Cystathionine β-Synthase Is Necessary for Axis Development in Vivo

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The cystathionine β-synthase (CBS) is a critical enzyme in the transsulfuration pathway and is responsible for the synthesis of cystathionine from serine and homocysteine. Cystathionine is a precursor to amino acid cysteine. CBS is also responsible for generation of hydrogen sulfide (H₂S) from cysteine. Mutation in CBS enzyme causes homocysteine levels to rise, and gives rise to a condition called hyperhomocysteinuria.

To date, numerous mouse knockout models for CBS enzyme has been generated, which show panoply of defects, reflecting the importance of this enzyme in development. In zebrafish, we and others have identified two orthologs of cbs, which we call cbsa and cbsb. Previous gene knockdown studies in zebrafish have reported a function for cbsb ortholog in maintaining ion homeostasis in developing embryos. However, its role in maintaining H₂S homeostasis in embryos is unknown. Here, we have performed RNA analysis in whole zebrafish embryos that showed a wide expression pattern for cbsa and cbsb primarily along the embryonic axis of the developing embryo. Loss-of-function analysis using a combination of approaches which include splice morpholinos and CRISPR/Cas9 genomic engineering show evidence that cbsb ortholog is responsible for anterior-posterior axis development, and cbsa function is redundant. Cbsb loss of function fish embryos show shortened and bent axis, along with less H₂S and more homocysteine, effects resulting from loss of Cbsb. Using a chemical biology approach, we rescued the axis defects with betaine, a compound known to reduce homocysteine levels in plasma, and GYY4137, a long term H₂S donor. These results collectively argue that cells along the axis of a developing embryo are sensitive to changes in homocysteine and H₂S levels, pathways that are controlled by Cbsb, and thus is essential for development.

Keywords: zebrafish, CRISPR, small molecules, methionine, homocystinuria, hydrogen sulfide, morpholino
INTRODUCTION

Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are two key enzymes that are involved in the biosynthesis of hydrogen sulfide (H₂S), a key signaling molecule that participates in various physiological functions in vertebrates (Huang and Moore, 2015). Endogenous H₂S biosynthesis is also catalyzed by the coordinated action of two other enzymes cysteine aminotransferase (CAT) and 3-mercaptopropionate sulfur transferase (3-MST). In addition to H₂S biosynthesis, CBS and CSE enzymes also participate in transsulfuration reactions involving cystathionine (precursor to cysteine) biosynthesis through a condensation reaction of serine and homocysteine (Jhee and Kruger, 2005). Hyperhomocysteinemia (HHcy), a condition with elevated homocysteine is associated with mutation in the CBS enzyme (Kraus et al., 1999). HHcy is classified based on levels of homocysteine that range from 10.8 to 12.4 mmol/L (normal), 15 to 30 mmol/L (moderate), 31 to 100 mmol/L (intermediate), or >100 mmol/L (severe) (Födinger et al., 1999). Neural tube defects resulting from failure of neural tube closure during the first 28 weeks of pregnancy remain the leading developmental disorder in these patients. Oral cleft, congenital heart defects and cardiovascular disorders were also observed in these patients. In adults, HHcy is associated with several conditions including diabetes, pulmonary embolism and Alzheimer’s disease. Thus, CBS remains an important target that requires further understanding (Majtan et al., 2016).

