

# Molecular cloning and functional characterization of a high-affinity zinc importer (*DrZIP1*) from zebrafish (*Danio rerio*)

Andong QIU, Majid SHAYEGHI and Christer HOGSTRAND<sup>1</sup>

School of Health and Life Sciences, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NN, U.K.

Zinc is a vital micronutrient to all organisms and a potential toxicant to aquatic animals. It is therefore of importance to understand the mechanism of zinc regulation. In the present study, we molecularly cloned and functionally characterized a zinc transporter of the SLC39A family [commonly referred to as the ZIP (Zrt- and Irt-related protein) family] from the gill of zebrafish (*Danio rerio*) (*DrZIP1*). *DrZIP1* protein was found to localize at the plasma membrane and to function as a zinc uptake transporter when being expressed in either chinook salmon (*Oncorhynchus tshawytscha*) embryonic 214 cells or *Xenopus laevis* oocytes. In comparison with pufferfish transporter proteins (*FrZIP2* and *FrECaC*) that are known to facilitate cellular zinc uptake, *DrZIP1* appears to have high affinity to bind and transport zinc, suggesting that it may

be a high-affinity zinc uptake transporter ( $K_m < 0.5 \mu\text{M}$ ) in fish. Orthologues of *DrZIP1* were also identified in both freshwater and seawater pufferfish (*Tetraodon nigroviridis* and *Takifugu rubripes*), indicating that these proteins may be functionally conserved among different fish species. *DrZIP1* mRNA is expressed in all the tissues examined in the present study and thus *DrZIP1* may be a constitutive zinc uptake transporter in many cell types of zebrafish.

**Key words:** chinook salmon (*Oncorhynchus tshawytscha*) embryonic 214 cells (CHSE-214 cells), *DrZIP1*, molecular cloning, pufferfish (*Tetraodon nigroviridis* and *Takifugu rubripes*), transport, zebrafish (*Danio rerio*).

## INTRODUCTION

Many macromolecules in cells require zinc as a vital structural cofactor for their functions in processes such as growth control, apoptosis, development and differentiation [1,2]. Due to its functional importance, even moderate zinc deficiency can cause problems including anaemia, loss of appetite, immune system defects, developmental problems and teratogenesis [3]. Conversely, excessive zinc accumulation can be toxic and has been linked to neurodegeneration [4,5]. Furthermore, zinc may be an environmental threat to aquatic organisms and is one among the elements with the highest frequency of water quality criteria violation in Europe and U.S.A. Thus zinc balance has to be precisely controlled to ensure that a proper amount of zinc is kept for the normal physiological functions in organisms.

Zinc uptake across the apical membrane of absorptive epithelial cells is an important step to regulate zinc homeostasis in vertebrates [6,7]. In fish, there are two routes of metal acquisition: from the diet through the intestine and from water through the gill. Teleost fish gill is an ion transporting epithelium that has long been used as a model to study ionoregulation [8]. Zinc uptake through the fish gill is considered to take place primarily in discrete 'mitochondria-rich' ionocytes, called chloride cells [6,9], and is a carrier-mediated transcellular process that follows Michaelis–Menten kinetics [6,10]. Kinetic studies suggest that there may be two different pathways for cellular zinc uptake in fish gill [10–12]. One of them is thought to be a lanthanum-sensitive  $\text{Ca}^{2+}$ -channel, partly on the basis of the observation that zinc and calcium compete for their entry to gill cells [10–12]. Similarly, competitive uptake of zinc and calcium was also described in the brush border

membrane of rat and piglet intestines [13–15]. Recently, we molecularly cloned the ECaC (epithelial calcium channel) genes from pufferfish (*Takifugu rubripes*) and zebrafish (*Danio rerio*) gills [16,17]. These fish ECaC genes seem to be mainly expressed in the gill and function to mediate the uptake of both calcium and zinc when expressed in both MDCK cells (Madin–Darby canine kidney cells) and *Xenopus laevis* oocytes [16,17]. Thus fish ECaC seems to be the candidate protein mediating the competitive pathway of zinc and calcium uptake in the gill. The facts that branchial zinc uptake cannot be completely inhibited by increased waterborne calcium and is less efficiently blocked by lanthanum compared with calcium [6,10], suggest the existence of another unknown zinc uptake pathway in fish gill. This assumption is further supported by the observation that there seems to be no obvious zinc uptake mediated by ECaC in fish intestine [18], as only a very low level of ECaC mRNA could be detected in pufferfish intestine and almost negligible ECaC mRNA was present in zebrafish and rainbow-trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) intestines [16,17]. As fish intestine appears to act as the bulk pathway for zinc uptake [19], there must be some unknown specific zinc uptake pathway mediating the intestinal zinc acquisition in fish. However, the molecular nature of the unknown zinc uptake pathways in fish gill and intestine remains unknown.

In recent years, the molecular characterization of zinc uptake transporters from evolutionarily diverse organisms (yeast, plants and mammals) has been achieved [20] and these proteins are from the large ZIP (Zrt- and Irt-related protein) family of metal transporters [21] that has been recently assigned the HUGO name, solute carrier family 39, with the approved symbol SLC39A [22].

Abbreviations used: Caco-2 cells, human Caucasian colon adenocarcinoma cells; CHSE-214 cells, chinook salmon (*Oncorhynchus tshawytscha*) embryonic 214 cells; ECaC, epithelial calcium channel; EGFP, enhanced green fluorescent protein; ZIP, Zrt- and Irt-related protein; LZT, LIV-1 subfamily of ZIP zinc transporters; MDCK cells, Madin–Darby canine kidney cells; MT, metallothionein; ORF, open reading frame; PKC, protein kinase C; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-PCR; TM domain, transmembrane domain.

<sup>1</sup> To whom correspondence should be addressed (email christer.hogstrand@kcl.ac.uk).

The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY314992 and AY529485.

**Table 1** Primers used in RT-PCR

Primer name	Nucleotide sequence (5' → 3')
DRF	TGAGCTGTTTCGCGAGGAG
DRR	CCYT CRAANACNGAGTG
DR5GSP	CGCAAGCCATGATAAACTCTG
DR5NGSP	AAGTGGGAAGCCATCATCAAG
DR3GSP	CTCAGTCAATGATCTGGAGGG
DR3NGSP	GGAGTGGACATCAGCTTCATGT
DRSQF	GGGGTAGTGGAGTGTCCAG
DRSQR	CTACAGATACTTGTATGCTCTGGTC
DRORFF	CGCCGAATTCGTCACGAATGGATTACCTGCTTC
DRORFR	ATATGGATCCTCAATGGTGTGGTGTGATGAGGCCAGAAAACACAGAGC
FRF	ATGGAATATCTGCTCCAGG
FRR	TCAGCCCAGAAACGACAG
FR5GSP	AGCCATGATAAACTCTGGAAAG
FR5NGSP	GACGCCCTCCAGCGAAACAGC
FR3GSP	ATGGAATATCTGCTCCAGG
FR3NGSP	CAGCTGTTTCGCTGGAGGC
UAP1	TGATCTAGAATTCGCGAAAGC
UAP2	AAGCAGTGGTATCAACGCAGAGT
ADP1	CTGATCTAGAATTCGCGAAGC(T) <sub>17</sub> V
DRRTF	GTCACGATGGATTACCTGCTTC
DRRTR	GTTCCAGCCAGAAAACACAGAG

The SLC39A family contains four separate subfamilies of metal transporter proteins [23]. The subfamily I is mainly composed of fungal and plant sequences, whereas the subfamily II consists of mammalian, nematode and insect genes [23]. The subfamily III (gufA subfamily) is a group of prokaryote and eukaryote proteins related to the gufA gene of *Myxococcus xanthus*, whose function is unknown. The fourth subfamily, LZT (LIV-1 subfamily of ZIP zinc transporters), consists of proteins related to the oestrogen-regulated gene, *LIV-1*, that facilitates cellular zinc uptake in glandular tissues [24]. *LIV-1* also exists in the zebrafish along with another LZT member *KE4* [23], suggesting that just like their mammalian counterparts, they may be involved in zinc homeostasis in zebrafish as well. Interestingly, the zebrafish *LIV-1* orthologue, LZT-Dr3 (SLC39A6), was recently shown to control the epithelia-mesenchymal transition in the gastrula organizer [25], underscoring the importance of characterizing the function of SLC39A proteins in zebrafish. Although members in the other ZIP subfamilies have not been identified from fish so far, it is probable that these ZIP homologues also exist in teleosts and participate in cellular zinc uptake. In the present study, to investigate the molecular mechanisms of zinc uptake in fish, we molecularly cloned and functionally characterized a ZIP homologue (*DrZIP1*) from zebrafish gill and this ZIP protein functioned as a high-affinity zinc uptake transporter when expressed in the heterologous expression systems, *X. laevis* oocytes and CHSE-214 cells [chinook salmon (*Oncorhynchus tshawytscha*) embryonic 214 cells].

