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Slc39a7/zip7 Plays a Critical Role in Development and Zinc Homeostasis in Zebrafish

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Abstract

Background: Slc39a7/Zip7, also known as Ke4, is a member of solute carrier family 39 (Slc39a) and plays a critical role in regulating cell growth and death. Because the function of Zip7 *in vivo* was unclear, the present study investigated the function of zip7 in vertebrate development and zinc metabolism using zebrafish as a model organism.

Principal Finding: Using real-time PCR to determine the gene expression pattern of zip7 during zebrafish development, we found that zip7 mRNA is expressed throughout embryonic development and into maturity. Interestingly, whole mount *in situ* hybridization revealed that while zip7 mRNA is ubiquitously expressed until 12 hours post-fertilization (hpf); at 24 hpf and beyond, zip7 mRNA was specifically detected only in eyes. Morpholino-antisense (MO) gene knockdown assay revealed that downregulation of zip7 expression resulted in several morphological defects in zebrafish including decreased head size, smaller eyes, shorter palates, and shorter and curved spinal cords. Analysis by synchrotron radiation X-ray fluorescence (SR-XRF) showed reduced concentrations of zinc in brain, eyes, and gills of zip7-MO-injected embryos. Furthermore, incubation of the zip7 knockdown embryos in a zinc-supplemented solution was able to rescue the MO-induced morphological defects.

Significance: Our data suggest that zip7 is required for eye, brain, and skeleton formation during early embryonic development in zebrafish. Moreover, zinc supplementation can partially rescue defects resulting from zip7 gene knockdown. Taken together, our data provide critical insight into a novel function of zip7 in development and zinc homeostasis *in vivo* in zebrafish.

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Introduction

Zinc is an essential trace element required for DNA synthesis, cell division, regulation of transcription, and protein synthesis. Approximately 2000 enzymes use zinc as a catalytic cofactor [1], and zinc binding motifs are found in up to 10% of the proteins encoded by the human genome [2] including zinc-finger-containing proteins, the most abundant protein superfamily in the mammalian genome. In this regard, zinc is an essential cofactor required for the activity of numerous proteins involved in cellular signaling pathways and biological processes including growth factors, cytokines, receptors, enzymes, and transcription factors [3,4,5,6]. In addition, zinc has been found to play a role in cell-mediated immunity and signal transduction, and as an antioxidant and an anti-inflammatory agent [7,8]. It is broadly

acknowledged that numerous disorders are the result of zinc deficiency such as poor appetite, growth retardation, skin lesions, mental lethargy, delayed wound healing, neurosensory disorders, and cell-mediated immune disorders [9,10,11].

Zinc homeostasis in single cells and in whole organism is regulated by two families of zinc transporters: zinc exporters (Slc30a/ZnT or CDF) and importers (Slc39a/Zip) [10,11,12,13,14,15,16,17]. In addition, the tissue-specific expression of each zinc transporter gene, the metals, hormones and cytokines that influence their expression, and the diseases that have been linked to their aberrant expression have been elucidated [16]. There are 10 ZnT family members in mammals. Deficiency in *ZnT2* or *ZnT4* causes reduced zinc concentrations in milk in mammals [18,19], while *ZnT5*-KO mice suffer from growth retardation and osteogenic problems [20]. ZnT5 and ZnT6 are

both localized to the Golgi apparatus [21] and uniquely form a heterodimer [22]. ZnT8 is specifically expressed in pancreatic β -cells and has been identified as a novel target autoantigen in patients with type 1 diabetes [23]. Furthermore, mutation of ZnT8 is associated with glucose intolerance and type 2 diabetes [24]. The Zip family can be divided into four subfamilies, named subfamilies I and II, *gufA* and LIV-1, with most mammalian Zip family members being classified into the LIV-1 subfamily. The LIV-1 subfamily contains nine members (*Zip4*, *Zip5*, *Zip6*, *Zip7*, *Zip8*, *Zip10*, *Zip12*, *Zip13*, and *Zip14*) [17]. With the exception of *Zip7* and *Zip13*, a feature of the LIV-1 subfamily are a potential metalloprotease motif (HEXPHEXGD) in the fifth transmembrane domain (TMD V) [17,25]. Mutations in the human *ZIP4* gene cause the inherited disorder acrodermatitis enteropathica [26], and our previous studies identified *Zip4* as a critical regulator of zinc homeostasis via a process distinct from zinc-stimulated endocytosis [27,28]. While *Zip5* expression is restricted to many tissues important for zinc homeostasis, including the intestine, pancreas, liver and kidneys, abundance of *Zip5* mRNA is not altered in response to changes in zinc concentration [29]. Rather, *Zip4* and *Zip5* are both dynamically regulated by several post-transcriptional, translational, and post-translational mechanisms [30]. Interestingly, zebrafish *zip6/liv1* controls the epithelial-mesenchymal transition (EMT) via activation of signal transducer and activator of transcription 3 (STAT3), suggesting that *zip6/liv1* may have an important role in cell migration [31,32]. The *Zip13* molecule is involved in the bone morphogenetic protein (BMP)/transforming growth factor beta (TGF- β) signaling pathway by controlling the nuclear localization of Smad proteins [33]. Understanding of the diverse functions of the Liv1 family continues to expand as more studies are conducted in model organisms.

Zip7 (Slc39a7, Ke4) also belongs to the LIV-1 subfamily of zinc transporters [34], and has been shown to play a critical role in maintaining the intracellular balance of zinc by affecting the redistribution of zinc from intracellular stores to the cytosol [35]. While ectopic expression of *Zip7* in cells results in an increase in intracellular zinc concentration [25], *Zip7* is localized to the membranes of endoplasmic reticulum (ER) and Golgi apparatus, but not to the plasma membrane; suggesting that *Zip7* functions to transport zinc from the ER and Golgi to the cytosol of mammalian cells [34] [36]. In addition, recent data suggests that *Zip7* acts at a critical link in zinc-mediated tyrosine kinase signaling, and may be involved in breast cancer progression [25]. A recent study identified protein kinase casein kinase II (CK2) as the kinase responsible for *Zip7* activation. CK2 could trigger cytosolic zinc signaling pathways through phosphorylation of *Zip7* and in turn, affect proliferative responses and cell migration [37].