The CBS pathway is a gateway to several essential biochemical processes including glutathione synthesis. Glutathione is a tripeptide that is synthesized from cysteine, and is vital for detoxification reaction in cells, and thus serves an anti-oxidant function. CBS generates key precursors such as cysteine, thus serving at a critical intersection point in pathways associated with endogenous detoxification mechanisms and biosynthesis of H₂S in the cell. It is therefore not surprising that CBS is critical for development and homozgyous CBS knockout in mice die in utero (Watanabe et al., 1995). Majority of published studies on CBS knockout mice is performed on heterozygous CBS background. Numerous defects ranging from vascular endothelial dysfunction (Lentz et al., 2000; Dayal et al., 2001), redox homeostasis (Vitvitsky et al., 2004), hyperkeratosis (Robert et al., 2004), endochondrial ossification (Robert et al., 2005), retinal neuron death (Ganapathy et al., 2009), lung fibrosis (Hamelet et al., 2007b), and hepatic steatosis (Hamelet et al., 2007a) have been reported. Majority of these defects have in part been attributed to elevated plasma homocysteine levels. A human CBS knock-in transgene in the endogenous mouse CBS locus has also been generated, which shows elevations in plasma and tissue levels of homocysteine but shows mild hepatopathy and no hepatic steatosis or fibrosis in contrast to classical models of homocystinuria (MacLean et al., 2010). In lower species, such as zebrafish, gene knockdown of either cbs or cse using antisense morpholino oligonucleotides (MOs) (Porteus et al., 2014) influenced the hypoxic ventilatory response, an adaptation under stress conditions that allows organism to intake and process oxygen at higher rates. The oxygen-sensing neuroepithelial cells in zebrafish showed an increase in intracellular calcium concentration. The same group later reported that cbsb knockdown using morpholinos (MOs) and not cse showed a reduction in calcium influx in larval zebrafish (Kwong and Perry, 2015). In addition to calcium, larval zebrafish containing gene knockdowns of cse and cbsb (Kümaj et al., 2015) showed better sodium uptake. Collectively, these results argue for a role for cbsb in maintaining H₂S and homocysteine homeostasis in developing embryos. However, the consequence of altering H₂S and homocysteine homeostasis via modulation of cbsb during zebrafish embryonic development is unknown. Here, we performed a systematic and detailed analysis into Cbs enzyme role in zebrafish embryonic development, and its role in maintaining H₂S and homocysteine homeostasis in vivo. Because of genome duplication in zebrafish, a significant portion of the genes in zebrafish have duplicates (Postlethwait et al., 2000). For the cbs gene, of the two orthologs cbsa and cbsb in zebrafish, we postulated that the cbsb gene was critical for embryonic development because of prior in situ hybridization (ISH) pattern data observed on zebrafish information network site. To investigate this hypothesis, we performed RNA analysis and whole mount ISH (WISH) across embryonic stages. We also performed loss-of-function analysis using MOs (Ekker, 2000) and CRISPR/Cas9 (Hruscha et al., 2013)-based genomic engineering approaches. Finally, we performed rescue for the loss-of-function phenotypes using small molecules that modulate the H₂S and cysteine biosynthesis pathway. Our study here points to a critical role for cbsb in embryonic axis development.

MATERIALS AND METHODS

Zebrafish

All zebrafish studies performed here were carried under the AUA protocol 320, which is approved by the MCW institutional animal care and use committee. Embryo stages were performed as per the zebrafish book and whole mount in situ hybridization (ISH) pattern data observed on zebrafish information network site. To investigate this hypothesis, we performed RNA analysis and whole mount ISH (WISH) across embryonic stages. We also performed loss-of-function analysis using MOs (Ekker, 2000) and CRISPR/Cas9 (Hruscha et al., 2013)-based genomic engineering approaches. Finally, we performed rescue for the loss-of-function phenotypes using small molecules that modulate the H₂S and cysteine biosynthesis pathway. Our study here points to a critical role for cbsb in embryonic axis development.

Chemicals

GYY4137 (P-(4-Methoxyphenyl)-P-4-morpholinyl-phosphinodithioic acid), Na₂H₂O₃ (sodium hydrosulfide hydrate) and betaine (Trimethylglycine) chemicals were purchased from Sigma Aldrich. 1 M Stock solution of GYY4137 was prepared in dimethylsulfoxide (DMSO) and stored at 4°C. 50 mg/ml solution of betaine was prepared in water and stored at 4°C. 1 M stock solution of Na₂H₂O₃ was prepared in water and used immediately.

Molecular Biology

Probes for sense and anti-sense cbsa and cbsb were generated using PCR-based methods. The primers for these probes include: Cbsa sense: Fwd 5′-CACCGAAAATATAGCCTACCATATAG GATGGAGCAGACCCACCA, Rev 5′-GG ACTTTTTTCT TCAATTTCGCA and antisense primers: Fwd 5′-GATGGAGA
CAGCCCCCACA, Rev 5′-CACCAGAAATTAACCCCTCCTAATA AAAGGGGCCACCTTTCTTCTAAATTTCCGA
Cbsb sense: Fwd 5′-CACCAGAAATTAACCCCTCCTAATTTAGG
GATCAACGGGATGCTGACGAT, Rev 5′-CAATCTCTACGCA CCCAGAGCA and antisense primers: Fwd 5′-ATCAACGGG
ATGCTGACGAT, Rev 5′-CACCAGAAATTAACCCCTCCTAATAA
GGCCACTTCTCACGACCCAGAGCA

