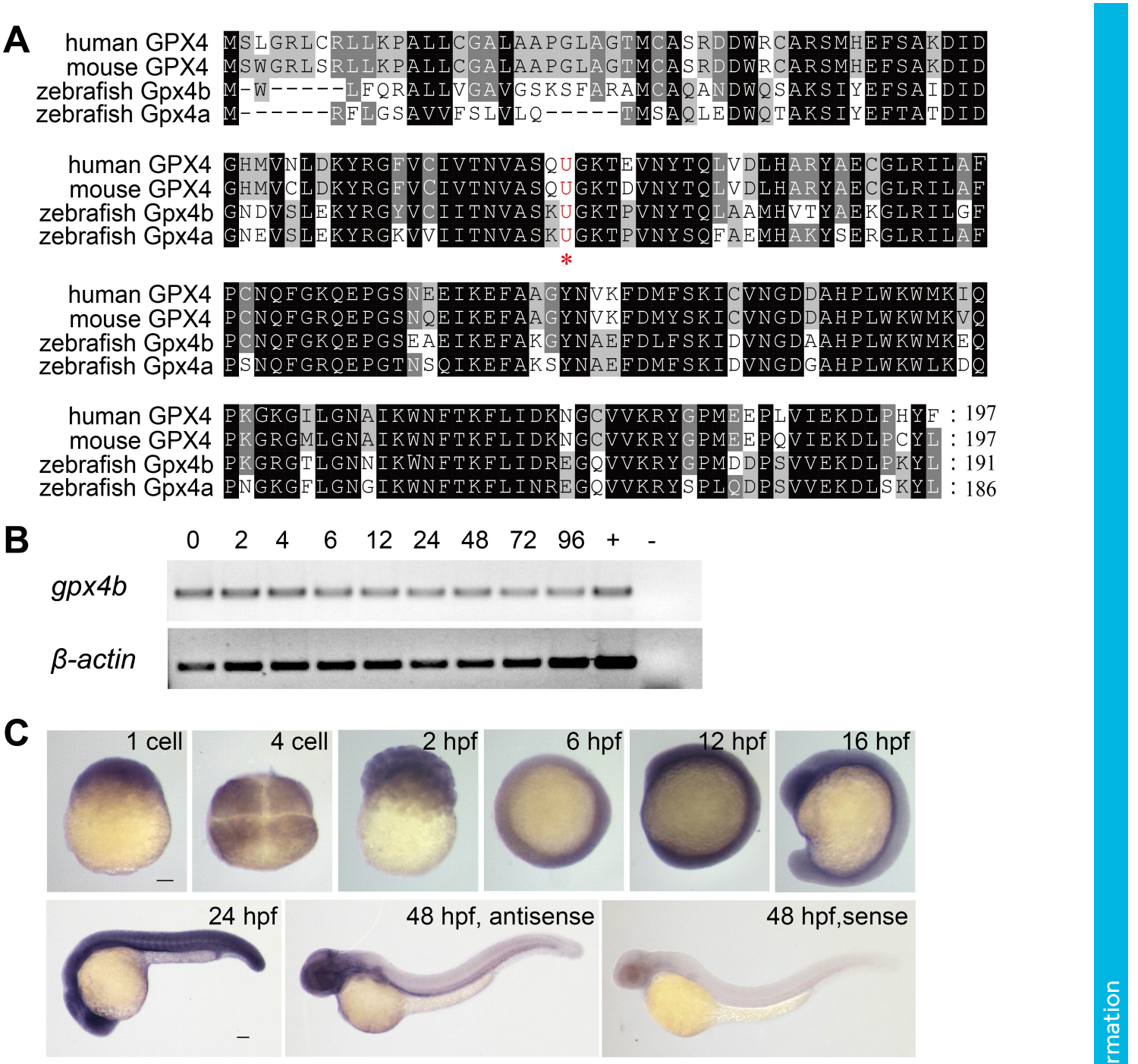
ChIP Assay.

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 (2×10^7) were used in each ChIP reaction. Chromatin in cell lysates was sheered to ~300–900 base pairs in length using a VCX 130 Sonicator (Sonics & Materials, Inc.; 18 × 10 s on, 17 × 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.

Supplementary references

Bai, Y., Tan, X., Zhang, H., Liu, C., Zhao, B., Li, Y., Lu, L., Liu, Y. and Zhou, J. (2014). Ror2 receptor mediates Wnt11 ligand signaling and affects convergence and extension movements in zebrafish. *J Biol Chem* **289**, 20664-20676.