## EXPERIMENTAL

### Cloning of the ZIP homologues from zebrafish (*DrZIP1*) and pufferfish (*FrZIP1*)

Total RNA was isolated from zebrafish gill with TRI Reagent (Sigma-Aldrich, Poole, Dorset, U.K.) and total RNA of pufferfish gill was obtained from HGMP-RC (Human Genome Mapping Project Resource Centre) of the MRC (Medical Research Council, Cambridge, U.K.). Reverse transcription was performed with powerscript reverse transcriptase (ClonTech Laboratories, Basingstoke, U.K.) using the primer ADP1 (Table 1).

Partial cDNA of *DrZIP1* was PCR-amplified from zebrafish gill with primers DRF and DRR (Table 1), and the unknown 5'- and 3'-ends of *DrZIP1* cDNA were subsequently amplified by RACE-PCR (where RACE stands for rapid amplification of cDNA ends). For 5'-RACE-PCR, the SMART oligonucleotide II (5'-AA-GCAGTGGTATCAAC GCAGAGTACGCGGG-3'; ClonTech Laboratories) was used in reverse transcription to produce the first-strand cDNA for 5'-RACE-PCR, and semi-nested 5'-RACE-PCR reactions were performed with gene-specific primers (DR5GSP and DR5NGSP; Table 1) and universal primer 2 (UAP2; Table 1). Semi-nested 3'-RACE-PCR was performed with gene-specific primers (DR3GSP and DR3NGSP; Table 1) and universal primer 1 (UAP1; Table 1). The full *DrZIP1* ORF (open reading frame) was then amplified by PCR with primers, DRSQF and DRSQR (Table 1) and a Platinum™ *Pfx Taq* polymerase (Invitrogen), and its sequence was verified through DNA sequencing on a capillary DNA sequencer (CEQ2000; Beckman, Coulter, High Wycombe, Bucks., U.K.).

MRC HGMP *Fugu* genomic database [26] was mined with the predicted *DrZIP1* protein sequence. Two genomic scaffolds, S000956 and M000624, were identified as they contain the same segments with high homology to *DrZIP1* protein sequence. Partial *FrZIP1* cDNA containing the predicted ORF was then amplified from pufferfish gill cDNA with primers, FRF and FRR (Table 1), and the 3'- and 5'-ends of *FrZIP1* cDNA were amplified by RACE-PCR as described by Dolphin et al. [27]. The 5'-end of *FrZIP1* cDNA was amplified by polyadenylic acid tailing and subsequent 5'-RACE-PCR with gene-specific primers (FR5GSP and FR5NGSP; Table 1) and universal primers (ADP1 and UAP2; Table 1). 3'-RACE-PCR was performed to amplify the 3'-end of *FrZIP1* cDNA with gene-specific primers (FR3GSP and FR3NGSP; Table 1) and UAP1 (Table 1). The full-length *FrZIP1* ORF was amplified with Platinum™ *Pfx Taq* polymerase (Invitrogen) and both of its strands were thoroughly sequenced on a capillary DNA sequencer (CEQ2000; Beckman) to verify its sequence information.

The nucleotide sequences of *DrZIP1* and *FrZIP1* cDNA have been submitted to GenBank® database (NCBI) with accession numbers AY314992 and AY529485 respectively.

### Computational analysis

The alignment of the ZIP proteins and the resulting prediction of phylogenetic relationship were generated with the CLUSTAL W method [28]. Hydrophobicity of *DrZIP1* and *FrZIP1* was analysed as described by Moller et al. [29]. Potential phosphorylation sites in *DrZIP1* and *FrZIP1* proteins were predicted with *PhosphoBase* v2.0 [30].

### Expression constructs

For functional expression of *DrZIP1*, the full-length *DrZIP1* ORF was subcloned into the EcoRI and SmaI sites of mammalian expression plasmid vectors, pIRES2EGFP (ClonTech) and pCI-neo (Promega, Chilworth, Southampton, U.K.), to generate pIRES2EGFP-*DrZIP1* and pCI-neo-*DrZIP1* respectively. The full ORF of *DrZIP1* was epitope-tagged by PCR at its C-terminus with a His<sub>6</sub> epitope and primers DRORFF and DRORFR (Table 1). The tagged *DrZIP1* ORF was then cloned into the EcoRI and BamHI sites of pIRES2EGFP to generate pIRES2EGFP-*DrZIP1*(H)<sub>6</sub>. Overlapping PCR was performed to tag the *DrZIP1* ORF at its C-terminus with the EGFP (enhanced green fluorescent protein) gene. The produced hybrid gene, *DrZIP1*EGFP, was then inserted into the EcoRI and NotI sites of the mammalian expression plasmid vector, pCI-neo (Promega), to generate

pCI-neo-*DrZIP1EGFP*. All the inserted genes in the plasmid constructs above were thoroughly sequenced to verify the reading directions and sequence information.

### Cell culture

CHSE-214 cells (obtained from A.T.C.C., Manassas, VA, U.S.A.), MDCK and Caco-2 cells (human Caucasian colon adenocarcinoma cells; a gift from Dr A. T. Mckie, King's College, London), were grown in minimum essential medium supplemented with 10% (v/v) fetal bovine serum (Sigma–Aldrich), 1% non-essential amino acids and 2% (v/v) penicillin/streptomycin (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.). MDCK and Caco-2 cells were cultured in six-well plates (Corning, Corning, NY, U.S.A.) at 37°C and 5% CO<sub>2</sub>, whereas CHSE-214 cells were grown at 20°C and 5% CO<sub>2</sub>.

### SDS-urea/PAGE and Western blotting

pIRES2EGFP-*DrZIP1(H)<sub>6</sub>* and pIRES2EGFP were transiently transfected into CHSE-214, MDCK and Caco-2 cells with Lipofectamine™ reagent (Invitrogen) respectively. After 48 h, these transfected cells were lysed in lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2% (w/v) CHAPS, 20 µg · ml<sup>-1</sup> leupeptin, 10 µg · ml<sup>-1</sup> pepstatin, 10 µg · ml<sup>-1</sup> aprotinin, 2 mM PMSF and 0.5 mM 4-[2-aminoethyl]-[1-benzenesulphonyl]-fluoride) and subsequently sonicated. Urea was then added to the cell lysate to a final concentration of 8 M.

Protein samples (20 µg total proteins/sample) were resolved by SDS-Urea/PAGE (8 M urea added to SDS/PAGE gel). The proteins in the gel were then blotted on to nitrocellulose membrane (Schleicher and Schuell, Elquevilly, France) and His<sub>6</sub>-tagged *DrZIP1* proteins were immunodetected by mouse anti-His antibody solution (1/2000 dilution) and secondary rabbit-anti-mouse IgG/horseradish peroxidase-conjugate (1/8000 dilution). ECL® Western blotting system (Amersham Biosciences, Little Chalfont, Bucks., U.K.) was used for the luminol-based detection of horseradish peroxidase-conjugated anti-mouse antibodies on the membrane.

### Subcellular localization of *DrZIP1* proteins

Recombinant expression plasmid, pCI-neo-*DrZIP1EGFP*, was transfected into CHSE-214 cells with Lipofectamine™ reagent (Invitrogen). Sub-localization of the expressed *DrZIP1EGFP* fusion proteins in transfected CHSE-214 cells was examined with an inverted phase-contrast fluorescence microscope (Nikon Eclipse 200, Nikon, Tokyo, Japan) 72 h after the start of transfection.