Primer sequences used for amplification include:
Cbsa, Fwd 5′-GATGAGACAGAACCCCA, Rev 5′-GACG
TTTCTCTGAATTTTCCGA
Cbsb, Fwd 5′-ATCAACGGGATGCTGACGAT, Rev 5′-CAAT
CTCACGACCCAGAGCA
Actin (actb1): Fwd 5′-GAAATTGTCCGTGACATCAA, Rev 5′-
CACTGTGTGGTGATACAGATT

Western Blotting and IF
For western blotting, 48 h post fertilization (hpf) CBSB-S1-injected, ComMO-injected and cbsb CRISPR embryos were dechorionated and then de-yolked in PBS. Total cell lysate was prepared in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. Measurement of protein concentration was performed using BCA assay kit from Pierce, Grand Island, NY, USA. SDS-PAGE and immunoblotting was performed using standard protocol. The cell lysates were separated on 10% glycine SDS-PAGE gel and transferred to PVDF membrane. Membranes were blocked in 5% BSA in TBS with 0.1% Tween-20 (TBST) for 1 h at room temperature followed by incubation with indicated primary antibodies in TBST with 5% BSA. Antibodies were purchased from following vendors. CBS (D8F2P) purified rabbit antibody (catalog #14782, Cell Signaling Technology, MA) was used at a 1:1000 dilution; anti-GAPDH antibody (Sigma-Aldrich, MO) was used at a 1:5,000 dilution at 4°C overnight. Horseradish peroxidase conjugated secondary antibodies were used and proteins were visualized by Clarity Western ECL Substrate (Bio-Rad). For the immunofluorescence experiment, CBS-S1-injected embryos, ComMO-injected embryos and cbsb-CRISPR embryos were dechorionated at 48 hpf and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C overnight. The following day, embryos were washed twice with PBST (PBS, 0.1% (v/v) Tween 20), permeabilized in ice-cold acetone for 10 min and washed in PBST. Embryos were blocked in 10% normal goat serum for 1 h and incubated with purified rabbit anti-homocysteine antibody (Catalog # ab15154, Abcam, MA) overnight at 4°C. Embryos were washed twice with PBST, incubated with the appropriate secondary antibody (Alexa fluor 568 goat anti-rabbit, Invitrogen, MA) for 2 h at RT, washed with PBST and imaged on a Zeiss AxioObserver Z1 fluorescence inverted microscope.

Morpholino Studies
Gene Tools, Inc. designed all the morpholinos. Sequence is provided (Figure 2C). Control MO sequence used in this study is 5′-CTCTTTACCTCAGTTACAATTTATA-3′. All MOs were each injected into Transgenic (Tg: flk:EGFP) (Choi et al., 2007) one cell stage embryos. The 1 mM MO stock solutions were diluted to a final concentration of 2 ng/mL (250 µM), and appropriate concentrations as shown in the figure panels were injected into each embryo. For phenotypic imaging, 30 and 52 hpf embryos were anesthetized in 0.02% tricaine, mounted on a depression slide, and imaged with a Zeiss Stemi 2000-C dissecting microscope.

H2S Measurement Studies
H2S was measured according to the published protocol (Papapetropoulos et al., 2009). Briefly, ComMO-injected and CBSB-S1-injected zebrafish embryos were collected by centrifugation and re-suspended in a total volume of 200 µL of PBS (pH 7.4), and 100 µL was then transferred directly into a tube containing zinc acetate (1% wt/vol, 187.5 µl) and NaOH (12%, 12.5 µL) to trap the H2S for 20 min at room temperature. The rest of the embryo suspension was used to estimate protein concentration by BCA assay. The reaction was terminated by adding 1 mL of H2O (pH 12.8), 200 µL of N, N-dimethyl-p-phenylenediamine sulfate (20 mM in 7.2 M HCl), and 200 µL of FeCl3 (30 mM in 1.2 M HCl). The mixture was incubated at room temperature in darkness for 15 min, and finally 600 µL of the mixture was added to a tube with 150 µL of trichloroacetic acid (10% wt/vol) to precipitate protein. The precipitated protein was removed by centrifugatation at 10,000 g for 5 min, and absorbance at 670 nm of the resulting supernatant (200 µL) was determined. The H2S concentration of each sample was calculated according to a calibration curve of NaHS.