Although *Zip7* has attracted much interest in numerous fields of research and many studies have been performed in primary cells and in cell lines, the *in vivo* functions of *Zip7* have not been determined due to a lack of a *Zip7* gene knockout animal model. *Zip7* orthologs include *CATSUP* in *Drosophila* [38] and *IAR1* in *Arabidopsis* [39]. Although knockout of *CATSUP* in *Drosophila* is lethal, testing partial loss of function mutants revealed *CATSUP* could downregulate tyrosine hydroxylase, a rate-limiting enzyme for production of dopamine in the brain [38]. Furthermore, *IAR1* has been suggested to transport zinc or other metals out of the ER and into the cytosol [39]. In this study, we have elucidated the *in vivo* role of *zip7* in the zebrafish vertebrate model. Previous studies revealed that zebrafish eyes highly express zinc transporters *znt4* and *zip1*, and have constitutively high levels of *zip7* [40]. We extended these findings and found that zebrafish *zip7* has essential roles in eye development. Morpholino-induced loss of *zip7* (*zip7*-

MO) resulted in decreased eye and head size as well as campylophthalmia. Together, our results suggest that *zip7* may play an important role in eye, brain, and skeletal development by regulating zinc transport during zebrafish development.

Results

The expression pattern of *zip7* mRNA during zebrafish embryogenesis

We used whole-mount *in situ* hybridization to determine the spatial distribution of *zip7* gene expression during zebrafish embryogenesis and found that at early stages of somitogenesis (approximately 12hpf), *zip7* mRNA was ubiquitously expressed (Figure 1A). At 24 hpf, *zip7* mRNA transcripts were still detected in the forebrain-proximal part of retina (Figure 1B), and at later stages of zebrafish development, embryos continued to express *zip7* mRNA around the retina (Figure 1D, 1E, and 1F). Temporal changes in *zip7* mRNA expression levels during development were quantified by real-time PCR between 0.2 hpf and 120 hpf, and in adult zebrafish (4 months). Analysis of the mRNA expression pattern of *zip7* (normalized to β -actin mRNA) showed that *zip7* mRNA is expressed during embryogenesis and continues to be expressed in adult zebrafish (Figure 1G). In addition, *zip7* mRNA is highly expressed in brain and eye of adult zebrafish (Figure 1H).

Silencing of *zip7* causes developmental defects in zebrafish

We took a gene knockdown approach to study *zip7* function in vertebrate development. *zip7*-MO were designed against the zebrafish *zip7* mRNA initiating ATG to block *zip7* protein translation. A *zip7*-GFP reporter assay confirmed that administration of *zip7*-MO effectively blocked *zip7* protein translation (Figure 2A). We tested a series of doses of *zip7*-MO and control MOs (4 ng, 6 ng, 8 ng, 10 ng, and 12 ng per embryo). While we did not detect abnormal development in embryos injected with control MO at any stage (Figure 2B), we did observe obvious developmental defects at multiple stages (24 hpf, 48 hpf, and 72 hpf) in embryos injected with *zip7*-MO. Among embryos injected with 10 ng or 12 ng *zip7*-MO, severe morphological abnormalities were observed at 72 hpf. Specifically, we observed death in a fraction of the *zip7*-MO-injected embryos, as well as many embryos with delayed growth that could not hatch and displayed a strikingly curved notochord and decreased eye size compared to controls (Figure 2C).

We then cloned the full-length zebrafish *zip7* gene and observed that injection of 225 pg *zip7* mRNA alone did not cause phenotypic changes in embryos (data not shown). To confirm the specificity of the *zip7*-MO, we co-injected 225 pg *zip7* mRNA with 10 ng *zip7*-MO and found that the developmental defects described above were partially rescued (Figure 2D). Statistical analysis (Figure 2E) showed that 72 hours after microinjection, approximately 94% of the fish displayed smaller eyes, spine bending, and head dysplasia compared to controls. Strikingly, 72 hours after *zip7* mRNA and *zip7*-MO were co-injected into fertilized eggs, the *zip7* knockdown phenotype was rescued suggesting that loss of *zip7* was specifically responsible for the developmental defects observed following *zip7*-MO injection.

Zinc supplementation can partially rescue development defects resulting from *zip7* silencing

We next tested whether supplementation with various concentrations of zinc (Zn^{2+} 25 μ M, 50 μ M, 75 μ M, or 100 μ M) could restore normal development in hatched embryos injected with

