Supplemental Material

H_2S promotes developmental brain angiogenesis via the NOS/NO pathway in zebrafish

SUPPLEMENTARY FIGURES AND LEGENDS

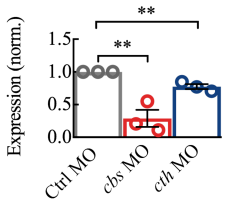

Figure S1. Expression of *cbs* and *cth* in zebrafish larvae was efficiently downregulated by *cbs* MO and *cth* MO, respectively. Summary of Western blot analyses. Error bars, SEM. $^{**}P < 0.01$ (unpaired two-tailed Student's *t*-test).

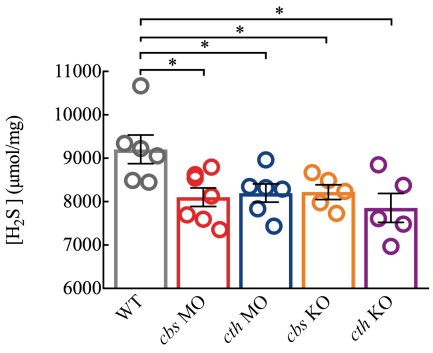

Figure S2. Production of H_2S in *cbs* and *cth* morphants and mutants was significantly reduced. The experiments were repeated 3 times. At least 3 samples were analyzed in each group at each time. Error bars, SEM. *$P < 0.05$ (unpaired two-tailed Student's *t*-test).

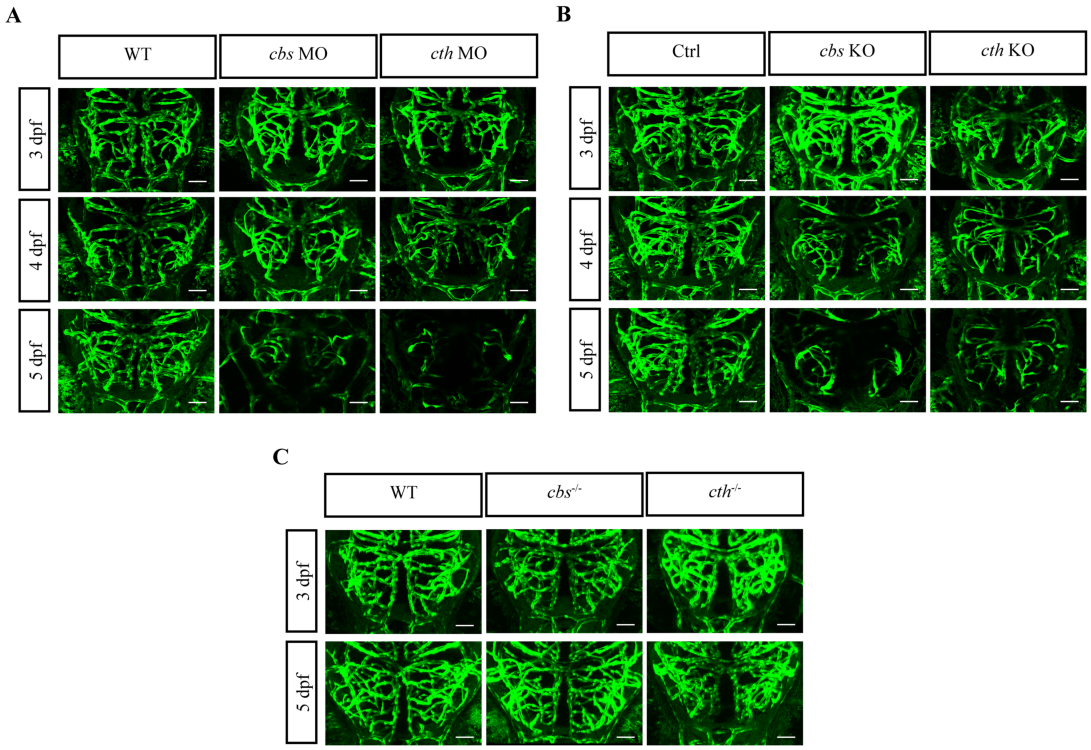

Figure S3. Representative brain vasculature long-term serial confocal images taken from Tg(Flk1:eGFP) larvae at 3 - 5 dpf.