Small Molecule Studies
CBSB-S1 injected or cbsb CRISPR embryos were treated with either 1 mM betaine or 1 mM GYY4137 or 1 mM NaHS at 6 hpf. All embryos were then developed to 30 or 52 hpf in the 28° C incubator. Pictures were taken as described earlier.

CRISPR Studies
CRISPR/CAS9 target sequences were designed using E-CRISP. (Heigwer et al., 2014). pTTS3-mCas9n was a gift from Wenbiao Chen (Addgene plasmid # 46757) (Jao et al., 2013). sgRNA and capped cas9 mRNA were synthesized by in-vitro transcription as previously described (Varshney et al., 2015) The cbsb sgRNA was validated by the Guide-it™ sgRNA Screening Kit from Clontech. One-cell stage embryos were injected with 250 pg of sgRNA and 250 pg of cas9 mRNA. Injected founder (F0) fish were fin-clipped at adulthood and screened for INDELs at the target location by Sanger sequencing. Two F0 founders containing the same 2 base pair deletion at the target site were bred to produce F1 mutants (lines C, H, and F). F1 mutants were raised to adulthood and in-crossed for production of F2 embryos, for which phenotypes were observed and quantified. For rescue, CRISPR embryos were placed in 1 mM betaine at 6 hpf. CRISPR phenotypes were evaluated at 5 dpf. All CRISPR lines were generated in the background of (Tg: flk:EGFP) (Choi et al., 2007) and CASPER (Zebrfish Information Resource Center), and is referred to as flk:EGFP CASPER in this study.
Quantification Details and Statistics
Embryo Length Determination
Body length was measured from the tip of the head to the end of the trunk. Scale bar calibration and length measurements were done with a Leica MZFL III microscope using a stage micrometer and Q-Capture PRO 7 software. T-test was performed using graph pad prism program on Figures 2E, 3F, 4D, 5, 6. Data are plotted as mean values and error bars indicate standard error of the mean. A standard t-test was used to evaluate statistical significance. A p-value <0.05 was considered significant and p-values <0.01, 0.001, 0.0001 were considered very significant.

RESULTS
Expression Analysis of cbsa and cbsb Genes during Embryonic Development
We first performed reverse transcription-based PCR (RT-PCR) using gene-specific primers for cbsa, cbsb and actin genes using RNA isolated from 2, 6, 10, 18, 24, 28, 36, 48, and 72 hpf embryos (Figure S1). Both cbsa and cbsb genes are maternally expressed and showed expression in 2 hpf embryo, which continued throughout all stages of development. The cbsa transcript is initially expressed minimally, but its expression begins to increase around 18 hpf (Figure S1). Notably, the cbsb expression peaks at end of gastrulation (10 hpf) stage, and the expression is constitutive in the remaining period of development (Figure S1). To detect the location of cbsb transcripts, we performed WISH using digoxigenin-labeled sense and antisense RNA probes at the bud stage, 18, 24, and 48 hpf (Figure 1). Cbsb expression is noticeably visible in the embryonic axis in the early tail bud stage (Figures 1A–C), an early indication of the importance of cbsb in axis development. At 18 hpf, cbsb expression is visible in the head region in the entire brain (Figure 1D) and in the somites in the body axis (Figure 1D'). At 24 hpf (Figures 1F,F'), cbsb expression pattern continues in the somites (Figure 1F), while in the head, expression shifts from the brain to the 4th ventricle highlighting the dynamic role of cbsb in early brain development. At this stage, sense probe controls do not show staining (Figure 1E). At 48 hpf, the expression of cbsb continues in both the brain and the 4th ventricle in the head as well as the somites in the trunk (Figures 1H,H'). WISH for cbsa was also performed at 24 hpf, a stage at which expression for cbsa was robust in RT-PCR results (Figure S1). Cbsa gene shows strong expression in the entire head, and in the trunk region (Figure 1G), particularly in the somites. Taken together, the expression data suggests that cbsa and cbsb genes are ubiquitously expressed throughout the head and in the somites, and suggest a role in head and axis development.