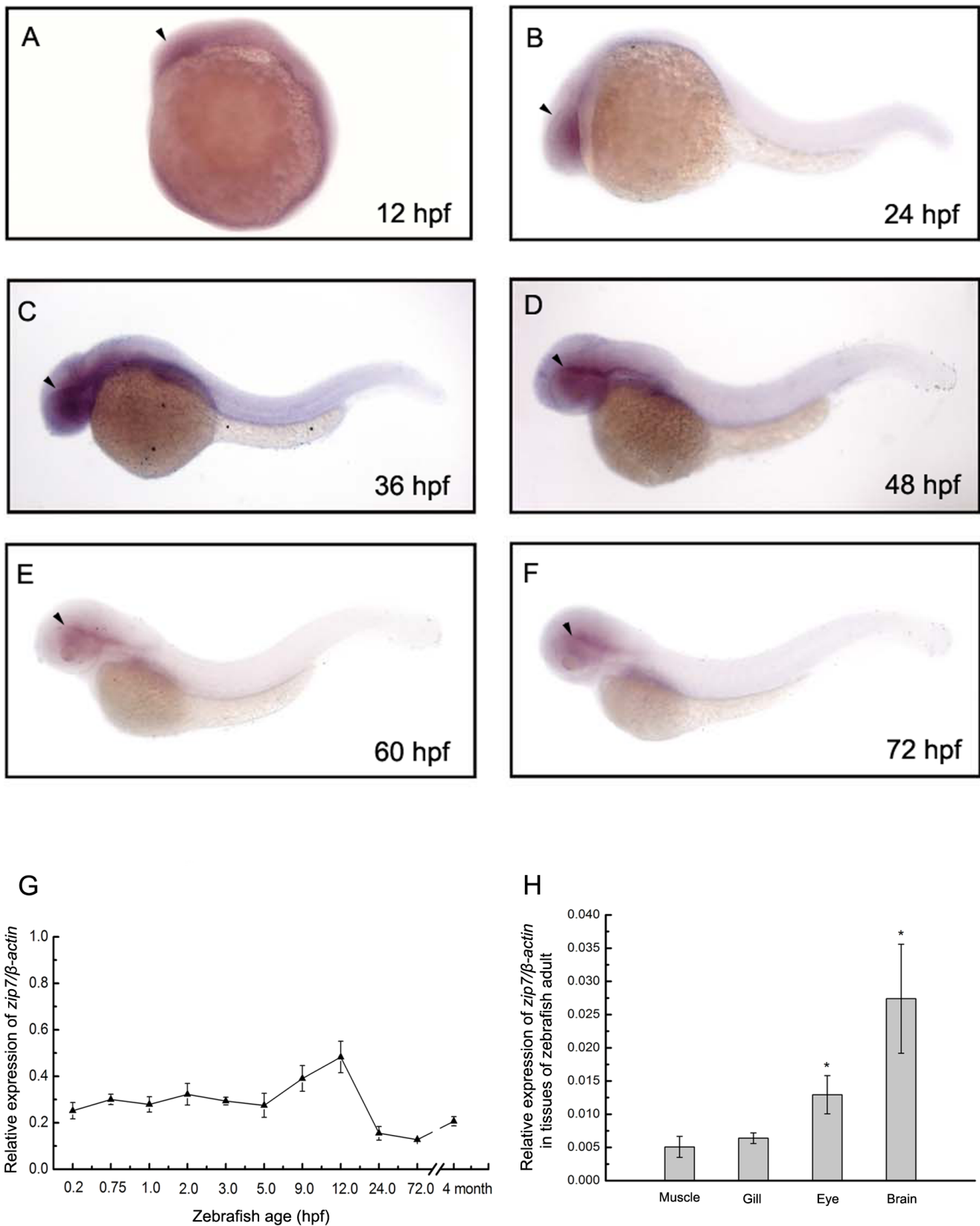


Figure 1. *Zip7* expression during zebrafish early embryonic development in wild type embryos and in tissues of adult zebrafish. (A–F) Lateral views (anterior to the left) of WISH of *zip7* expression at 12 hpf, 24 hpf, 36 hpf, 48 hpf, 60 hpf, 72 hpf, embryos. Arrows indicate the eye. (G) The *zip7* mRNA expression pattern assayed by qRT-PCR normalized to β -actin transcripts in zebrafish embryos across development and at the adult stage. (H) Normalized *zip7* mRNA expression levels in tissues of zebrafish adult. * $P < 0.05$ versus muscle (1-way ANOVA, Dunnett's multiple comparison test).

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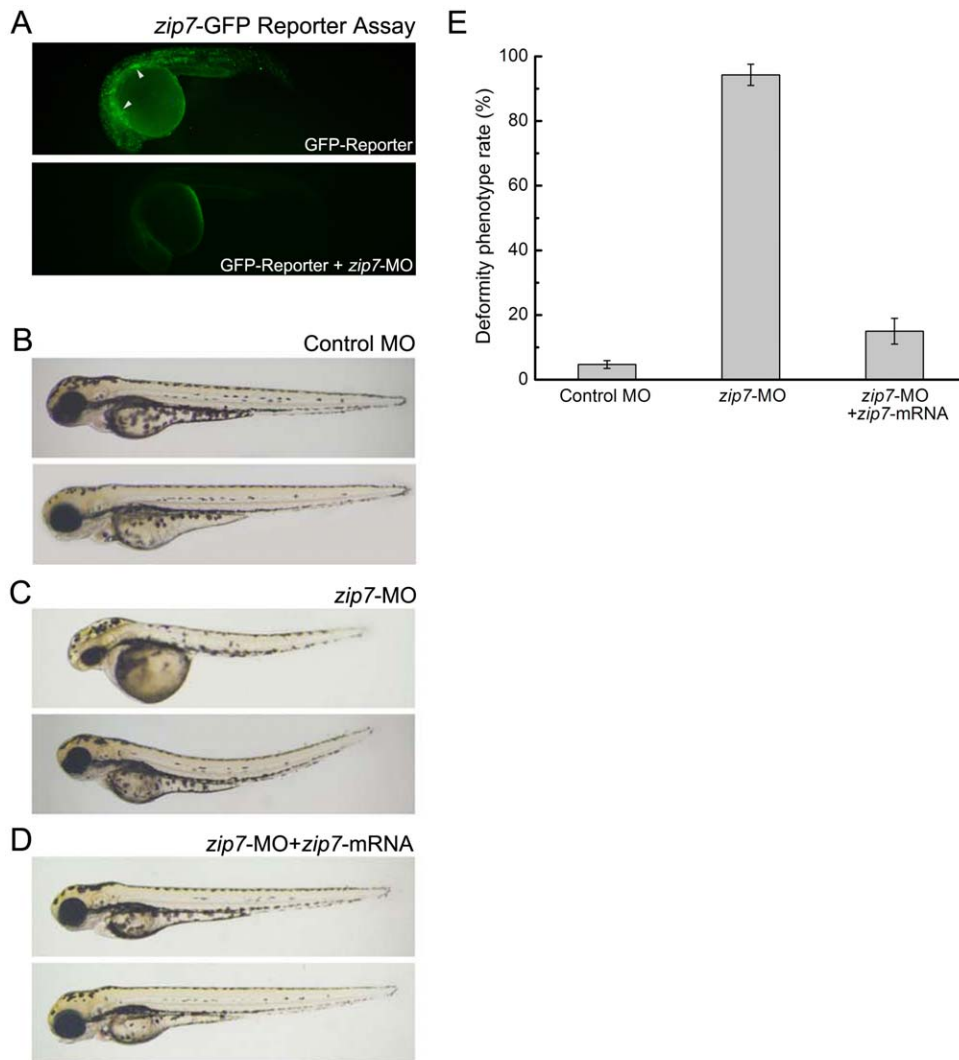


Figure 2. Morphological phenotypes of *zip7* loss with MO microinjection and/or *zip7* mRNA rescue at 72 hpf embryos. (A) Fluorescent images of live 24 hpf, GFP-Reporter control embryos ($n = 57/60$) and GFP-Reporter+*zip7*-MO morphant embryos ($n = 62/62$) indicate inhibition of *zip7*-GFP reporter expression (green, white arrows) by *zip7*-MO. (B–D) Lateral views (anterior to the left) of embryos at 72 hpf (B) Wild type with control-MO microinjection (2 nL, 10 ng). (C) Wild type with *zip7*-MO microinjection (2 nL, 10 ng). (D) Wild type with *zip7*-MO (1 nL, 10 ng) + *zip7* mRNA (bottom, 1 nL, 225 pg) microinjection. (E) Statistical analysis of microinjection of *zip7*-MO and *zip7* mRNA phenotypes. doi:10.1371/journal.pone.0042939.g002

10 ng *zip7*-MO. Strikingly, we found that, in fact, the defects we observed in zebrafish morphology during early embryonic development upon *zip7* knockdown could be rescued with zinc supplementation. In this regard, while the phenotypes of embryos at 72 hpf injected with control MO (Figure 3A) or *zip7*-MO (Figure 3B) were consistent with our previous results (Figure 2), we observed that the morphological defects in *zip7*-MO-injected embryos hatching in the presence of 50 μ M zinc at 72 hpf were partially rescued (Figure 3C). Moreover, when embryos were microinjected with *zip7*-MO and then cultured in different concentrations of zinc solution, we found that in the presence of 50 μ M or 75 μ M zinc, approximately 80% of the *zip7*-knockdown embryos showed rescue (Figure 3D). However, the phenotype of *zip7*-knockdown embryos could not be restored in the presence of 25 μ M zinc ion due to a failure to hatch, and concentrations higher than 100 μ M would be toxic (data not shown). Thus, hatching in the presence of 50 to 75 μ M zinc could supply an

appropriate zinc environment for the development of *zip7*-MO zebrafish embryos.