### Monitoring of intracellular weakly bound Zn<sup>2+</sup> level and luciferase assay

The zinc reporter plasmid pMT-793 [Rainbow trout MT-A promoter (793 bp) with six metal-responsive elements driving luciferase gene; kindly provided by Dr P. E. Olsson] was co-transfected together with expression plasmid constructs with Lipofectamine™ reagent (Invitrogen). After 72 h, these transfected cells were treated with 30 µM ZnSO<sub>4</sub> in minimum essential medium (Sigma–Aldrich) for 24 h. Then, the cells were lysed with report lysis buffer (Promega) and the supernatant of the cell lysate was collected for luciferase assay. Luciferase activities were assayed with luciferase assay reagent (Promega) in an LKB 1251 Lumino-meter. Protein concentration was quantified with Bradford reagent (Sigma–Aldrich) to normalize the luciferase activities.

### Stable transfection and <sup>65</sup>Zn<sup>2+</sup> accumulation assay in CHSE-214 cells

CHSE-214 cells were transfected with expression plasmids, pIRES2EGFP-*DrZIP1* and pIRES2EGFP with Lipofectamine™ reagent (Invitrogen) and clonally derived stable transfectant CHSE-214 cell lines were generated by the combined selection of G418 and green fluorescence.

The cells with 80% confluence in six-well plates were washed with prewarmed uptake buffer (150 mM KCl, 100 mM glucose and 10 mM Hepes, pH 7.0) [31] three times and then preincubated in prewarmed uptake buffer for 10 min at 20°C before assays. For time-dependent <sup>65</sup>Zn<sup>2+</sup> accumulation, CHSE-214 cells were incubated in 1 ml of prewarmed uptake buffer (150 mM KCl, 100 mM glucose and 10 mM Hepes, pH 7.0), containing 0.5 µM ZnCl<sub>2</sub> and 0.033 µCi of <sup>65</sup>Zn<sup>2+</sup> at 20°C for 1, 5, 10, 20, 40, 60 and 90 min respectively. Concentration-dependent <sup>65</sup>Zn<sup>2+</sup> accumulation was measured by incubating CHSE-214 cells in 1 ml of prewarmed uptake buffer containing ZnCl<sub>2</sub> at concentrations of 0.033, 3, 10 or 20 µM plus <sup>65</sup>Zn<sup>2+</sup> tracer at 20°C for 40 min. Assays were stopped by removing the uptake buffer and washing the cells three times in ice-cold washing buffer (150 mM KCl, 100 mM glucose, 10 mM Hepes and 5 mM EDTA, pH 7.0). The cells in each well were then lysed in 1 ml of 1 M NaOH. After a thorough mixing, 50 µl of cell lysate from each well was diluted 50 times for protein quantification with Bradford reagents and 0.9 ml was taken for radioactivity counting in an LKB1282 CompuGamma counter.

### *DrZIP1* expression and <sup>65</sup>Zn<sup>2+</sup> flux in *X. laevis* oocytes

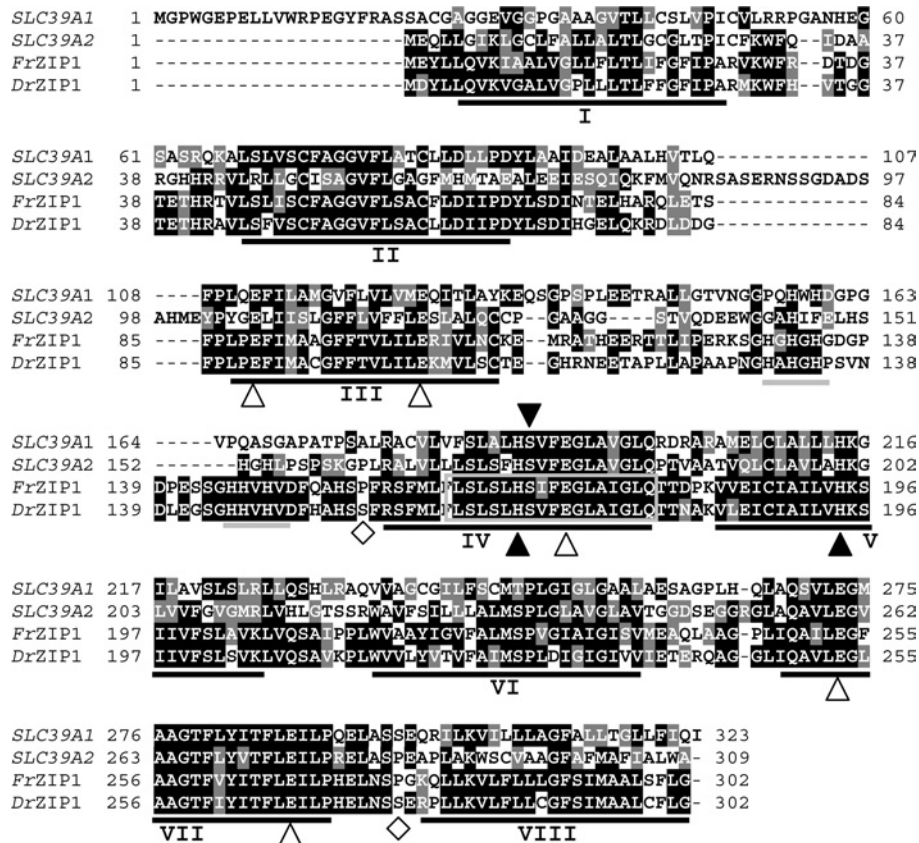
The full-length *DrZIP1* ORF was excised from pIRES2EGFP-*DrZIP1* and inserted into pSPT-18 plasmid vector (Roche Biochemicals, Welwyn Garden City, U.K.) to generate pSPT-18-*DrZIP1*, in which transcription of *DrZIP1* is under the control of the T7 promoter. The capped *DrZIP1* mRNA was synthesized by *in vitro* transcription using mMACHINE™ T7 Ultra kit® (Ambion, Austin, TX, U.S.A.).

*X. laevis* oocytes at developmental stage IV were isolated and the capped *DrZIP1* mRNA (~30 ng/oocyte) were injected into them. Some oocytes were injected with nuclease-free water as control. After 72 h, <sup>65</sup>Zn<sup>2+</sup> influx was assayed by incubating 12 injected oocytes of each group in 500 µl of influx buffer (73 mM NaCl, 1 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub> and 15 mM Hepes, pH 7.0) containing 1.26 µM <sup>65</sup>Zn<sup>2+</sup> (6 µCi · ml<sup>-1</sup>) at 18°C for 2 h. The flux of oocytes were washed thoroughly six times in ice-cold stop buffer (influx buffer supplemented with 100 µM ZnCl<sub>2</sub>) and dispersed individually into each 0.5 ml tube. The radioactivity of each oocyte was counted in an LKB1282 CompuGamma counter.

The statistical significance of differences between groups was determined by Student's *t* test (SPSS software, SPSS, San Rafael, CA, U.S.A.) and groups were considered significantly different at *P* < 0.05.

### RT-PCR (reverse transcriptase-PCR)

Total RNA was isolated from gill, brain, intestine, kidney, eye, ovary, skin, fin, heart and muscle of ten zebrafish individuals. RT-PCR reactions were performed to amplify *DrZIP1* fragments by using *DrZIP1*-specific primers, DRF and DRR (Table 1). The cycle conditions were: an initial cycle of 2 min at 94°C, 30 cycles of 0.5 min at 94°C, 0.5 min at 60°C and 1 min at 72°C, followed by a final extension cycle of 2 min at 72°C. House-keeping gene, zebrafish β-actin (GenBank® accession no. AF057040), was analysed by RT-PCR (25 cycles) as a control.



**Figure 1** Structural relationship of *DrZIP1* and *FrZIP1* with human *SLC39A1* and *SLC39A2*

The predicted *DrZIP1* and *FrZIP1* proteins were aligned with human *SLC39A1* and *SLC39A2*. The eight predicted TM domains are underlined and numbered I–VIII. Shaded residues represent positions of identity or similarity among the sequences as compared with *DrZIP1* and *FrZIP1*. Two potential histidine-rich metal binding motifs between TM III and TM IV are underlined in grey. The signature sequences are highlighted in grey. ▲, conserved histidine and serine residues; △, glutamate residues conserved between *DrZIP1*, *FrZIP1*, *SLC39A1* and *SLC39A2*; ◇, potential phosphorylation sites of PKC.