Loss of Function of cbsb but Not cbsa Affects Embryonic Axis Development
To investigate function of cbsa and cbsb, we employed two strategies, morpholinos (MOs) and CRISPR/Cas9 genomic engineering methods. We designed MOs that targeted cbsa and cbsb (Figure 2A) genes at specific splice junctions [cbsa: intron 4-exon 5 (CBSA-S1); cbsb: intron 2-exon3 (CBSB-S1), exon3-intron3 (CBSB-S2), and intron4-exon5 (CBSB-S3)]. MO

FIGURE 1 |ISH analysis of cbsa and cbsb genes in zebrafish embryos. (A–G) are digoxigenin-labeled whole mount in situ hybridization for cbsa and cbsb RNA. Panels (A–C) are bud stage, (D,F,H) are 18, 24, and 48 hpf embryos respectively. Panels (D–H') are high power images of the respective (D,F,H). Panel (E) is a cbsb sense probed 24 hpf embryo, and Panel (G) is a cbsa antisense probed embryo at 24 hpf. Scale bars indicate 0.25 mm.
sequences are provided (Figure 2C). Efficacy of MOs were determined at the RNA (Figure 2B) and protein (Figure 2D) level. CBSA-S1 indeed induced an alteration in cbsa transcript with no change in cbsb transcript (Figure 2B). Of the three splice MOs for cbsb, CBSB-S1 injected embryos produced a smaller alternate band (Figure 2B). The band was extracted and sequenced, which revealed that exon 3 was missing (data not shown). Importantly, cbsa mRNA in CBSB-S1-injected embryos did not show consistent change relative to WT or conMO lanes (Figure 2B, and data not shown). Similarly, CBSB-S2 and -S3 MOs did target cbsb and not cbsa (Figure 2B), but concentrations of MOs required to achieve this effect was high. Therefore, for cbsb, we eventually settled on CBSB-S1 MO, which produced consistent loss of native cbsb full-length transcript. We also investigated protein changes in the embryo lysates, and observed consistent down regulation of CBSB protein at 24 and 48 hpf (Figure 2D). Because CBS is an enzyme the catalyzes the production of H₂S gas via a reaction where cysteine combines with homocysteine to form cystathionine (Wallace and Wang, 2015), we measured H₂S levels in CBSB-S1 MO-injected and control MO (ConMO)-injected embryos. CBSB-S1 MO1 knockdown embryos showed reduction in H₂S levels compared to their control counterparts (Figure 2E, \( \ast \ast P < 0.01 \)).

The WISH analysis suggested redundant functions for cbsa and cbsb in embryonic development. CBSA-S1 alone injected embryos showed no phenotype (data not shown). CBSB-S1 when injected alone into 1-cell stage zebrafish embryos showed a shortened anterior-posterior axis and head edema (Figures 3B–D) compared to ConMO-injected embryos at 30 hpf (Figure 3A). Therefore, we concluded that all effects were observed due to cbsb loss-of-function. A dose-dependent increase in severity of the bent axis and brain edema phenotypes was observed (Figures 3B–D), which was quantified (bent axis, Figure 3E, \( \ast \ast \ast \ast P < 0.0001 \); brain edema, Figure 3F, \( \ast \ast \ast \ast P < 0.0001 \)), and correlated well with percentage mortality (Figure 3G, \( \ast \ast P < 0.01 \)). At \( \sim \)50 hpf, the axis defects were pronounced in CBSB-S1-injected embryos (Figure 4A) compared to ConMO-injected embryos (Figure 4B), and phenotype expressivity varied from moderate to severe phenotypes (Figure 4C and Figure S2). The axis length was measured and showed decrease in CBSB-S1-injected embryos compared to controls (Figure 4D, \( \ast \ast \ast \ast P < 0.0001 \)). We also investigated the vasculature grossly since we injected CBSB-S1 into transgenic embryos where VEGFR2 promoter or fms-like kinase (FLK) drives enhanced green fluorescent protein (EGFP) [Tg (flk:EGFP)] expression (Choi et al., 2007) in the vasculature. We did not observe much changes to the major vessels in CBSB-S1-injected embryos compared to ConMO-injected embryos (Figure S3). These results suggest that cbsb is important for axis development.
Figure 3 | Dose-dependent effect of cbsb morpholinos at 30 hpf. Phase contrast images of 30 hpf embryos injected with 4 ng of control (Con) MO (A); 1 ng cbsb splice1 (CBSB-S1) MO (B); 2 ng (CBSB-S1) (C) and 4 ng (CBSB-S1) (D) are shown. Arrow shows brain edema. Panels (E,F) quantifies the number of CBSA-S1 MO-injected embryos displaying bent axis, and brain edema (black arrows) compared to ConMO-injected embryos. n = 186 surviving embryos for CBSB-S1 MO and n = 272 surviving embryos for ConMO, which were a total of four experiments ****P < 0.0001 for both bent axis phenotype and brain edema phenotype. (G) Percentage survival observed over a 5 day period for three different doses of CBSB-S1-injected (1 ng, n = 70; 2 ng, n = 70; 4 ng, n = 70) and ConMO-injected embryos (4 ng, n = 70), n = 6 time points, **P < 0.01 for comparison between ConMO and each of the respective CBSB-S1 MO doses. Scale bars indicate 0.25 mm.