Silencing of *zip7* can result in abnormal zinc distribution in zebrafish

Zinc intensity maps generated with SR-XRF showed zinc distribution and concentrations in zebrafish embryos at 72 hpf. Data were imaged at 30 μ m X-Y resolution in two- and three-dimensions using Igor Pro Folder software (Figure 4). Zinc mainly distributes in eyes, gills, brain and yolk of wild type embryos. In this regard, in contrast to the images of wild type (Figure 4A and 4E) or control MO-injected embryos (Figure 4B and 4F), zebrafish with *zip7*-MO injection showed a loss of zinc in eyes (Figure 4C and 4G). However, zebrafish injected with *zip7*-MO hatching in the presence of zinc (50 μ M) showed the presence of zinc in eyes (Figure 4D and 4H). Quantitation and statistical analysis showed no evident difference in the whole embryo zinc content (Figure 4I). However, it was noteworthy that zinc content in eye of *zip7*-MO

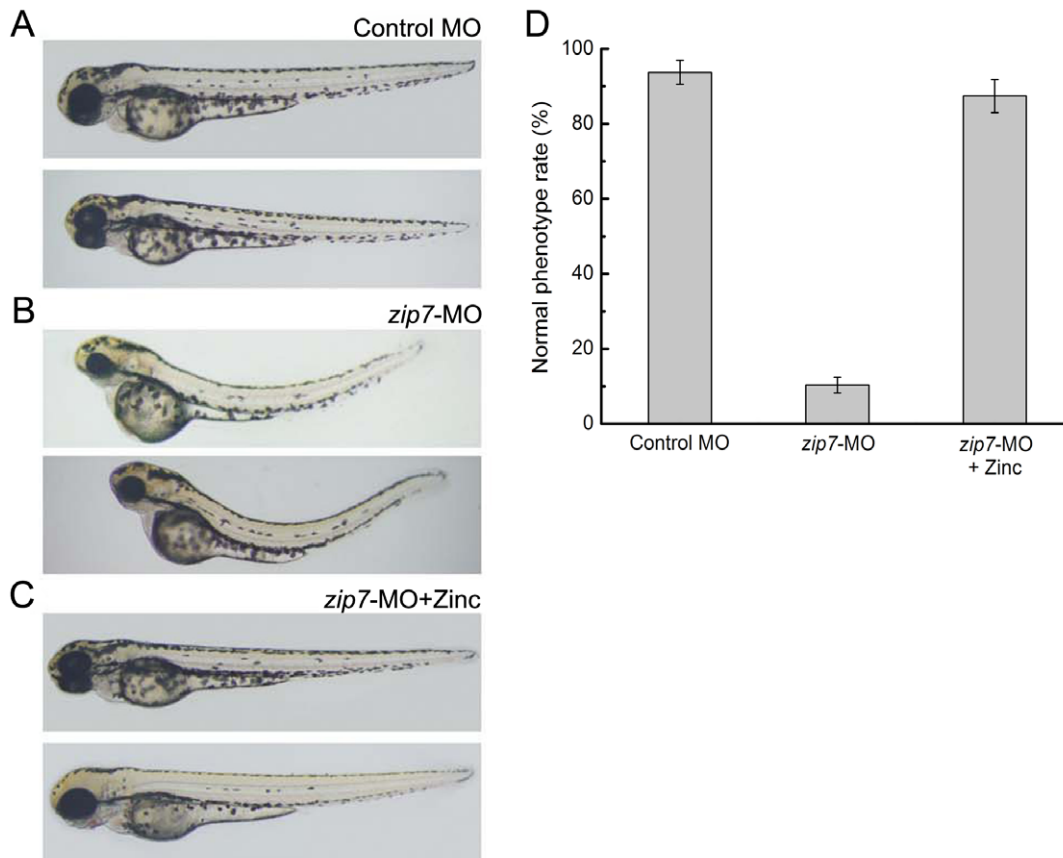


Figure 3. Morphologic phenotypes of *zip7*-deficient embryos and Zn^{2+} (50 μM) rescue at 72 hpf. Lateral views (anterior to the left) of embryos at 72 hpf. (A) Wild type with control-MO microinjection (2 nL, 10 ng). (B) Wild type with *zip7*-MO microinjection (2 nL, 10 ng). (C) Wild type with *zip7*-MO microinjection (2 nL, 10 ng) hatching in the presence of Zn^{2+} (50 μM). (D) Statistical analysis of microinjection *zip7*-MO and Zn^{2+} phenotype. doi:10.1371/journal.pone.0042939.g003

was remarkably lower than wild type and control MO, while addition of exogenous zinc restored it to a normal level (Figure 4J). Together, these data indicate that *zip7* could play a critical role for zinc transportation in the eyes.

Silencing of *zip7* affects the expression of other zinc transporters

We analyzed the expression levels of two zinc transporter family members, the *zip* and *znt* families, in *zip7*-MO and control MO embryos at 3 dpf (Figure 5A and B). Compared with the control MO embryos, the *zip7* transcript level in *zip7*-MO embryos decreased markedly. However, the expression of *zip3*, *zip6*, *znt2*, *znt5* and *znt6* increased significantly. The transcript levels of other *zip* and *znt* family members were not different between *zip7*-MO and control MO embryos.

Discussion

In the present study, we investigated the function of Zip7 in development using zebrafish as a vertebrate model organism. In the zebrafish model system, several experimental approaches can be utilized, including RNA *in situ* hybridization, morpholino injections, and analysis of mutant and transgenic fish lines. Incorporation of these diverse approaches can lead to improved understanding of the *in vivo* regulation of key molecular pathways with conserved roles in vertebrate zinc homeostasis. For example,

results from a study of *zip6/liv1* using the zebrafish model suggest that *zip6/liv1*, as a downstream target of STAT3, is required for nuclear translocation of the Zn-finger transcription factor Snail, which regulates the epithelial-mesenchymal transition (EMT) during early zebrafish development [31].