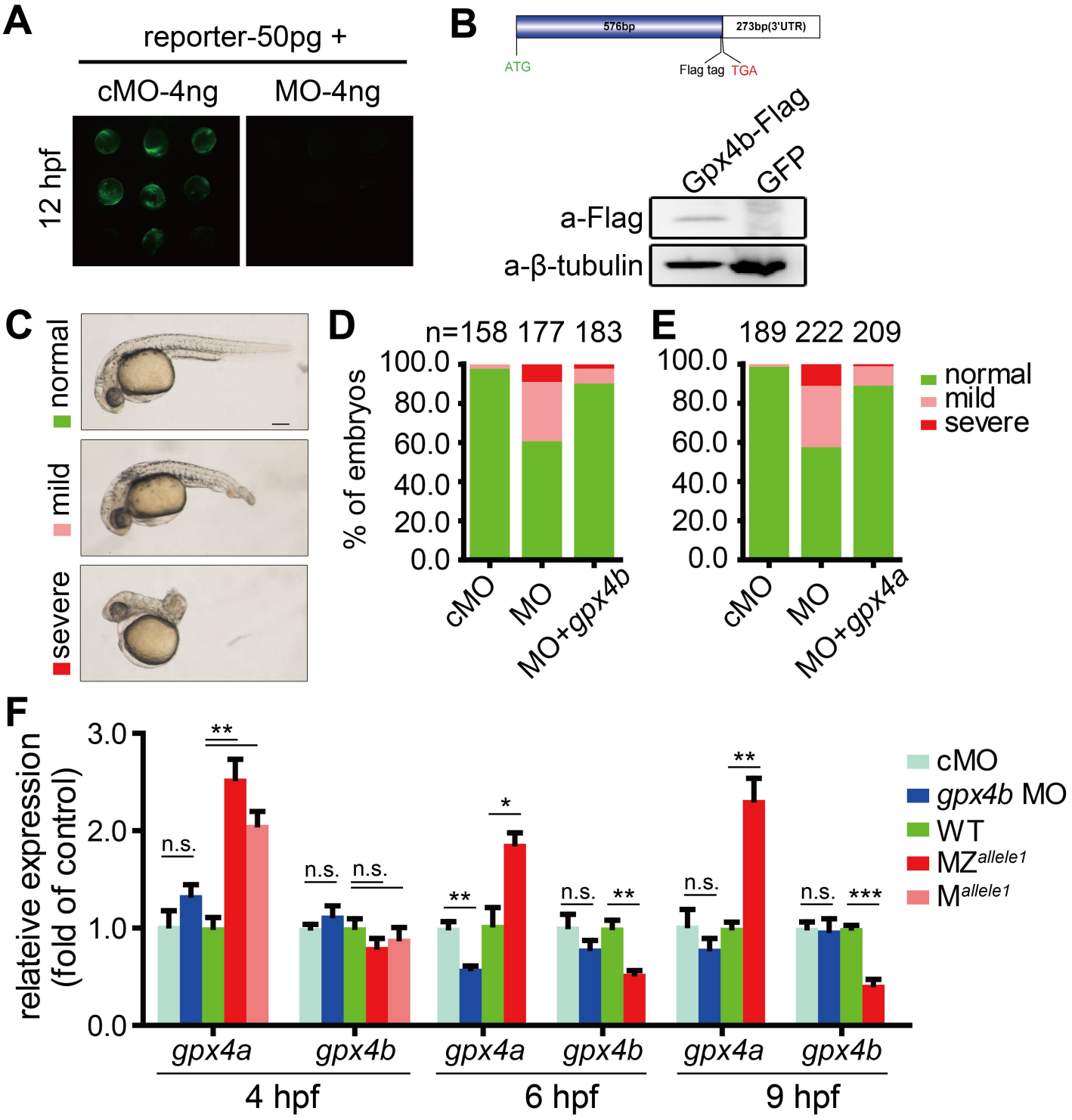
Rong, X., Chen, C., Zhou, P., Zhou, Y., Li, Y., Lu, L., Liu, Y., Zhou, J. and Duan, C. (2014). R-spondin 3 regulates dorsoventral and anteroposterior patterning by antagonizing Wnt/beta-catenin signaling in zebrafish embryos. *PLoS One* **9**, e99514.