**cbsb CRISPR-Modified Embryos Phenocopy cbSb MO**

To complement MO loss-of-function analysis, we generated guide RNAs (gRNAs) that target exon 4 of cbsb gene (Figure 4A and Figure S4A). Guide RNA efficacy was demonstrated (Figure S4B), and gRNA + Cas9 injected fish were genotyped for genomic modifications (Figure S4C). Indeed, 35.4% of the gRNA/Cas9-injected F0 fish showed 2 bp deletions, and several showed deletions and point mutations (Figure S4C). Also, cbsb RNA was lower and no change in cbta RNA was observed in cbsb CRISPR fish when compared to control fish (Figure 4C). The CBS protein level was lower (Figure 2D) in cbsb CRISPR fish compared to control fish. We raised the F0 cbsb CRISPR fish and generated three independent cbsb CRISPR fish lines (C, H, and K). Three distinct axis phenotypes were observed (Figure 5), which were categorized as mild, medium and severe. Phenotypes were readily visible at 48 hpf with a bent axis and tail. In extreme cases, the axis was severely truncated (Figure 5, severe phenotype) similar to CBSB-S1 embryos (Figure S2). The phenotype progressed and became evident by 72 and 120 hpf. It is noteworthy that the bent in the axis resulted in alterations of tail to upward or downward orientation. Quantification of the axis phenotype severity for CRISPR fish in three independent lines (Figure 5D) showed ~30% of defective embryos per line. Based on t-test analysis of the data from 5 independent experiments (Figure 5D), the differences between the percentage of embryos with bent axis in each of the cbsb CRISPR lines (C, *P < 0.05; H and K, ***P < 0.001) was found to be statistically significant compared to the percentage of embryos with bent axis in the control flk:EGFP CASPER line. These results show that cbsb is mainly responsible for axis development in zebrafish.

**Rescue of cbsb Axis Phenotype with Small Molecules**

Because CBSB is a critical enzyme in cysteine & H2S metabolism (Figure S5), we investigated whether small molecules that influence these pathways would rescue the axis phenotype. We treated CBSB-S1-injected embryos with small molecules that rescue loss or gain of specific products in the CBSB enzyme pathway. Hydrogen sulfide (H2S) is an important byproduct of cysteine metabolism, and was observed to be lower in CBSB-S1 knockdown embryos (Figure 2E). We treated CBSB-S1-injected embryos at 6 hpf with two H2S donor compounds [Sodium hydrosulfide (NaHS), & GYY4137 (Figure S6)], and investigated whether replenishment of H2S would rescue the cbsb morphant phenotype. NaHS is an immediate H2S releasing compound while GYY4137 is a slow-releasing H2S donor. Intriguingly, GYY4137 (Figures 6C,C’) rescues the head edema and axis defects at 52 hpf while NaHS (Figure 6D) does not rescue these phenotypes suggesting that a long-term release of H2S is necessary for CBS enzyme function. Cbs deficiency also causes increase in homocysteine levels (Jhee and Kruger, 2005). Therefore, drug betaine that lowers
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DISCUSSION

This manuscript reports the importance of Csb enzyme in maintaining H₂S homeostasis during embryonic zebrafish development. The salient features include, the characterization of the expression of cbsa and cbsb transcripts during embryonic development, the loss-of-function analysis using two complementary independent approaches, and finally the rescue of the axis phenotype with small molecules that are used for treatment of CBSB enzyme deficiency in the clinic.