(*A*) Representative projected confocal images taken from *cbs* or *cth* morphants.

(*B*) Representative projected confocal images taken from *cbs* or *cth* F0 mutant larvae.

(*C*) Representative projected confocal images taken from *cbs* or *cth* homo-mutant larvae.

Confocal images were taken at 3 - 5 dpf of the same larva (*A* - *C*).

Scale bar, 50 μm (*A* - *C*).

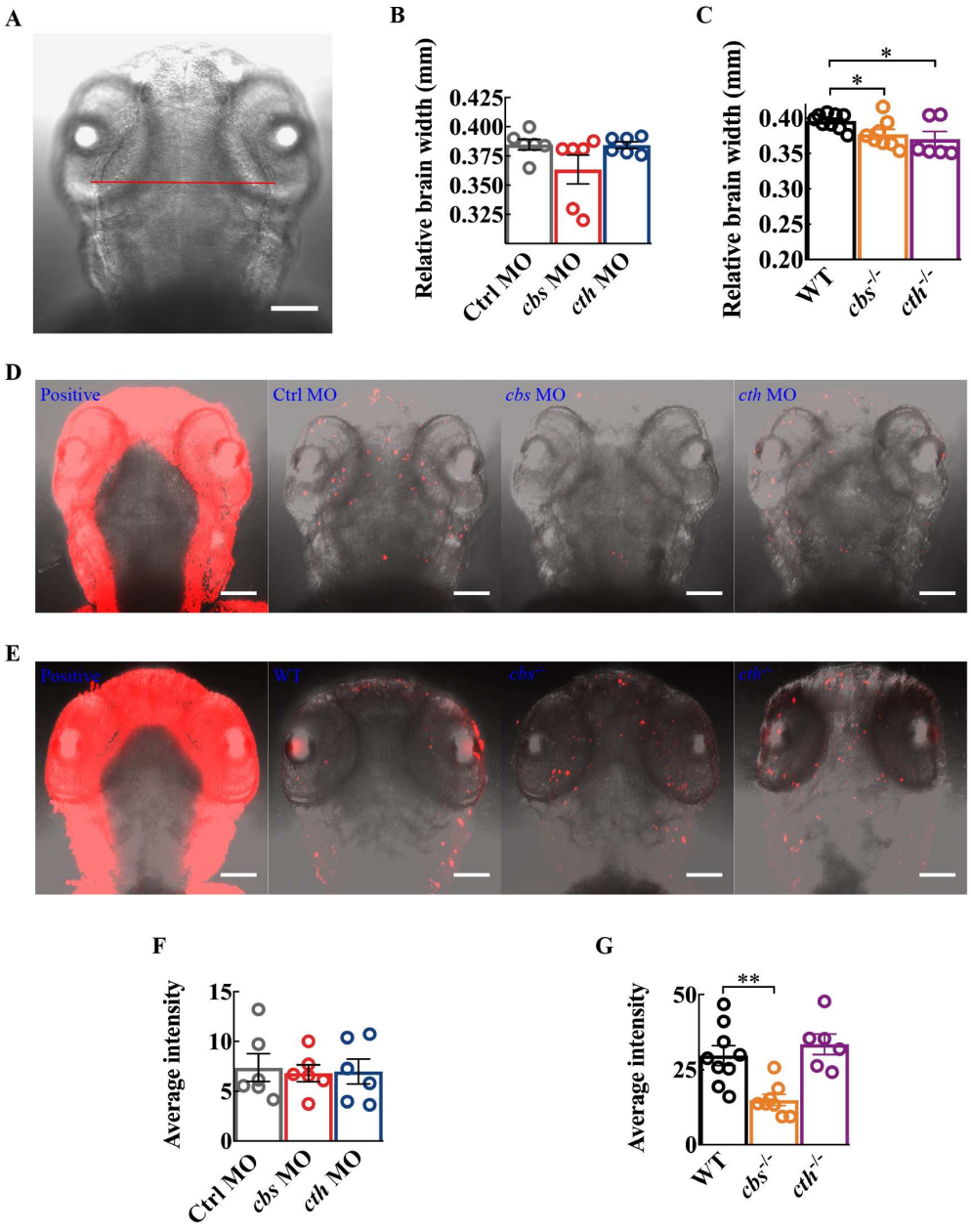

Figure S4. Brain size and cell apoptoss in *cbs* or *cth* morphants and homo-mutants.

(*A*) Image of a 3-dpf larva showing the measurement of brain size, with the width of the optic tectum delineated with a red line as relative brain width. (*B* and *C*) Summary of the relative brain width of *cbs* or *cth* morphants (*B*) and homo-mutants (*C*). (*D* and *E*) Representative projected confocal images of TUNEL signals. (*D*) From left to right: positive control, Ctrl MO,

cbs MO, *cth* MO. (*E*) From left to right: positive control, WT, $cbs^{-/-}$, $cth^{-/-}$. (F and *G*) Summary of average TUNEL signal intensity of *cb*s or *cth* morphants (*F*) and homo-mutants (*G*). Scale bar, 100 μm (*A*, *D, E*). Error bars, SEM. **P* < 0.05, ***P* < 0.01 (unpaired two-tailed Student's t-test).

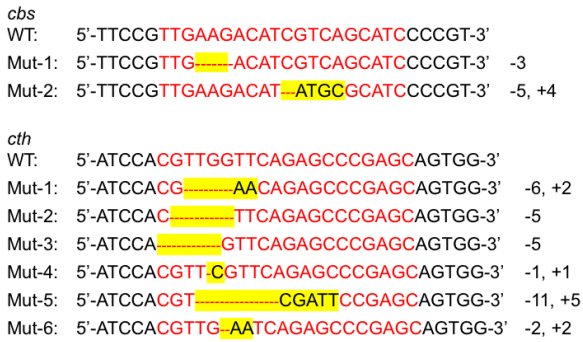

Figure S5. Mutations of the *cbs* and *cth* F0 mutants. All the indel mutations are highlighted in yellow, and sgRNA target sequences are shown in red.

Figure S6. Mutations of the *cbs* and *cth* homo-mutants. All the indel mutations are highlighted in yellow.