Supplementary Fig. 1

Fig. S1 Amino acid sequence alignment of GPX4/Gpx4 and spatiotemporal expression pattern of zebrafish *gpx4b*.

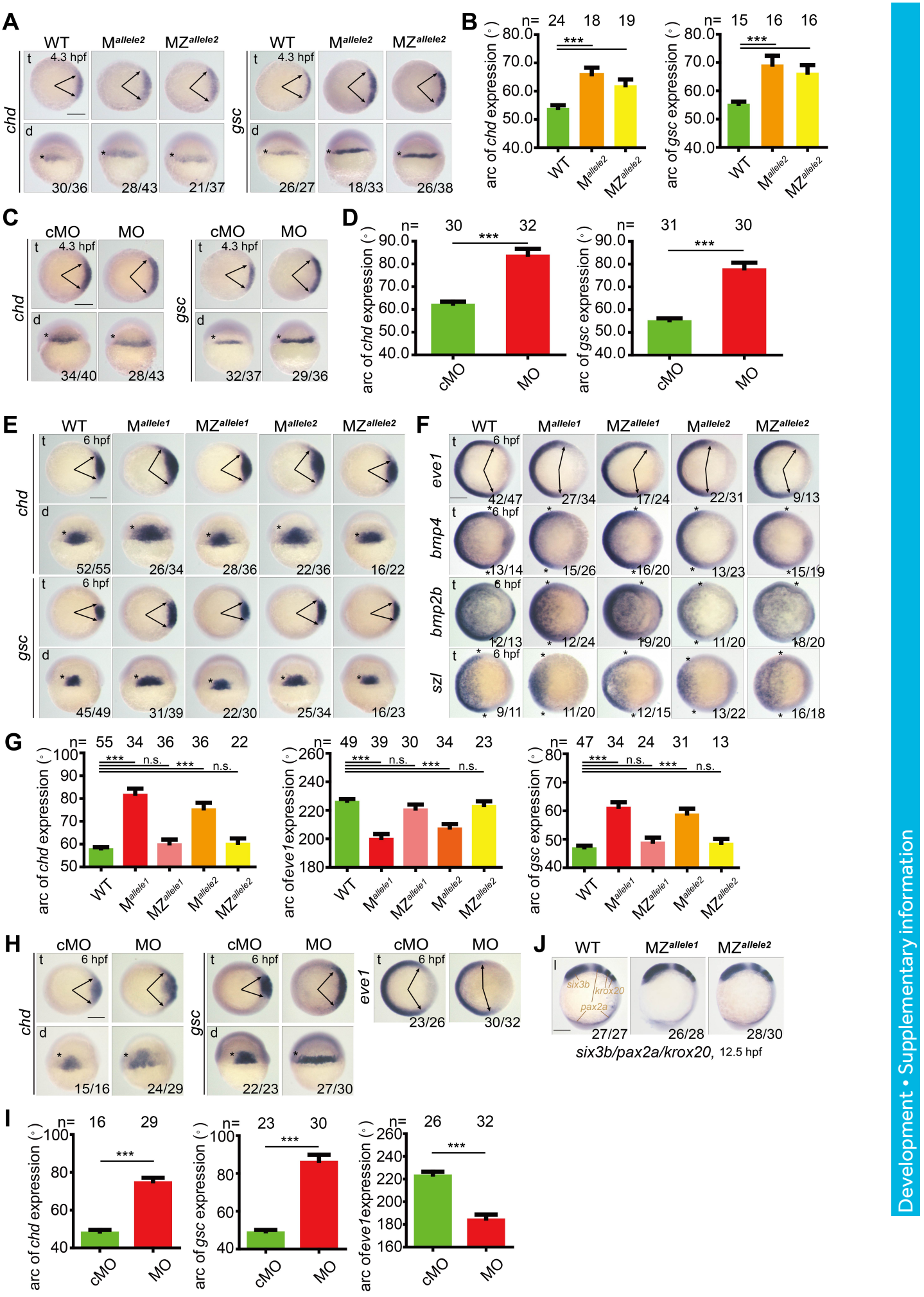
(A) Amino acid sequence alignment of human, mouse, and zebrafish GPX4/Gpx4. Identical amino acids are indicated in black and similar amino acids are indicated in grey. The Sec residue (U) is indicated with an asterisk and highlighted in red. The accession numbers are as follows: human GPX4 NP_002076.2, 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.