The mouse *Zip7* gene was discovered while characterizing genes in the major histocompatibility complex on chromosome 17 [41]. Human *ZIP7* was mapped to the HLA class II region on chromosome 6 [42]. However, the precise function of Zip7 in whole body zinc homeostasis is not clear. Our results using RT-PCR revealed that in zebrafish, *zip7* began to be expressed early in embryonic development, and its expression continued throughout embryonic development and into maturity. These results were further confirmed by a whole-mount *in situ* hybridization assay that showed *zip7* expression gradually decreased and became restricted to developing embryos, and in adult zebrafish was found highly *zip7* expression in the brain and eye. Previous study also found *zip7* was mainly expressed in eye, brain, ovary, liver, gill and intestine in zebrafish [40]. The expression pattern of *zip7* may suggest that *zip7* performs a specific function in these organs. In addition, the mouse and human *ZIP7* mRNAs were also detected in many cDNA libraries including embryo, mammary gland, ovary, uterus, cervix, testis, prostate, tongue, larynx, stomach, pancreas, bladder, eye, pituitary, bone, bone marrow, skin, and peripheral nervous system [36]. According to the human *ZIP7* gene expression atlas available in BioGPS (<http://www.biogps>).

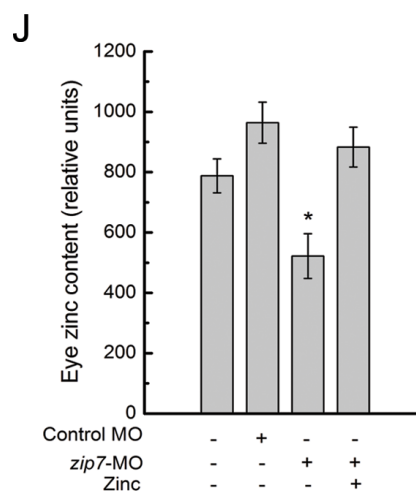
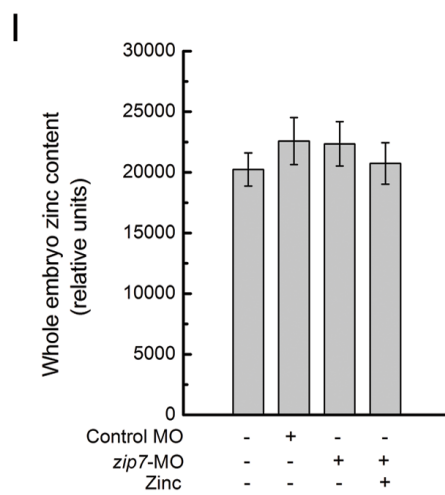
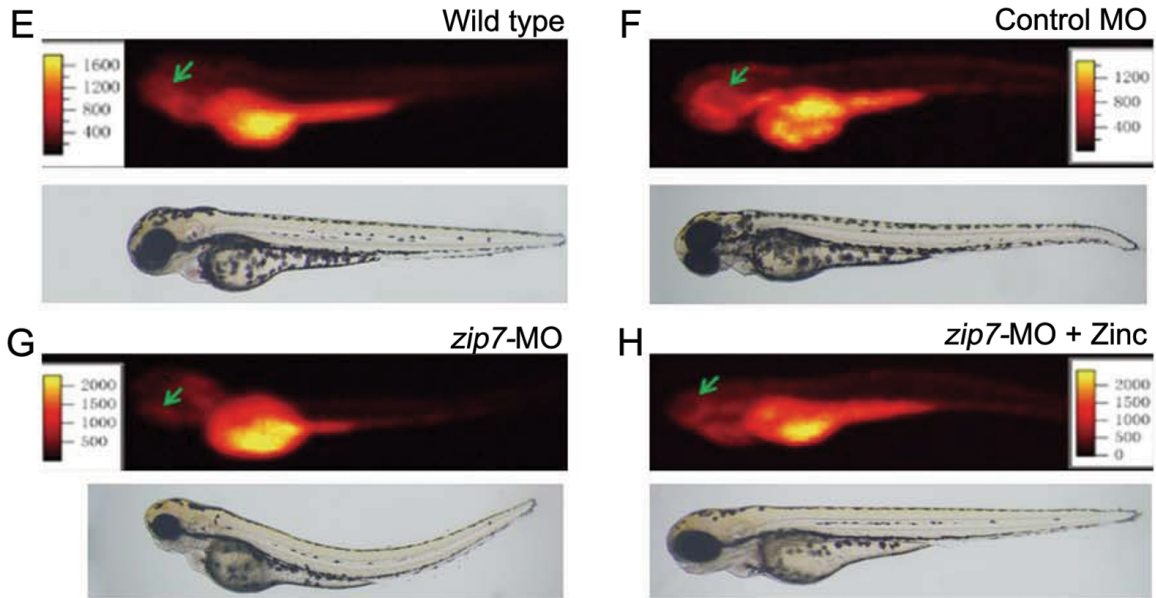
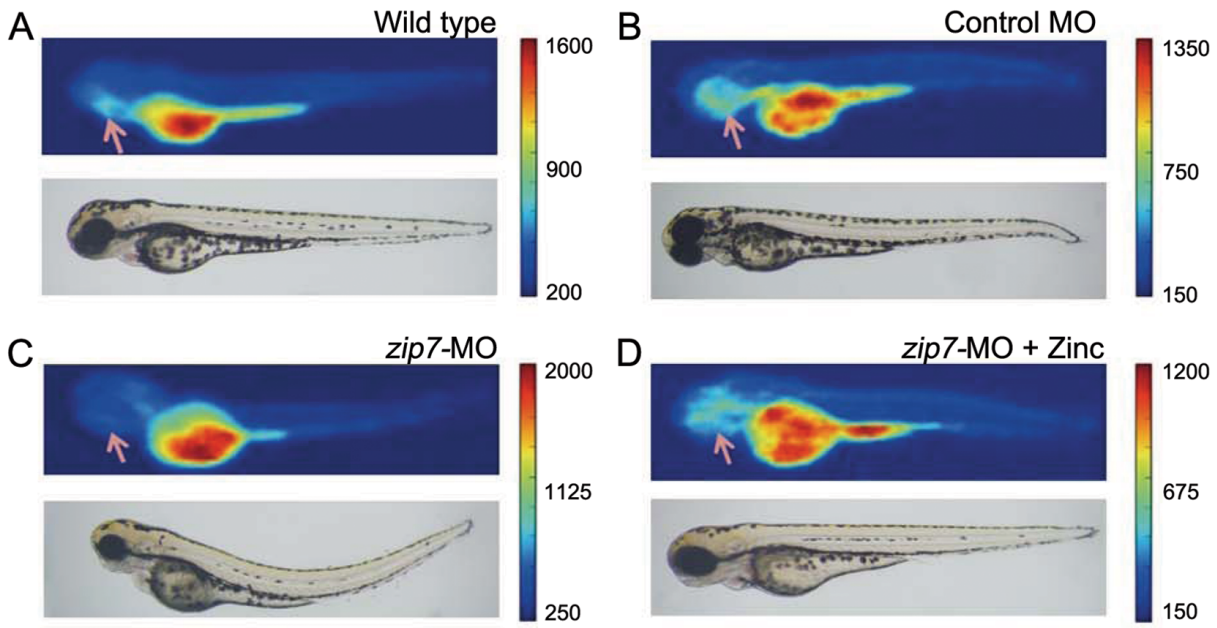