Total RNA of pufferfish gill, gut, kidney and ovary was obtained from HGMP-RC of the MRC. The level of *FrZIP1* mRNA was analysed by RT–PCR using *FrZIP1*-specific primers, FRF and FRR (Table 1). Cycle parameters: an initial cycle of 2 min at 94 °C, 30 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 2 min, followed by a single cycle of 72 °C for 2 min. As a control, a 0.5 kb fragment of  $\beta$ -actin (Ensembl ID: SINFRUG00000081338) was amplified by RT–PCR (25 cycles) using primers which are specific to sequences in exons three and five respectively of pufferfish  $\beta$ -actin.

The expression levels of *DrZIP1* and *FrZIP1* mRNA were normalized to  $\beta$ -actin mRNA by using SigmaGel software (Jandel Scientific, Madera, CA, U.S.A.).

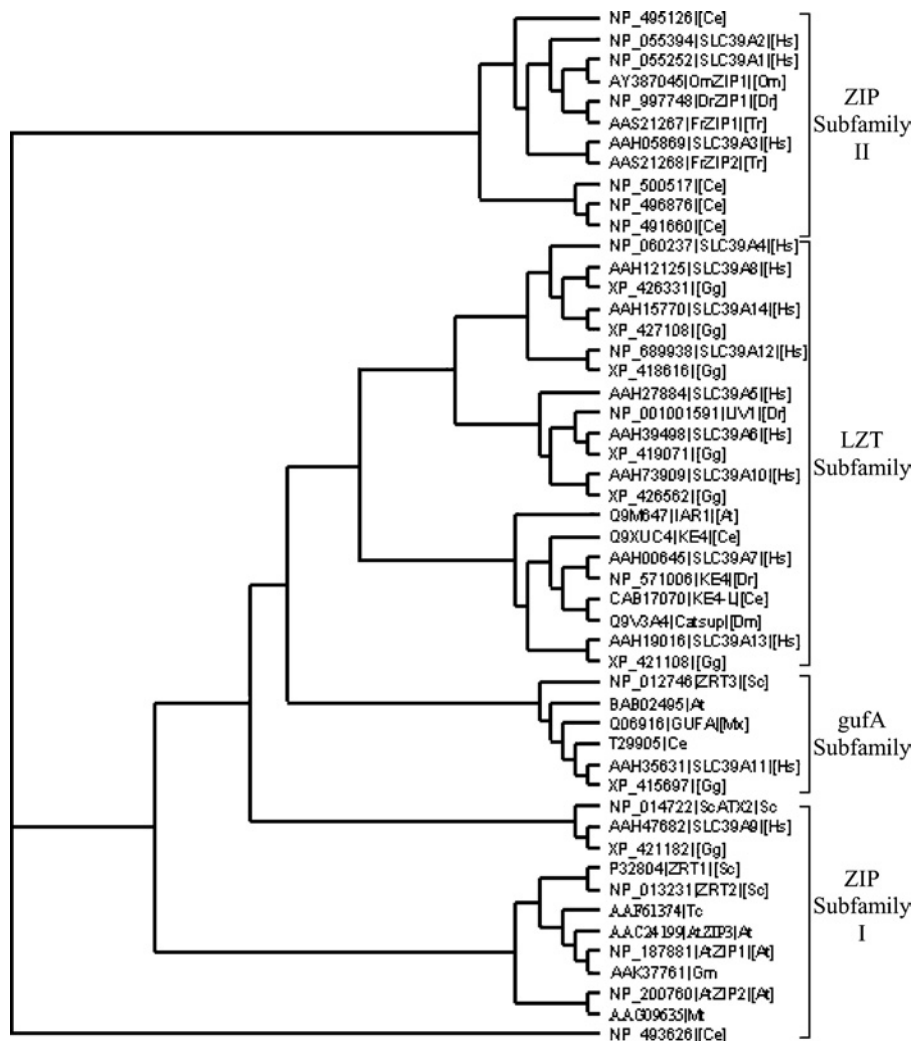
## RESULTS

### Structural characterization of *DrZIP1*

A zebrafish cDNA, which is predicted to encode a 302 amino acid protein of 33 kDa with obvious homology to mammalian ZIP transporters (Figure 1), was molecularly cloned by the combination of database mining and degenerate PCR, and we termed it *DrZIP1*. The predicted *DrZIP1* protein shows significant identity with members of the ZIP family, for example, approx. 38% with the human ZIP1 and ZIP2 transporters (*SLC39A1* and *SLC39A2*) (Figure 1). Furthermore, when plotted in a phylogenetic tree of the *SLC39A* family, *DrZIP1* is found to be more similar to human

*SLC39A1*, *SLC39A2* and *SLC39A3* proteins compared with members of other ZIP clades, including zebrafish LIV-1 and KE4 in the LZT subfamily and fungal and plant ZIPs in the ZIP subfamily I. *DrZIP1* forms a cluster together with human *SLC39A1*–3, rainbow trout ZIP1 (*OmZIP1*) and pufferfish ZIP1 (*FrZIP1*) and ZIP2 (*FrZIP2*) proteins in ZIP subfamily II (Figure 2).

Like the functionally characterized ZIP proteins (*Zrt1*, *Zrt2*, human *SLC39A1* and *SLC39A2*, etc.), *DrZIP1* protein is predicted to have eight TM (transmembrane) domains and both the N- and C-terminus located on the extracellular surface of the cell (Figure 1). A number of other structural features in *DrZIP1* are conserved in other functional ZIPs. Two potential metal binding motifs containing three histidine residues (... HA/GHGH... and ... HHVHV...) are found in the histidine-rich variable region between TM III and IV (Figure 1). These motifs are conserved in many of the characterized ZIP members including human *SLC39A1* and *SLC39A2* (Figure 1) and are proposed to play important roles in metal binding. Just like the functionally characterized ZIP proteins, TM IV and V of *DrZIP1* protein are amphipathic and each possesses a central, fully conserved histidine residue on its hydrophilic side (Figure 1). These histidine residues are considered to be providing an intramembranous zinc-binding site and to be of functional importance [32]. TM IV of *DrZIP1* proteins contain a serine residue that is conserved in most of the characterized ZIP family members (Figure 1). The conserved histidine and serine residues are predicted to lie on the polar face of amphipathic  $\alpha$ -helices with a possible role in metal ion



**Figure 2** Phylogenetic relationship of *DrZIP1* and *FrZIP1* with some representative members of the SLC39 family

Four subfamilies of the SLC39 family are named by Taylor and Nicholson [23]. *DrZIP1* and *FrZIP1* are members of the ZIP subfamily II. *At*, *Arabidopsis thaliana* (thale cress); *Ce*, the nematode worm *Caenorhabditis elegans*; *Dm*, *Drosophila melanogaster* (fruitfly); *Dr*, *Danio rerio* (zebrafish); *Gg*, *Gallus gallus* (chicken); *Gm*, *Glycine max* (soybean); *Hs*, *Homo sapiens* (human); *Mt*, *Medicago truncatula* (barrel medic); *Mx*, the Gram-negative bacterium *M. xanthus*; *Om*, *Oncorhynchus mykiss* (rainbow trout); *Sc*, *S. cerevisiae* (baker's yeast); *Tc*, *Thlaspi caerulescens* (stone cress); and *Tr*, *Ta. rubripes* (seawater pufferfish).

transport. Additionally, in the *DrZIP1* protein, there are correspondingly two aspartate residues in TM II, two glutamate residues in TM III and VII, and one aspartate residue in TM IV and V respectively (Figure 1). The aspartate residues in TM III, IV and VII are also conserved in human SLC39A1 and SLC39A2 proteins (Figure 1). Aspartate and glutamate residues are moderately strong biological ligands for zinc ions [33]. The *DrZIP1* protein has very short, cytoplasmic N- and C-termini, which also feature the functionally characterized ZIP proteins (Figure 1). Importantly, a typical signature sequence of the SLC39A family is identified in the TM IV of *DrZIP1* (LSLSLHVSFVFEGLAIG) protein (Figure 1). All these structural key features together with the overall amino acid sequence suggest that *DrZIP1* belongs to the SLC39 family and may function as a zinc uptake transporter just like the functionally characterized ZIP proteins in yeast, plant and mammals.