Supplementary Fig. 3

Fig. S3 Dorsoventral and anteroposterior patterning in *gpx4b* M and MZ mutants and morphants.

(A) Expression of dorsal organizer markers in $M^{allele2}$ and $MZ^{allele2}$ 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.

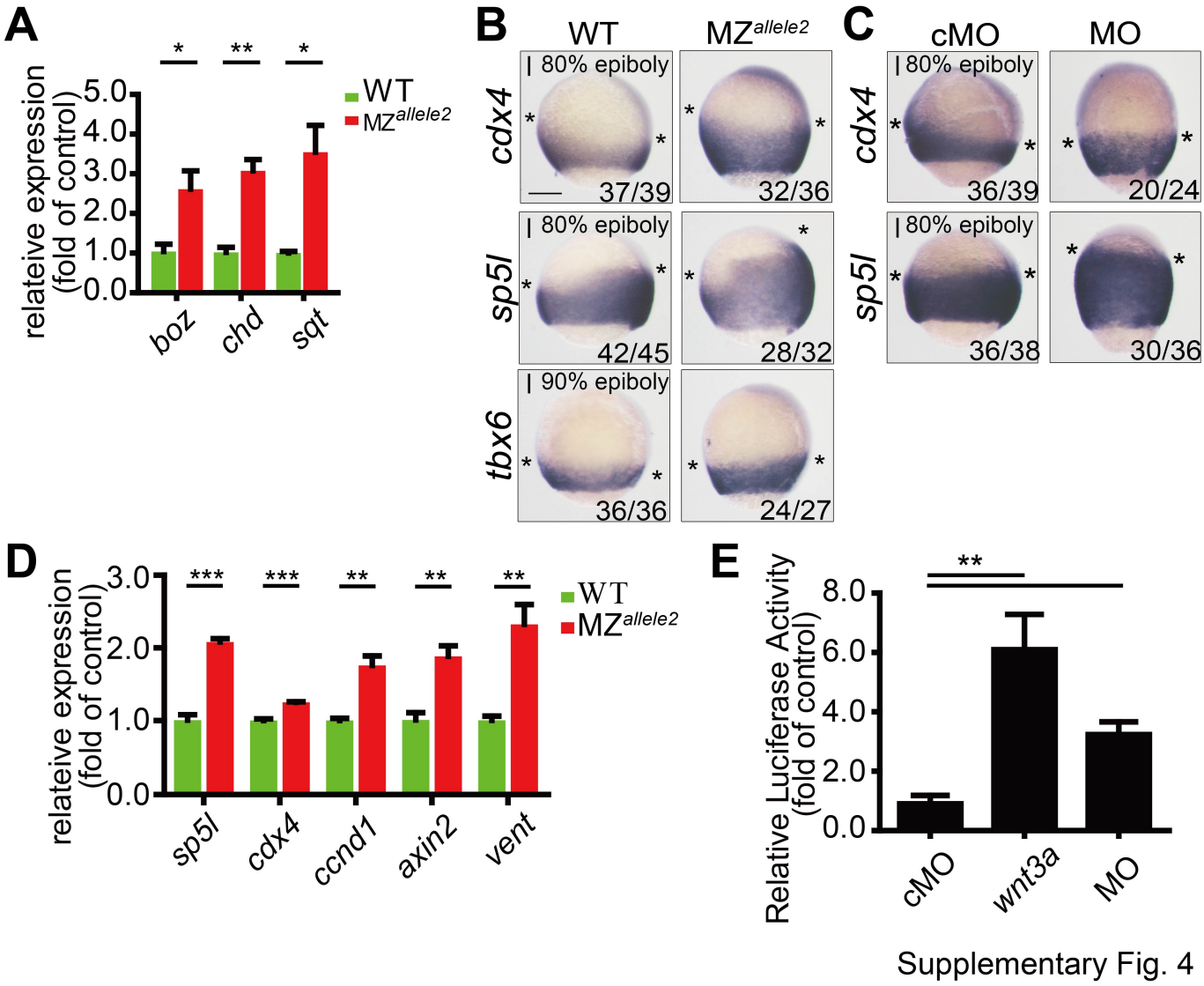
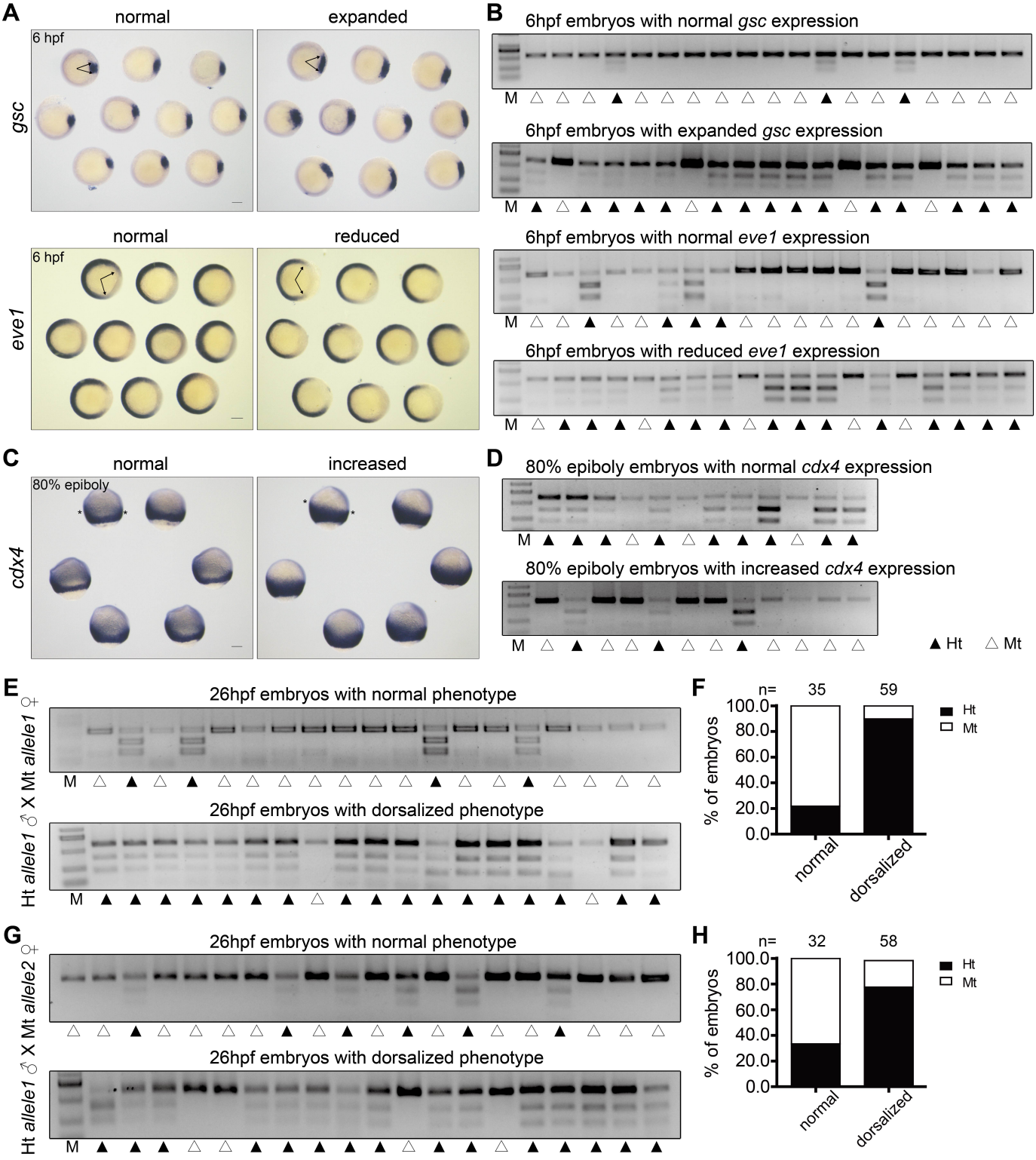