Figure 4. SR-XRF images and quantitative analysis of Zinc in *zip7*-Deficient Embryos and Zn^{2+} (50 μM) rescue. Lateral views (anterior to the left) of embryos at 72 hpf, arrows indicate eye. (A and E) Wild type without any microinjection, (B and F) wild type with control-MO microinjection (2 nL, 10 ng), (C and G) Wild type with *zip7*-MO microinjection (2 nL, 10 ng), (D and H) Wild type with *zip7*-MO microinjection (2 nL, 10 ng) and hatching in the presence of Zn^{2+} (50 μM). (A–D) Two-dimensional images of SSRF, (E–H) Three-dimensional images of SSRF. Quantitation and statistical analysis of zinc densities in wild type, control-MO, *zip7*-MO, and *zip7*-MO+zinc embryos (I and J). (I) Relative zinc content in the whole embryo, and (J) relative zinc content in the eye. *Statistical differences with corresponding wild type (*t* test, $P < 0.05$). The zinc relative content was acquired by d4/d2/d1 (d4: photon counting of zinc correspondence, d2: electronic counting of light intensity, and d1: irradiation time). doi:10.1371/journal.pone.0042939.g004

org/#goto=genereport&id=7922), *ZIP7* is highly expressed in prostate, pituitary gland, retina, smooth muscle, lung and colon, which is similar to zebrafish *zip7* expression.

Furthermore, we analyzed embryos lacking *zip7* using antisense morpholino (*zip7*-MO) oligonucleotides (MOs), which are the most widely used anti-sense knockdown tools in the zebrafish (*Danio rerio*) community. MOs are typically employed as oligomers of 25

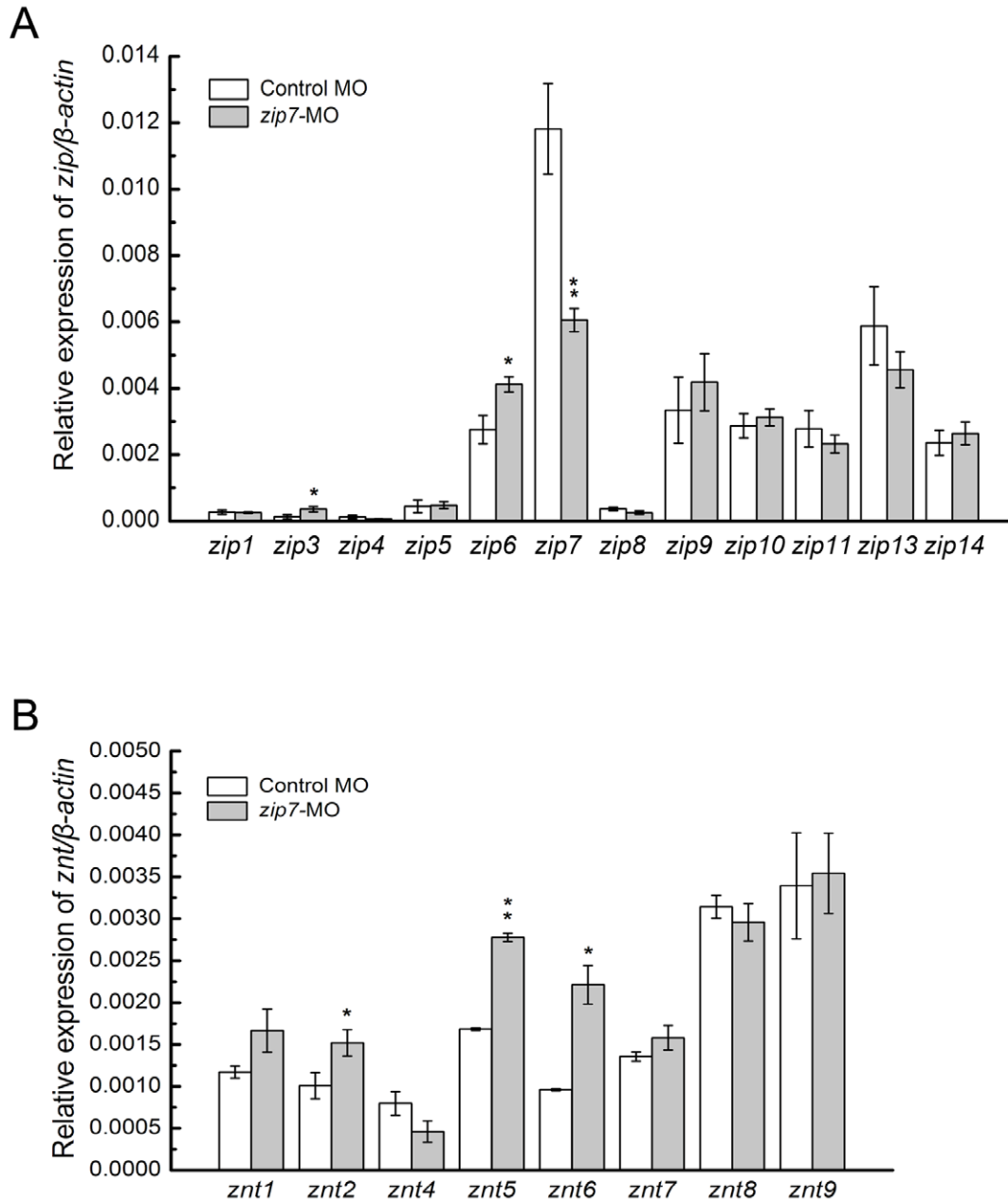


Figure 5. The expression of *zip* and *znt* family members in *zip7*-MO embryos. (A) Relative mRNA levels of *zip* family members in wild type embryos and *zip7*-MO embryos at 3 dpf. (B) Relative mRNA levels of *znt* family members in wild type embryos and *zip7*-MO embryos at 3 dpf. For qRT-PCR analysis, results were normalized to the internal control, β -actin, and presented as relative expression level calculated by the $2^{-\Delta\Delta Ct}$ method. Results are presented as mean \pm SEM. * $P < 0.05$, ** $P < 0.005$. doi:10.1371/journal.pone.0042939.g005

morpholine bases that are targeted via complementary base pairing to the RNA of interest. A neutrally-charged phosphorodiamidate backbone results in molecules with high binding affinity for RNA, thereby facilitating steric hindrance of proper transcript processing or translation [43]. In our studies, embryos receiving injections of *zip7*-MO displayed retarded embryonic development accompanied by smaller heads, smaller eyes, shorter palates and spinal lordosis, whereas the injection of control-MO did not result in any obvious phenotype. We also carried out rescue experiments in which we co-injected *zip7*-mRNA with *zip7*-MO, and found that the *zip7* mRNA expression was comparable to that found in wild type embryos. These results suggest that *zip7* plays a critical role during zebrafish embryonic development.