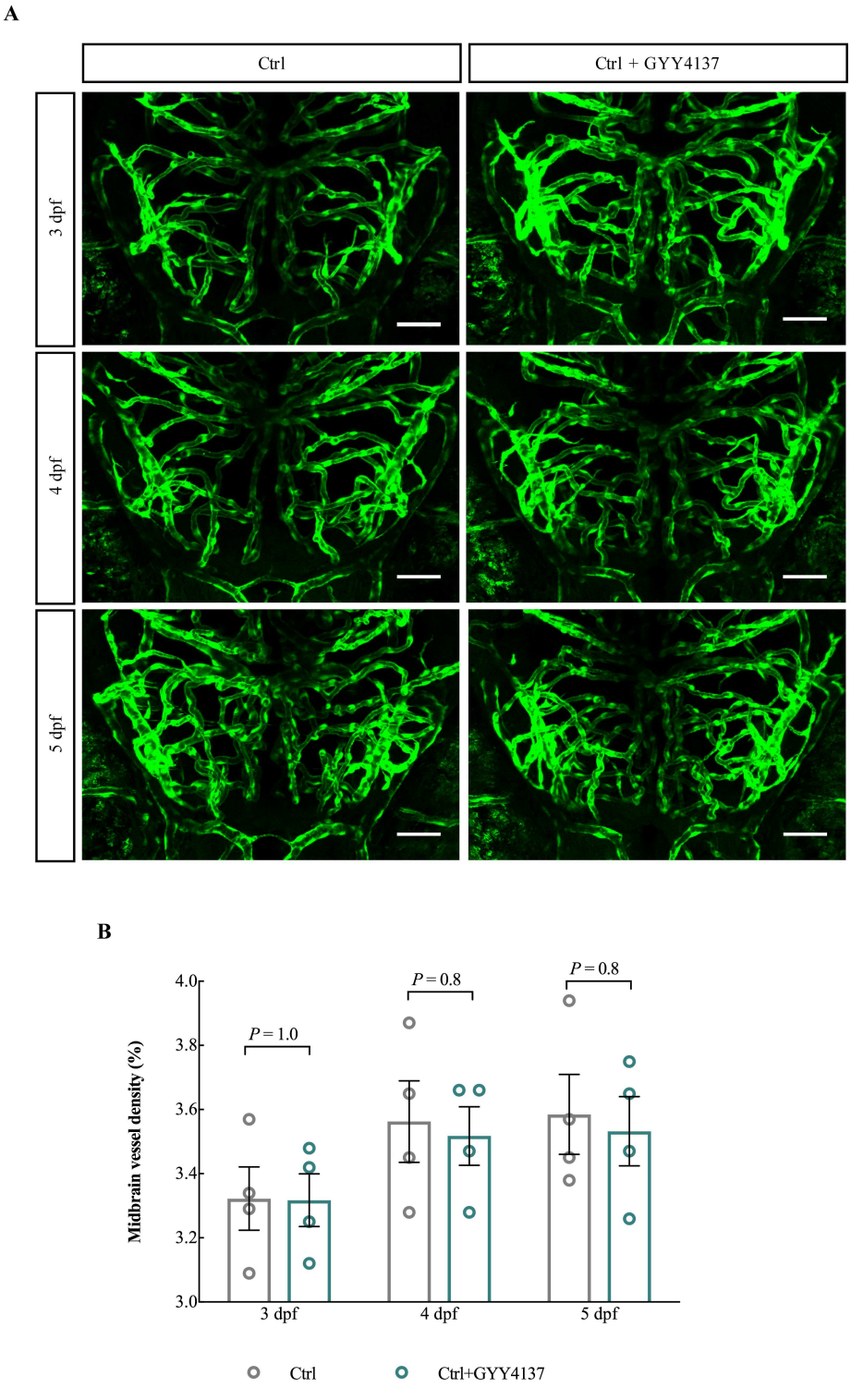

Figure S7. GYY4137 did not change the midbrain vessel density of the control fish.

(*A*) Representative projected confocal images showing that GYY4137 treatment did not change the midbrain vessel density of the Ctrl fish. Confocal images were taken at 3 - 5 dpf of the same larva.

(*B*) Summary of the midbrain vessel density of Ctrl fish and fish treated with GYY4137. Four embryos were examined for each group.

Scale bar, 50 μm (*A*). Error bars, SEM. (unpaired two-tailed Student's t-test for *B*).

SUPPLEMENTARY METHODS

Zebrafish husbandry

The adult zebrafish (Dario rerio) were maintained with an automatic fish housing system at 28°C following standard protocols. Zebrafish embryos and larvae were raised in 10% Hanks' solution[1] under a 14 h-10 h light-dark cycle, and 0.003% 1-phenyl-2-thiourea (PTU) (Sigma, P7629) was added to the Hanks' solution since 24 hpf to prevent pigment formation. The Tg(Flk1:eGFP) zebrafish were described previously[2]. Euthanasia was performed by rapid freezing.