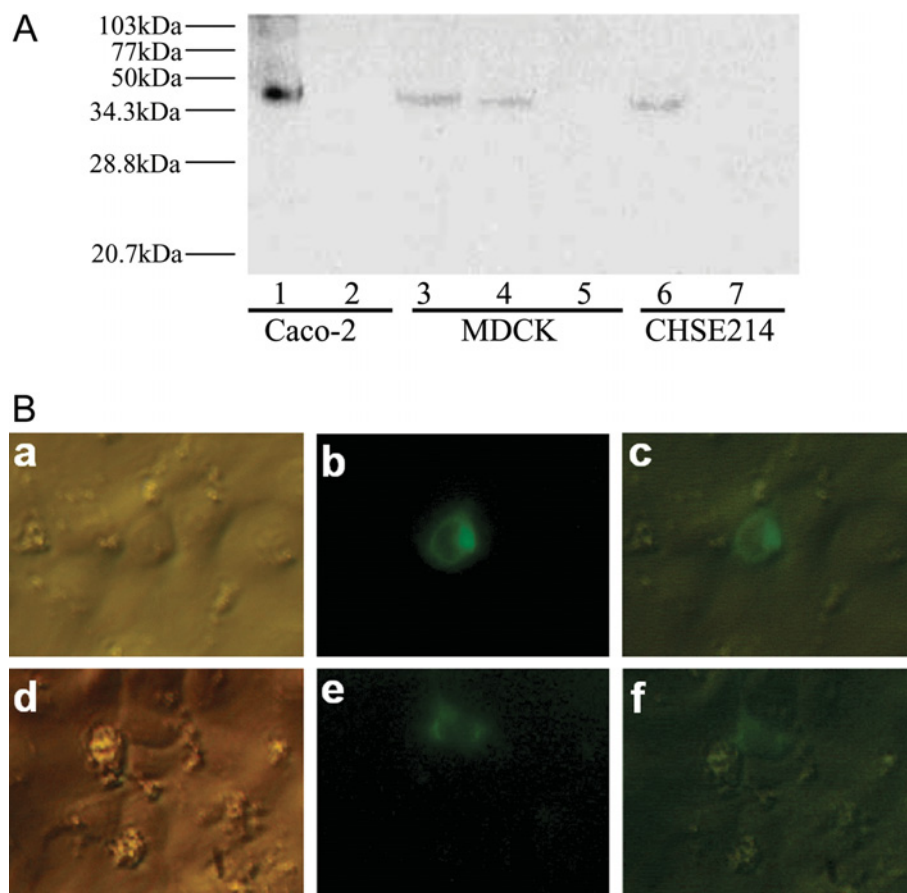
As shown in Figure 1, *DrZIP1* protein has two potential PKC (protein kinase C) phosphorylation sites (residues 153–159 between TM III and IV and 275–281 between TM II and VIII), suggesting the possible existence of post-translational regulation

on *DrZIP1* functions. However, there is no predictable N-glycosylation site in *DrZIP1* protein (Figure 1).

On the basis of mining of pufferfish genomic database (<http://fugu.hgmp.mrc.ac.uk>), genes orthologous to *DrZIP1* can also be identified in seawater and freshwater pufferfish, *Takifugu rubripes* (Contig FS: S000956) and *Tetraodon nigroviridis* (FS\_CONTIG\_7691\_2). Thus *ZIP1* seems to be present in both freshwater and seawater fish. The molecularly cloned full-length *FrZIP1* cDNA contains an ORF of 909 bp encoding a protein of 302 amino acids with a predicted mass of 33.4 kDa (Figure 1). It is therefore not surprising that the predicted *FrZIP1* protein shares approx. 73% identity with *DrZIP1* (Figure 1).

#### Protein expression of *DrZIP1* and its subcellular localization

To examine if *DrZIP1* protein can be expressed in cultured cells, we tagged the *DrZIP1* ORF with a His<sub>6</sub> epitope at its C-terminus to generate *DrZIP1*(H)<sub>6</sub>, which was subsequently inserted into pIRES2EGFP to create pIRES2EGFP-*DrZIP1*(H)<sub>6</sub> for expression from the cytomegalovirus promoter. CHSE-214, MDCK and



**Figure 3** Expression of *DrZIP1* protein in cultured cells and its subcellular localization in CHSE-214 cells

(A) Detection of *DrZIP1*(H)<sub>6</sub> protein expressed in Caco-2, MDCK and CHSE-214 cells by SDS-Urea/PAGE and Western blotting. Lanes 1, 3 and 6: *DrZIP1*(H)<sub>6</sub>-expressed cells; lane 4: stable MDCK-*DrZIP1*(H)<sub>6</sub> cells; and lanes 2, 5 and 7: negative control cells transfected with pIRES2EGFP. (B) Subcellular localization of *DrZIP1* proteins expressed in CHSE-214 cells. The plasmid construct, pCI-neo-*DrZIP1*EGFP, was transfected into CHSE-214 cells, and the subcellular localization of the expressed *DrZIP1*EGFP protein in CHSE-214 cells was examined with an inverted fluorescent microscope. a and d, bright light; b and e, blue light; c and f, overlapping of pictures from both bright and blue light.

Caco-2 cells were transfected with pIRES2EGFP-*DrZIP1*(H)<sub>6</sub>, and *DrZIP1*(H)<sub>6</sub> protein was subsequently examined by SDS-Urea/PAGE and Western blotting. As shown in Figure 3(A), a single protein band was detected in lanes containing proteins from pIRES2EGFP-*DrZIP1*(H)<sub>6</sub>-transfected Caco-2, MDCK and CHSE-214 cells, but there is no such band in other lanes containing proteins from vector-only transfected Caco-2, MDCK and CHSE-214 cells. When compared with protein markers, the molecular mass of the detected *DrZIP1*(H)<sub>6</sub> protein is approx. 40 kDa, which is a little bigger than the 33.6 kDa of the predicted *DrZIP1* protein (Figure 3A).

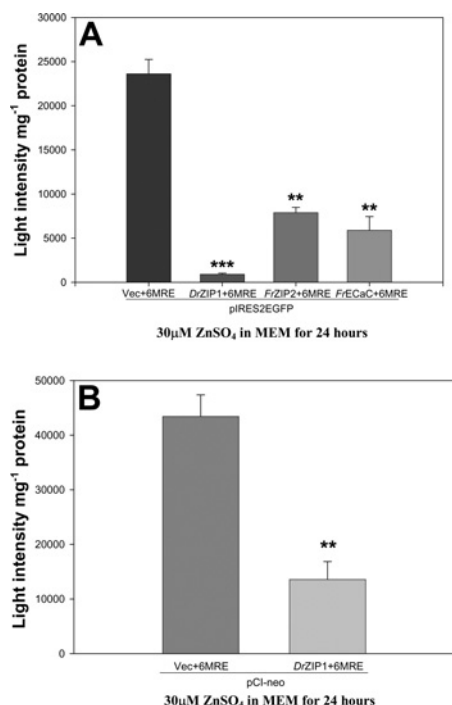
The immunodetection of *DrZIP1*(H)<sub>6</sub> protein suggests that *DrZIP1*(H)<sub>6</sub> can be successfully expressed as a full-size protein in Caco-2, MDCK and CHSE-214 cells. As the only difference between expression constructs pIRES2EGFP-*DrZIP1*(H)<sub>6</sub> and pIRES2EGFP-*DrZIP1* is the His<sub>6</sub> epitope, which was tagged at the C-terminus of *DrZIP1* ORF, it can be supposed that a full-length *DrZIP1* protein can also be produced in these cells.