Furthermore, we explored how Zip7 affected the developmental process in zebrafish through zinc rescue experiments. Addition of 50 μ M zinc to hatching *zip7*-MO-injected embryos resulted in normal development, which indicated that *zip7* mediates development through zinc⁺ instead of directly influencing other signal pathways. To further verify our results, we measured the distribution of zinc in whole embryos by Synchrotron radiation microbeam X-ray fluorescence (SR-XRF). SR-XRF is highly specific and sensitive for identification, characterization, and distribution analysis of metals and nonmetals in a given cell type or tissue [44]. SR-XRF is one of the few techniques capable of providing spatially resolved *in vivo* metal abundance data on a sub-micrometer scale, without the need for chemical fixation, coating, drying or even sectioning of samples [45]. The SR-XRF has been used to detect the relative contents and distributions of many trace metals in biological samples [46]. We found that the distribution of zinc between *zip7*-MO fish and wild type fish was highly different. Compared with the wild type fish, the distribution of zinc in *zip7*-MO-treated embryos was disordered and severely reduced in eyes. Quantitation and statistical analysis showed no significant difference in the whole embryo zinc content, while zinc content was severely reduced in the *zip7*-MO eye. These data indicate that *zip7* is a critical zinc transporter in zebrafish eye and is essential for zinc homeostasis during eye development. In contrast, the distribution of zinc in *zip7*-MO embryos hatching in the presence of 50 to 75 μ M zinc resembled that observed in wild type embryos. This result suggested *zip7*-MO zinc content could be restored to normal levels by exogenous zinc supplementation, which was critical for zebrafish embryonic development.

Taken together, our results show that Zip7 has a very vital effect on embryonic development by regulating the absorption and distribution of zinc, however the specific details of this process remain unclear. We continued to detect the expression of other zinc transporters when *zip7* was silenced. qRT-PCR analysis revealed the expression of *zip3*, *zip6*, *znt2*, *znt5* and *znt6* were significantly increased in the absence of *zip7*. Zip3 localizes to cell bodies of the retina that also express PKC (protein kinase C) [47]. Zip6 localizes at the plasma membrane of rat neurons, suggesting a role for Zip6 in neuronal zinc uptake [48], and Zip6 has been shown to control the epithelial-mesenchymal transition in the zebrafish gastrula organizer [31]. ZnT2 expression was restricted to tissues with unique zinc requirements, such as mammary and prostate glands, where it mainly localized to the zymogen granules. In addition, ZnT2 expression level could be affected by dietary zinc content [49,50]. ZnT5 and ZnT6 have both been shown to localize to the Golgi apparatus, and often form hetero-oligomers that function to activate alkaline phosphatases in the early secretory pathway [21,51]. Together, these studies show that the same tissues or organelles expressing *zip7* co-express *zip3*, *zip6*, *znt2*, *znt5* or *znt6*. Silencing of *zip7* could change zinc levels and affect the expression of other zinc transporters, ultimately leading

to a more serious zinc imbalance. Exogenous zinc supplementation could rectify this vicious circle, and other zinc transporters could execute their normal roles. Thus, the defect caused by *zip7* silencing can be compensated for. Previous studies revealed that zinc is essential for normal cell growth and development and is involved in protein, nucleic acid, carbohydrate and lipid metabolism, as well as in the control of gene transcription, growth, and differentiation. Intracellular zinc signals are classified into transcription-independent early zinc signaling (Ezs) and transcription-dependent late zinc signaling (Lzs) [52]. Many cytosolic proteins may have zinc-binding potential are expected to be closely involved in a wide range of physiological responses including development, immune function, cancer progression, and hard and connective tissue disorders [5]. Zinc itself affects a variety of signaling molecules including PKC, Ca²⁺/calmodulin-dependent protein kinase II, Erk1/2, cAMP-dependent protein kinase, protein tyrosine phosphatase, and caspase-3 [5]. In addition, zinc also acts as an intracellular second messenger [3,5]. It has been suggested that ZIP7 protein is localized to the Golgi apparatus and the endoplasmic reticulum, which are critical organelles in the redistribution of zinc from intracellular stores to the cytosol [36,53]. As such, zinc release has downstream effects on cell signaling and hence, zinc is indeed a second messenger. An important component of zinc action in cells is the ability to inhibit protein tyrosine phosphatase activity, resulting in activation of mitogen-activated protein kinases, such as ERK1/2, c-Jun N-terminal kinase, and p38, as well as the tyrosine kinases Src and epidermal growth factor receptor. Thus, Zip7 is a central hub in cell signaling, regulating cell growth and differentiation as well as embryonic development [5,25].

In summary, our results reveal that *zip7* plays indispensable roles in maintaining zinc homeostasis and organism development especially in eyes, brain, and gills. These findings will be helpful for the understanding of mechanisms of zinc homeostasis and diseases resulting from defects in proper zinc homeostasis.

Materials and Methods

Fish husbandry and embryo preparation

Adult male and female zebrafish (*Danio rerio*) were maintained under a 14 hour light/10 hour dark cycle at 28.5°C with recirculating deionized water. Embryos were collected from natural matings and staged matings according to Kimmel [54]. All zebrafish experimental protocols were approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, and Chinese Academy of Sciences.

qRT-PCR analysis

Pooled embryos or adult tissues were homogenized in TRIzol Reagent (Invitrogen) to extract total RNA according to the manufacturer's instructions and treated with DnaseI (Promega). RNA concentration and purity were assessed by spectrophotometry. 2.0 μ g of RNA was reverse-transcribed with M-MLV reverse transcriptase (Promega) and oligo (dT) 18 primers (Takara) as recommended. PCR was performed by using CFX96™ Real-Time System (Bio-Rad) and iQ™ SYBR Green Supermix (Bio-Rad) as described in the manufacturer's manual. The reaction proceeded as follows: 95°C for 5 min, 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. β -actin was used as an internal reference to normalize the PCR reaction. Primer sequences are listed in Table 1. The primers for genes (*zip1*, *zip3*, *zip4*, *zip6*, *zip9*, *zip11*, *zip13*, *znt1*, *znt2*, *znt4*, *znt5*, *znt6*, *znt7*, *znt8* and *znt9*) have been reported previously [55].