Western blotting

Protein sample preparation and western blotting were performed as previously described[1]. Protein sample preparation was conducted at 3 dpf. The primary antibodies were anti-CBS (Aviva, ARP45746_T100), anti-CTH (Santa Cruz, sc-374249), anti-VEGF (R&D, MAB1247), anti-β-actin (Immunoway, YT0099) anti-ERK1/2 (Cell Signaling, 9107), and anti-p-ERK1/2 (Cell Signaling, 4370).

RNA preparation and Real-time PCR

Total RNAs of zebrafish embryos were extracted by using TRIzol reagent according to the manufacturer's instructions (Invitrogen, 15596018). The extracted total RNA was used to generate the first-strand cDNA by using PrimeScript reverse transcriptase (Takara, 2680A) with random primer. The real-time PCR with SYBR Premix Ex Taq II (Takara) was performed

on the cDNA to detect the relative expression of *nos1*, *nos2a*, and *nos2b*. The relative RNA amount was calculated with the ΔΔCt method and normalized with *β-actin* (*actb1*) expression (as an internal control). The primers used for real-time PCR are as follows.

nos1 primers:

forward: 5′-ACACAGTGGATCTGGAGCAC-3′

reverse: 5′-GCCGCACCAAATTTCTCTCC-3′

nos2a primers:

forward: 5′-AACATTTTGGAGCGCGTTGG-3′

reverse: 5′-CGGCAACATTGATAGCCACG-3′

nos2b primers:

forward: 5′-AAGCCCCGACTCTACTCCAT-3′

reverse: 5′-TGGACCTTTTCCCTCCTGTG-3′

actb1 primers:

forward: 5′-AAGCCCCGACTCTACTCCAT-3′

reverse: 5′-TGCTCAATGGGGTATTTGAGGG-3′

Measurement of H_2S production

Total H_2S production in the zebrafish larvae was examined at 3 dpf with the H_2S measurement kit (Sino Best Biological Technology, YX-C-C000). Protein samples were extracted with the extraction buffer and then mixed with solution I - IV. Absorbance at 665 nm was measured and total H_2S production was calculated according to the provided formula.

Whole-mount TUNEL assay

Zebrafish embryos at 3 dpf were fixed in 4% paraformaldehyde (PFA) overnight at 4 ℃. Genotyping of mutant larvae was conducted before they were fixed with PFA. TUNEL staining was conducted using the *In Situ* Cell Death Detection Kit, TMR red (Roche Diagnostics GmbH, 12156792910). Procedures were performed as previously described[3]. The dorsal view of whole-mounts with Z stack (3 μm per step) images were captured with an Olympus Fluoview 1000 confocal microscope (Olympus, Japan). XLumplfl 20× (W/IR; NA, 0.95) objective lenses were used. Raw images were processed with ImageJ.

Brain size measurement

Since larval brains are too small to be dissected and weighed, we measured the width of the optic tectum from dorsal confocal images using ImageJ. The width of the optic tectum predicts the mass of the brain with 79% accuracy in zebrafish[4], thus the measurement is regarded as a predictor of overall brain size[5].

REFERENCES

[1] Xu B, Zhang Y, Du XF, et al. Neurons secrete miR-132-containing exosomes to regulate brain vascular integrity. *Cell Res* 2017;27(7):882-97.
[2] Roman BL, Pham VN, Lawson ND, et al. Disruption of acvrl1 increases endothelial cell number in zebrafish cranial vessels. *Development* 2002;129(12):3009-19.
[3] Du W-J, Zhang R-W, Li J, et al. The Locus Coeruleus Modulates Intravenous General Anesthesia of Zebrafish via a Cooperative Mechanism. *Cell reports* 2018;24(12).
[4] Näslund J. A simple non-invasive method for measuring gross brain size in small live fish with semi-transparent heads. *PeerJ* 2014;2:e586.
[5] Chen Y-C, Harrison PW, Kotrschal A, et al. Expression change in Angiopoietin-1 underlies

change in relative brain size in fish. *Proceedings Biological sciences* 2015;282(1810).