As a hypothesized zinc uptake transporter, *DrZIP1* proteins were predicted to function mainly at the plasma membrane. To test this hypothesis, we tagged the *DrZIP1* ORF at the C-terminus with an EGFP gene to produce hybrid gene, *DrZIP1*EGFP, which was subsequently cloned into pCI-neo to generate recombinant expression plasmid pCI-neo-*DrZIP1*EGFP. After 3 days from the transfection with pCI-neo-*DrZIP1*EGFP, a bright rim of green

fluorescence was observed at the periphery of CHSE-214 cells that were expressing *DrZIP1*EGFP, whereas the other cells without *DrZIP1*EGFP expression showed no fluorescence (Figure 3B). This result suggested plasma membrane localization of the EGFP-tagged *DrZIP1* proteins in CHSE-214 cells. As EGFP itself is a soluble protein [34] and *DrZIP1* is predicted to be a membrane protein on the basis of computational analysis, the sorting of *DrZIP1*EGFP proteins in cultured cells may mainly depend on the structural properties of *DrZIP1*. Thus the subcellular localization of *DrZIP1*EGFP at plasma membrane of CHSE-214 cells probably represents the real location of *DrZIP1* proteins expressed in CHSE-214 cells. In the cell shown in Figure 3(B), panels b and c, there appears to be a concentrated spot of potentially intracellular fluorescence in addition to the pericellular rim. It is not known whether or not this perhaps represents a pool of intracellular *DrZIP1*.

#### ***DrZIP1* as a zinc-binding protein of high affinity**

The hypothesized role of *DrZIP1* as a zinc uptake transporter suggests that *DrZIP1* protein can bind to Zn<sup>2+</sup> and enable it to cross the plasma membrane into cells, and therefore increase the cellular zinc accumulation. To examine the assumed zinc-binding and transporting properties of *DrZIP1*, we chose a zinc reporter system to detect the effects of *DrZIP1* expression on intracellular



**Figure 4** Effects of *DrZIP1* expression on luciferase activities in CHSE-214 cells

(A) Luciferase activities in CHSE-214 cells that were co-transfected with zinc-reporter plasmid, pMT793, and one among pIRES2EGFP-*DrZIP1*, pIRES2EGFP-*FrZIP2*, pIRES2EGFP-*FrECaC* and pIRES2EGFP, and subsequently treated with 30  $\mu$ M ZnSO<sub>4</sub> for 24 h. (B) Luciferase activities in CHSE-214 cells, that were co-transfected with pMT793 and either pCI-neo-*DrZIP1* or pCI-neo, and subsequently treated with 30  $\mu$ M ZnSO<sub>4</sub> for 24 h. Values are means  $\pm$  S.E.M. ( $n = 3$ ). Significant differences between experimental groups ( $P < 0.05$ ) were determined using Mann–Whitney  $U$  test.

weakly bound Zn<sup>2+</sup> concentration, and this system includes a zinc-sensitive reporter plasmid, pMT793, and CHSE-214 cells. pMT793 contains a luciferase reporter gene driven by a rainbow trout MT (metallothionein)-A promoter (793 bp) [35]. The MT-A promoter contains six metal-responsive elements as transcription enhancers that are very sensitive to intracellular weakly bound Zn<sup>2+</sup> and can be greatly activated by elevated intracellular weakly bound Zn<sup>2+</sup> through MTF-1 (metal-responsive transcription factor-1) [35,36]. Thus the fluctuation of intracellular weakly bound Zn<sup>2+</sup> can be represented as the change of luciferase activities in the pMT793-transfected cells. CHSE-214 cells are chosen due to their deficiency of functional expression of zinc-binding proteins and MT, and consequently high sensitivity to zinc [37,38].

To express *DrZIP1* in CHSE-214 cells, the full-length ORF of *DrZIP1* was inserted into the mammalian expression vector, pIRES2EGFP, to generate pIRES2EGFP-*DrZIP1*. As a control, pufferfish ECaC (*FrECaC*) and ZIP2 (*FrZIP2*) were also cloned into pIRES2EGFP to generate pIRES2EGFP-*FrZIP2* and pIRES2EGFP-*FrECaC* respectively. Both *FrECaC* and *FrZIP2* have been shown to stimulate zinc uptake when individually expressed in MDCK cells.

pIRES2EGFP-*DrZIP1*, pIRES2EGFP-*FrZIP2*, pIRES2EGFP-*FrECaC* or pIRES2EGFP was co-transfected with pMT793 into CHSE-214 cells respectively. After 72 h, the transfected cells were treated with 30  $\mu$ M ZnSO<sub>4</sub> for 24 h and luciferase activity was subsequently assayed to examine the effects of *DrZIP1*, *FrZIP2* and *FrECaC* expression on the intracellular weakly bound Zn<sup>2+</sup> concentration of CHSE-214 cells. As shown in Figure 4(A), luciferase activities were 28 times lower in pIRES2EGFP-*DrZIP1*-

transfected CHSE-214 cells compared with pIRES2EGFP-transfected CHSE-214 cells, suggesting that the expression of *DrZIP1* largely decreased the weakly bound Zn<sup>2+</sup> concentration in CHSE-214 cells. Just like *DrZIP1*, expression of *FrECaC* and *FrZIP2* also exhibited similar effects on the weakly bound Zn<sup>2+</sup> level when expressed in CHSE-214 cells by lowering luciferase activities approx. four and three times in comparison with pIRES2EGFP-transfected CHSE-214 cells (Figure 4A). Comparatively, *DrZIP1* showed approx. eight and six times higher ability to decrease the luciferase activity compared with *FrZIP2* and *FrECaC* in CHSE-214 cells respectively (Figure 4A). To exclude the possibility of the pIRES2EGFP effects on the luciferase activity in CHSE-214 cells, we used another mammalian expression plasmid vector, pCI-neo, to replace pIRES2EGFP for the overexpression of *DrZIP1* from cytomegalovirus promoter. Concordantly, expression of *DrZIP1* in pCI-neo still decreased luciferase activities by approx. three times in CHSE-214 cells when compared with control cells transfected with pCI-neo (Figure 4B). These results suggest that *DrZIP1*, *FrZIP2* and *FrECaC* proteins have significant zinc-binding abilities that can decrease intracellular weakly bound Zn<sup>2+</sup> level when CHSE-214 cells are exposed to excessive zinc, and that *DrZIP1* protein displays much higher affinity to bind zinc compared with *FrZIP2* and *FrECaC*.

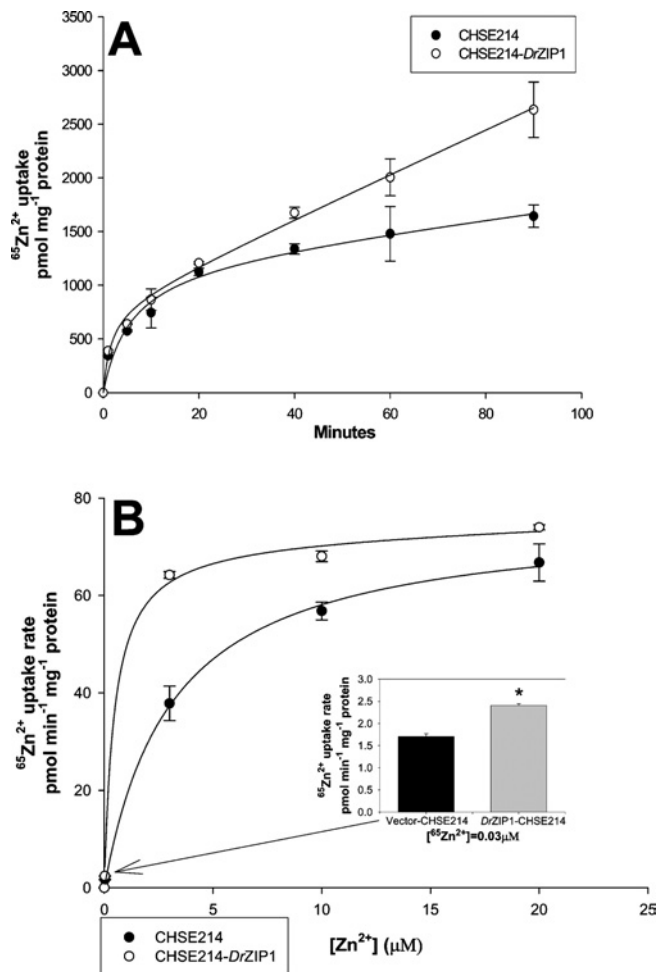
#### Functional characterization of *DrZIP1* expressed in CHSE-214 cells

To examine whether the expression of *DrZIP1* can directly stimulate zinc uptake, we generated stable transfectant CHSE-214 cells overexpressing *DrZIP1*, hereafter referred to as CHSE-214-*DrZIP1*. The stable overexpression of *DrZIP1* in CHSE-214 cells was demonstrated on the basis of the examination of EGFP proteins using an inverted fluorescence microscope. As we failed to generate the vector-only stable transfectant CHSE-214 cell line, the untransfected CHSE-214 cells, referred to as CHSE-214 cells, were thus used as the control in the assays of <sup>65</sup>Zn<sup>2+</sup> accumulation. CHSE-214 cells may share the same properties of zinc uptake as vector-only stable transfectant CHSE-214 cells as it has been shown that transfection with GFP alone has no effects on zinc uptake activity in human K562 erythroleukaemia cells [39].