The RNA expression pattern for cbsa and cbsb across developmental stages shows that cbsb is the dominant gene in terms of its levels, which later also is the case for function. The expression of cbs is observed as early as bud stage (10 hpf) along the developing embryonic axis, and continues through 24 and 48 hpf along the anterior-posterior axis of the embryo. Csb expression is prominent in the brain and somite muscle, two tissues that are highly susceptible to changes in H₂S levels during normal physiology.
Our approach to assess function of cbsb was to use complementary methods such as MOs and CRISPR-Cas9. Given the evidence that MOs induced phenotypes do not replicate fully in genetic mutants (Kok et al., 2015), we only focused on phenotypes that were observed routinely under both loss-of-function conditions. One main difference between the two approaches is that the splice1 MO (CBSB-S1) targets exon 3 while cbsb CRISPR targets exon 4 of cbsb gene. In the morphants, we did observe head edema and axis defect. Because cbsb CRISPR embryos only showed anterior-posterior shorter axis defect, we reasoned that axis defect as the primary defect for loss of cbsb function during development. It is reasonable to argue that head edema is a secondary effect related to extremely high levels of homocysteine (Figure S7) and methionine in the brain, which is presumed to occur with CBS enzyme deficiency (Figure S5). However, our results and those from others collectively argue against the secondary effect possibility. For example, patients with CBS deficiency who were treated with betaine therapy showed cerebral edema (Yaghmai et al., 2002). Consistent with this observation, treatment of CBSB-S1 embryos with betaine, a molecule known to reduce homocysteine levels and increase methionine levels in plasma failed to rescue head edema (Figure 6F). Thus, the edema seems to be an upstream effect of homocysteine levels in the brain, perhaps increased methionine. Homocysteine and methionine are also products of folate-mediated one carbon cycle, and evidence exists in zebrafish that folate pathway disruption in zebrafish (Lee et al., 2012) causes developmental delays including shortened axis development. Folate also known as vitamin B9 is a co-factor for enzymes involved in transmethylation reactions and de novo synthesis of purines and thymidylate. These products are critical for DNA replication and thus cell cycle-associated events in a developing embryo. Pathway disruption in folate leads to disruption in thymidylate levels, which in turn affects dTTP thus lengthening the cell cycle in the developing embryo (Lee et al., 2012). This delay in S-phase along with the observed increase in apoptosis in these embryonic cells in zebrafish results in fewer cells in the embryo that are necessary for organism development including axis formation. Our results suggest that alteration of products (homocysteine and methionine) shared by the transsulfuration and folate pathways by the transsulfuration and folate pathways predictably show similar consequence in a developing embryo, namely shortened axis development. This interconnection provides one mechanistic explanation to our findings, and requires further exploration in the context of embryonic development.

Previous work has shown that small molecule slow releasing H2S donor GYY4137 treated for 4 days in zebrafish larvae significantly reduced sodium uptake (Kumai et al., 2015), and this reduced sodium uptake was rescued when CBS or CSE was pharmacologically inhibited. In our study, GYY4137 rescues both axis and edema defects associated with loss of cbsb. Taken together, this implies that GYY4137 influences CBSB enzyme directly or indirectly to influence sodium uptake.
uptake in cells of a developing embryo, and this influence is critical for restoring homeostasis in embryo, which includes proliferation and apoptosis of cells that are necessary for axis formation. In addition to H$_2$S, gases such as nitric oxide (NO), and carbon monoxide (CO) collectively referred to as gasotransmitters interact leading to oxidative stress in the developing embryo (Olson et al., 2012). These gases have already been implicated in control of breathing and ion regulation in fish (Perry et al., 2016). NO also has profound effects on neuromuscular properties in zebrafish (Jay et al., 2014), which directly correlates to trunk axis structural integrity. Thus, future work will focus on identifying the interactions of gasotransmitters and the mechanisms that they associate with in facilitating embryonic growth and development.

In summary, we have identified a novel phenotype for cbsb in zebrafish using multiple approaches, and have demonstrated that cbsb is partly responsible for anterior-posterior axis development in zebrafish. A combination of homocysteine and H$_2$S regulation is responsible for the axis development, and structures along the axis of the embryos are highly susceptible to this regulation.

**AUTHOR CONTRIBUTIONS**

SP designed and performed experiments, interpreted data, wrote, and edited the manuscript; CK designed and performed experiments, and interpreted data, wrote, and edited the manuscript; AD designed and performed experiments, and interpreted data; SE-B designed and performed experiments, and interpreted data; NL performed experiments and interpreted data; RB provided intellectual input, direction in experimental design, and edited the manuscript; PM provided intellectual input, direction in experimental design, provided resources, and
ACKNOWLEDGMENTS
We thank Developmental Vascular Biology Program members for their input and suggestions.
SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00014/full#supplementary-material
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.