Fig. S4 Wnt activity in *gpx4b* MZ^{allele2} mutants and morphants.

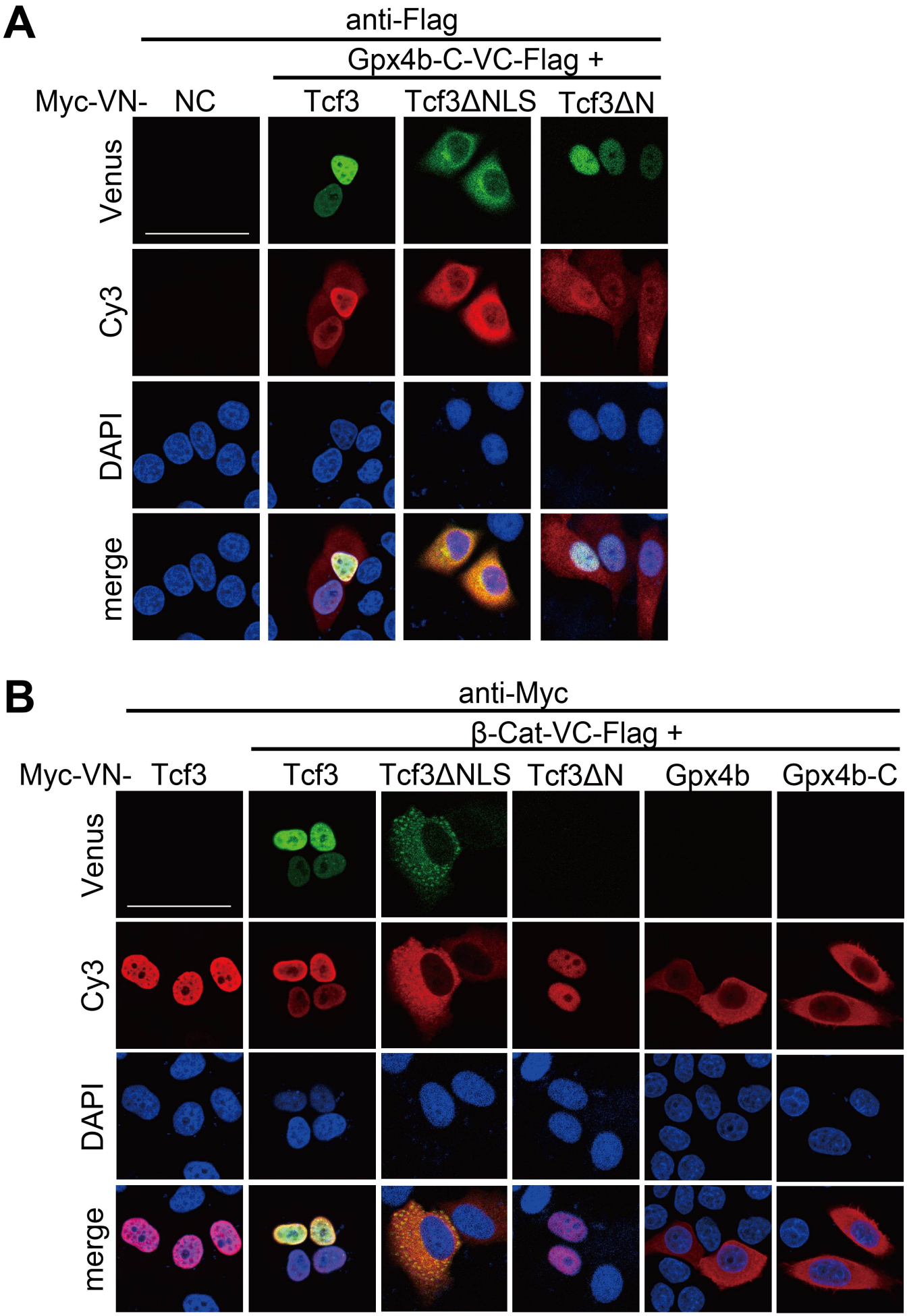
(A) The expression of *boz*, *chd*, and *sqt* mRNA in each indicated group of embryos at 4.3 hpf as analyzed by qRT-PCR. **(B)** Expression of direct zygotic Wnt markers *cdx4*, *sp5l*, and *tbx6* in MZ^{allele2} mutants compared to WT embryos at 9 hpf as revealed by WISH. **(C)** Effects of *gpx4b* 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. l, 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^{allele2} 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 *gpx4b* mutants assessed by genotyping.

(A) Representative expression of *gsc* and *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 *gsc* and *eve1* mRNA expression domains. **(B)** Genotyping results of individual embryos with indicated expression domain as shown in A. **(C)** Representative expression of *cdx4* in progeny of allele1 homozygous female cross to heterozygous male at 80% epiboly as revealed by WISH. Asterisks indicate the edges of the *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 μm. M, marker; Ht, heterozygous mutant; Mt, homozygous mutant.



Supplementary Fig. 6

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 sequence information.

Click here to Download Table S1