Examination of <sup>65</sup>Zn<sup>2+</sup> accumulation in CHSE-214 cells indicated the presence of endogenous zinc uptake systems. The endogenous zinc uptake is a saturable process in a time- and concentration-dependent fashion (Figures 5A and 5B). When assayed over a range of zinc concentrations (0.03–20  $\mu$ M), this endogenous system showed Michaelis–Menten kinetics with an apparent Michaelis constant  $K_m$  of 3  $\mu$ M zinc and a  $J_{max}$  (the maximum flux of substrate in an enzymatic assay) of 76 pmol Zn<sup>2+</sup> · min<sup>-1</sup> · (mg of protein)<sup>-1</sup> (Figure 5B).

Similarly, <sup>65</sup>Zn<sup>2+</sup> accumulation in CHSE-214-*DrZIP1* cells is also a saturable process in a time- and concentration-dependent way. <sup>65</sup>Zn<sup>2+</sup> accumulation was significantly increased in CHSE-214-*DrZIP1* cells that accumulated approx. 1.6-fold higher zinc compared with CHSE-214 cells over 90 min (Figure 5A). Zinc accumulation in CHSE-214-*DrZIP1* cells was also a concentration-dependent and saturable process with an apparent  $K_m$  of 0.5  $\mu$ M zinc and a  $J_{max}$  of 75 pmol Zn<sup>2+</sup> · min<sup>-1</sup> · (mg of protein)<sup>-1</sup> (Figure 5B). In comparison with wild-type CHSE-214 cells, expression of *DrZIP1* markedly stimulated zinc uptake (1.5-fold) in CHSE-214-*DrZIP1* cells at low zinc concentration in the uptake buffer (< 2.5  $\mu$ M), whereas this stimulation decreased at higher zinc concentration (> 2.5  $\mu$ M). Furthermore, the  $K_m$  of zinc accumulation in CHSE-214-*DrZIP1* cells is approx. six times lower than the endogenous level in CHSE-214 cells, whereas the  $J_{max}$  of zinc accumulation in both cells is similar, suggesting that expression of *DrZIP1* increased the affinity of CHSE-214 cells for





**Figure 5** Functional expression of *DrZIP1* in CHSE-214 cells

(A) Zinc accumulation was assayed by incubating CHSE-214-*DrZIP1* and CHSE-214 cells in uptake buffer containing  $0.5 \mu\text{M}$   $\text{ZnCl}_2$  plus  $0.033 \mu\text{Ci}$   $^{65}\text{Zn}^{2+}$  at  $20^\circ\text{C}$  for either 1, 5, 10, 20, 40, 60 or 90 min. (B) Zinc accumulation rate was determined by incubating cells in the uptake buffer containing a range of substrate concentrations at  $20^\circ\text{C}$  for 40 min. Values are means  $\pm$  S.E.M. ( $n = 3$ ). Significant differences between experimental groups ( $*P < 0.05$ ) were determined using Mann-Whitney *U* test.

zinc 6-fold. Thus when expressed in CHSE-214 cells, *DrZIP1* can function as a zinc uptake transporter possibly with high affinity for zinc.

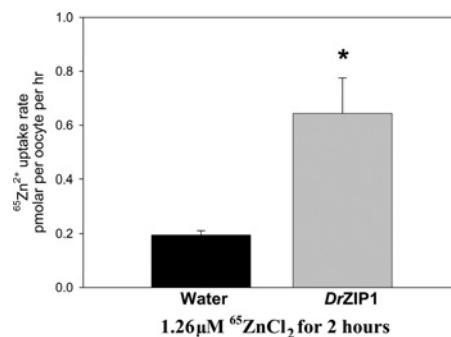
#### Functions of *DrZIP1* expressed in *X. laevis* oocytes

For cross-examination of the zinc-transporting activity of *DrZIP1*, a classical robust expression system, *X. laevis* oocytes, was employed to express *DrZIP1* followed by  $^{65}\text{Zn}^{2+}$  influx assays.

As shown in Figure 6, *DrZIP1*-injected oocytes accumulated approx. three times higher  $^{65}\text{Zn}^{2+}$  compared with water-injected ones after being incubated in a flux buffer containing  $1.26 \mu\text{M}$   $^{65}\text{Zn}^{2+}$  at  $18^\circ\text{C}$  for 2 h. Thus *DrZIP1* functioned as a zinc uptake transporter when expressed in *X. laevis* oocytes.

#### Expression distribution of *DrZIP1* and *FrZIP1* mRNA in various tissues

*DrZIP1* mRNA is ubiquitously expressed in all tissues of ten individual zebrafish herein examined. The highest level of *DrZIP1* mRNA was detected in the ovary (Figure 7A), whereas substantial *DrZIP1* mRNA levels were also detected in the heart, eye, kidney



**Figure 6** Functional characterization of *DrZIP1* in *X. laevis* oocytes

A total of 12 oocytes injected with either water or *DrZIP1* mRNA were incubated in influx buffer containing  $1.26 \mu\text{M}$   $^{65}\text{ZnCl}_2$  ( $6 \mu\text{Ci} \cdot \text{ml}^{-1}$ ) at  $18^\circ\text{C}$  for 2 h. Values are expressed as means  $\pm$  S.E.M. ( $n = 12$ ). Significant differences between experimental groups ( $*P < 0.05$ ) were determined with Student's *t* test.

and brain (Figure 7A). There were relatively modest levels of *DrZIP1* mRNA in the intestine and the lowest levels of *DrZIP1* mRNA were found in gill and skin (Figure 7A).

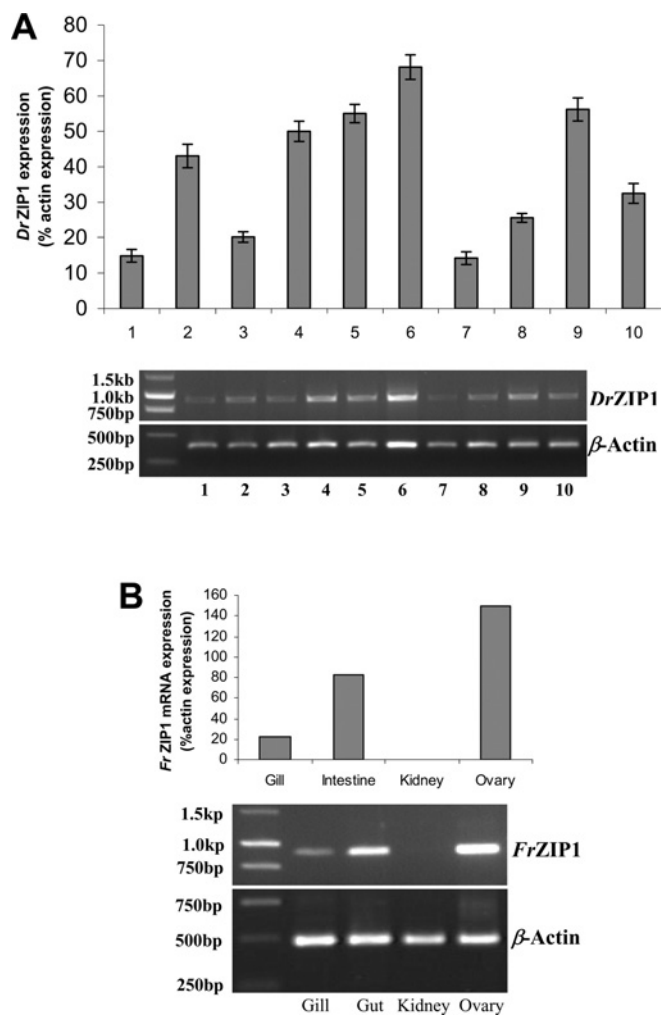
Similarly, in pufferfish, the highest level of *FrZIP1* mRNA was detected in the ovary and the lowest in the gill (Figure 7B). As in the zebrafish, the *FrZIP1* mRNA level was higher in the intestine compared with the gill (Figure 7B). However, extremely low levels of *FrZIP1* mRNA seem to be present in the kidney (Figure 7B).