Table 1. Primers for qRT-PCR analysis.

Gene name	Forward Primer (5'–3')	Reverse Primer (5'–3')
<i>zip7</i>	TTGGTCTGTGGGTGCTAGGT	AGCAGAGGGAGAGTGGGAAT
<i>zip1</i>	GGTGAGAGTTGGAGCTCTGG	AGTGGGAAGCCATCATCAAG
<i>zip3</i>	CGTATACGGCTGATGTGGTG	AGGCCTGTGTAAACCACTG
<i>zip4</i>	CAGACATGCTTCTACGCTG	GCCCGATCTGGTCTTCATAA
<i>zip5</i>	TGCAACGTCTGTCCTTCAG	TCCCAAACCCAAACTACCAA
<i>zip6</i>	GTCATCATGGGAGACGGACT	GGCAAAATCACCGAGTTCAT
<i>zip8</i>	TCCCCGCCTGCCCTTACACTT	CAGTCCAATGAAGTACATCAAAACTT
<i>zip9</i>	TCGGAATGTGACGAGCCTTCGC	ACATGTATCTCGGAGATCGCGTG
<i>zip10</i>	TCACCTGCACATGTTGTTCT	ACATCCAAACCCATCCTGAA
<i>zip11</i>	TCAGGCCCTGTGGGACTC	GCCACAGCCACTGGGAGGA
<i>zip13</i>	GGAGACCAACCAAGGAAGT	GTCTTTGGGAGGGTGACAAA
<i>zip14</i>	TAGGGGATGTTGAGGTCAG	T CGCTGTCTATACGGGACT
<i>znt1</i>	GAAGGCTGCCGATATGTGTC	AGGACATGCAGGAAAACACC
<i>znt2</i>	TCGGTGGCACAGATCAGAGATT	ACCGTGGCCACAGGACTCA
<i>znt4</i>	CATCTGCTGGAGGGTGTGA	CTGCAGTTGTACCCGTGAGT
<i>znt5</i>	TATCTCCAGTGGGAAGCTGG	ATCACTGCACACCCCATTTT
<i>znt6</i>	CCATCGCTCCGTCCTG GGGAA	ACCGCCAGCACCTCGAAACG
<i>znt7</i>	CCCTTCTGAATGCTACCAA	CACCGACCTGTGTGAAGATG
<i>znt8</i>	ATCGTCTTGATGGAAGGCA	TTTCTCGAAGCACCTCCTGT
<i>znt9</i>	CCTGTTTTGGTTGGCAAAGT	GAATGCTCTCTGCCCTTCGTC
<i>β-actin</i>	CTCTCCAGCCTTCCTCTCT	CTTCTGCATACGGTCAGCAA

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Plasmids constructions

The *zip7* coding region was amplified from whole-genome cDNA and cloned into the pCS2+ vector to generate *pCS2+–zip7*. Injection of *zip7* mRNA (*in vitro* transcribed by mMACHINE[®] High Yield Capped RNA Transcription Kit) was performed to rescue *zip7* knockdown. For whole-mount *in situ* hybridization studies, a PCR-amplified region containing 431 bp of the last exon and the 3'UTR of *zip7* was inserted into the pCS2+ vector to generate the *pCS2+–zip7*-probe.

Microinjection of morpholino-oligonucleotides (MOs) and mRNA

Antisense morpholino oligonucleotides against *zip7* mRNA were designed and synthesized by Gene Tools, LLC. The sequences of the translational blocking *zip7*-MO were 5'-GCGATTTGCTAAAGACCCTGATTGT-3' (–3 to –22, using the nucleotide of the start codon as the reference). The sequence of the mismatched control MO for *zip7* was 5'-CCTCTTACCTCAGT-TACAATTTATA-3'. The dosage for morpholino injection was 10 ng per embryo.

Capped sense RNA was synthesized using the mMACHINE kit (Ambion) from *pCS2+–zip7*. Microinjections were carried out using the Harvard Apparatus PLI-90 microinjector. For double *zip7*-MO injections and *pCS2+–zip7* mRNA and *zip7*-MO injections, embryos were injected separately with 1 nL of each at appropriate concentration.

GFP reporter assay

The *zip7*-MO designed against *zip7* targeted the *zip7* ATG start site to block its translation. To assay the effectiveness of *zip7*-MO, a 195-bp *zip7* cDNA fragment (27 bp upstream and 165 bp

downstream of ATG start site) was fused with GFP cDNA and cloned into PCS2+ vector. The *zip7*-GFP reporter construct, which contains the *zip7*-MO target site, was injected to one-cell stage, wild type embryos together with or without *zip7*-MO. GFP was detected by fluorescent microscope at 24 hours after injection.

Whole mount *in situ* hybridization

Antisense RNA probes were synthesized with DIG RNA Labeling Kit (AP6/T7) (Roche) from the cDNA in the pCS2+ vector. Embryos were selected at 12 hpf, 24 hpf, 36 hpf, 48 hpf, 60 hpf, or 72 hpf. Embryos beyond 24 hpf were treated with 0.003% phenylthiourea to prevent melanization, and all the embryos were removed from chorions. The steps of whole mount *in situ* hybridization referred to Sun Y [56]. Finally, the embryos were photographed using the Nikon SMZ1500 Zoom Stereomicroscope.

Full scale scanning of zebrafish by SR-XPF

We detected the distribution of zinc in whole embryos injected with *zip7*-MO or not, and cultured with zinc or not, by SR-XRF. The absolute contents and distribution of zinc in zebrafish embryos 72 hpf were analyzed with SR-μXPF at the beamline BL15U at Shanghai Synchrotron Radiation Facility (Shanghai, China). The continuous synchrotron X-rays were monochromatized by a Si double crystal [44]. A monochromatic X-ray beam with photon energy of 12 keV was used to excite the zebrafish. The zinc distribution in the zebrafish was continuously scanned at a step of 30 μm for both x and y directions. Each spot was irradiated for 1.5 s. The results were analyzed using the Igor Pro Folder program and Originlab OriginPro 8.5 software.

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Author Contributions

Conceived and designed the experiments: FW. Performed the experiments: GY Y. Zhang JY. Analyzed the data: YY FZ ZZ AW XY Y. Zhou. Wrote the paper: GY Y. Zhang JY FW.

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