## DISCUSSION

Zinc is both a vital metal nutrient and a problematic toxicant to aquatic biota. To understand the interplay between nutrition and toxicity, it will be important to determine the mechanisms that regulate zinc uptake into the body and organs. In yeast, plants and mammals, ZIP members (SLC39A) have been identified to play prominent roles in cellular zinc uptake [20]. However, it remains to be studied which proteins are functionally responsible for cellular zinc uptake in fish, although fish ZIP homologues have been predicted to exist in fish to perform similar roles [19]. In the present study, to understand the molecular mechanism of zinc uptake in fish cells, we cloned a novel ZIP homologue from zebrafish gill, *DrZIP1*. Orthologues of *DrZIP1* were also identified in both seawater and freshwater pufferfish (*Ta. rubripes* and *Te. nigroviridis*) genomes, as *Takifugu ZIP1* (*FrZIP1*) shares a high amino acid identity (73%) with *DrZIP1* throughout the protein, especially in the predicted TM domains. Thus fish ZIP1 may be well conserved and functionally important among fish species.

*DrZIP1* belongs to the ZIP family and is a zinc uptake transporter in fish based on its structural and functional properties. Similar to the ZIP members in yeast, plants and mammals, *DrZIP1* contains all the typical conserved structural features of the ZIP family, including eight TM domains, a potential cytoplasmic metal binding domain between TM III and IV and functionally important histidine residues in TM IV and TM V. Moreover, *DrZIP1* protein also shares a significant amino acid similarity with the known members of the ZIP family, especially mammalian ZIP proteins such as human SLC39A1 and SLC39A2. As both human SLC39A1 and SLC39A2 have been functionally characterized as zinc uptake transporters when expressed in human erythroleukaemia K562 cells [31,39], *DrZIP1* may play a similar role in cellular zinc transport in fish. Furthermore, functional expression analysis suggested that *DrZIP1* protein can be expressed as a membrane protein in cultured cells and identified to





**Figure 7** Tissue distribution of *DrZIP1* and *FrZIP1* mRNA in zebrafish and pufferfish

(A) *DrZIP1* mRNA levels, zebrafish gill (1), brain (2), intestine (3), kidney (4), eye (5), ovary (6), skin (7), fin (8), heart (9), and muscle (10) were determined by RT-PCR for 30 cycles. Upper panel: relative amount of *DrZIP1* mRNA to  $\beta$ -actin mRNA in various tissues; and lower panel: expression pattern of *DrZIP1* and  $\beta$ -actin mRNA in agarose gels. Values are means  $\pm$  S.E.M. ( $n = 10$ ), significant difference ( $P < 0.05$ ) between groups was tested with ANOVA followed by post-hoc test. (B) Levels of *FrZIP1* mRNA in pufferfish gill, intestine, kidney and ovary were determined by RT-PCR for 30 cycles. Upper panel: relative amount of *FrZIP1* mRNA to  $\beta$ -actin mRNA in various tissues; and lower panel: expression pattern of *FrZIP1* and  $\beta$ -actin mRNA in agarose gels.

be located at the plasma membrane in CHSE-214 cells, a fish embryonic cell line. The plasma membrane localization of *DrZIP1* protein provided the functional basis at cellular level for its role in cellular zinc uptake. The role of *DrZIP1* as a zinc uptake transporter is subsequently confirmed by the experimental facts that expression of *DrZIP1* stimulated zinc uptake in both CHSE-214 cells and *X. laevis* oocytes.

Binding zinc, at least temporarily, should be an essential structural property of zinc transporters to facilitate the transport of zinc across cell membrane and this property may therefore influence the affinity of zinc transporters for zinc. Like the known zinc uptake transporters, *FrZIP2* and *FrECaC* [16,17], *DrZIP1* behaved like a zinc-binding protein by decreasing the intracellular weakly bound zinc level when being overexpressed in zinc-treated CHSE-214 cells. However, in comparison with *FrZIP2* and *FrECaC* proteins, *DrZIP1* exhibited much higher binding affinity

for zinc (decreasing luciferase activities eight and six times when compared with *FrZIP2* and *FrECaC* respectively), suggesting that it may represent a zinc uptake transporter with much higher affinity for zinc in fish. Consistently, the function of *DrZIP1* as a high-affinity zinc importer is also supported by the observation that stable expression of *DrZIP1* increased the endogenous affinity of CHSE-214 cells for zinc approx. six times by decreasing the  $K_m$  from 3 to 0.5  $\mu$ M.

*DrZIP1* markedly stimulated zinc uptake of CHSE-214 cells at lower zinc levels but no difference in zinc influx between *DrZIP1* and mock transfected cells was observed at higher extracellular zinc concentrations. Thus *DrZIP1* enhanced zinc affinity of CHSE-214 cells but had no effect on the endogenous maximal capacity of zinc uptake. A possible reason for this feature of *DrZIP1* is the post-translational modification of its zinc uptake activity in response to increased external zinc levels, just like the yeast high-affinity zinc uptake transporter, Zrt1. In yeast *Saccharomyces cerevisiae*, after zinc treatment, Zrt1 protein can be quickly ubiquitinated, recruited into the endocytic pathway and removed from the cell surface [40,41]. Without this inactivation, total cellular zinc levels could increase from less than 100  $\mu$ M to nearly 100 mM within 1 h of treating a zinc-limiting yeast cell with zinc [42]. Similarly, mouse ZIP4 protein expressed in human embryonic kidney 293 cells is post-translationally inactivated in response to prompt zinc treatment [43]. The proposed post-translational inactivation of *DrZIP1* protein after zinc treatment is supported by the existence of two predicted PKC phosphorylation sites in *DrZIP1* protein, including a serine residue between TM III and IV. In yeast, the phosphorylation of a serine residue between TM III and IV of Zrt1 protein in response to zinc is proposed to be the pathway of the ubiquitinated inactivation of Zrt1 protein [42]. However, it remains to be demonstrated how the functions of *DrZIP1* are post-translationally regulated.

The ubiquitous expression of *DrZIP1* mRNA, coupled with its function as a zinc importer, suggests that *DrZIP1* may be a major constitutive zinc uptake transporter in many cells of the zebrafish. The highest level of ZIP1 mRNA exists in the ovary of both zebrafish and pufferfish, which is consistent with the observation that high levels of zinc are accumulated in fish ovary during oocyte development [44,45]. Moreover, the levels of the main zinc-binding proteins, MT, began to increase at the onset of vitellogenesis, and levels peaked (approx. 7-fold of basic levels) when spawning occurred [46,47]. Substantial levels of *DrZIP1* mRNA also exist in heart, eye, kidney and brain of zebrafish. Eye and brain are known to have a high-concentration of zinc [6,48] and kidney is an important organ to reabsorb zinc from the urine in freshwater fish since there is an extremely low urinary excretion rate for zinc [49]. In contrast, we found no *FrZIP1* mRNA in pufferfish kidney. To counteract the passive diffusion of salts from seawater, marine teleosts such as the pufferfish kidney excrete divalent ions [50]. There appears to be no evidence for renal zinc reabsorption in marine fish. In both fish species, ZIP1 mRNA is expressed at higher levels in the intestine when compared with the levels in gill. During standard conditions of divalent ions, the intestine is thought to act as the major pathway for zinc uptake and the gill to supplement absorption when dietary zinc levels become limited [19]. With decreasing dietary zinc levels, the gill becomes increasingly important, especially when waterborne zinc levels are increased [51]. Thus it can be speculated that ZIP1 mRNA in fish gill could be up-regulated when dietary zinc becomes limited and availability of waterborne zinc is increased.

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