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Zinc transporter ZIP10 forms a heteromer with ZIP6 which regulates embryonic development and cell migration

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Short title: ZIP6:ZIP10 heteromer induces EMT, cell migration and proliferation

Keywords: SLC39A6, SLC39A10, cancer, zebrafish, mouse, human

Abbreviation list: PrP, prion protein; Snail1, snail family zinc finger 1; EMT, epithelial–mesenchymal transition; STAT, signal transducer and activator of transcription; ZIP, Zrt- and Irt-like proteins; GSK, glycogen synthase kinase; CDH1/E-cadherin, Epithelial cadherin; IRT1, iron(ii) transport protein 1; β-TRCP, beta-transducin repeat containing E3 ubiquitin protein ligase; HNK-1, human natural killer-1; Cx43, Connexin-43; SMA, survival of motor neuron 1, telomeric GATA, GATA binding protein; PLα, proximity ligation assay; EDTA, Ethylenediaminetetraacetic acid; BSA, bovine serum albumen; PFA, paraformaldehyde; DAPI, 4',6-diamidino-2-phenylindole; Morpholino, morpholino-modified antisense oligonucleotide; mRNA, messenger ribonucleic acid; TMT, tandem mass tag; ESI, electrospray ionisation; qPCR, quantitative real-time polymerase chain reaction; ISH, in situ hybridisation; PBS, Phosphate buffered saline; PMSF, phenylmethane sulfonyl fluoride; SDS, Sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FACS, Fluorescence-activated cell sorting; SEM, standard error of the mean; ER, endoplasmic reticulum; PM, plasma membrane; CHO, Chinese-hamster ovary

SUMMARY STATEMENT

Zinc is involved in cell migration during embryo development and in cancer. We show that a zinc transporter consisting of two proteins, ZIP6 and ZIP10, stimulates both cell migration and division in mammalian cells and in the zebrafish embryo.

ABSTRACT

There is growing evidence that zinc and its transporters are involved in cell migration during development and in cancer. In the present study, we show that zinc transporter ZIP10 (SLC39A10) stimulates cell motility and proliferation, both in mammalian cells and in the zebrafish embryo. This is associated with inactivation of GSK-3α and -3β and downregulation of E-cadherin (CDH1). Morpholino-mediated knock-down of zip10 causes delayed epiboly and deformities of the head, eye, heart and tail. Furthermore, zip10 deficiency results in overexpression of cdh1, zip6 and stat3, the
latter gene product driving transcription of both zip6 and zip10. The non-redundant requirement of Zip6 and Zip10 for epithelial to mesenchymal transition (EMT) is consistent with our finding that they exist as a heteromer. We postulate that a subset of ZIPs carrying PrP-like ectodomains, including ZIP6 and ZIP10, are integral to cellular pathways and plasticity programs, such as EMT.
INTRODUCTION
As early as 1923 it was reported that tumour tissues of different origin contained aberrant levels of zinc [1]. Thirty-two years later, Tupper et al. published that the uptake of $^{65}$Zn(II) was higher in mouse mammary tumour tissue compared to non-tumour tissue [2]. The biological explanation for these observations has only been revealed recently as zinc(II) is now known to be an intracellular signalling ion regulating major signalling pathways [3-6]. Several research articles have demonstrated a role of zinc(II) signals in the increased phosphorylation of kinases involved in pathways stimulating cell proliferation and migration [5, 7-9]. However, rather than activating kinases directly, it appears that zinc(II) acts primarily through the inhibition of protein phosphatases that dephosphorylate kinases [10-14].

Zinc signals can be initiated by release of zinc from proteins, notably metallothioneins, or by influx into the cytosol through zinc transporters [3, 5, 15, 16]. There is a growing body of literature linking dysregulation of zinc transporters of the SLC39A (ZIP) family to various forms of cancer. Elevated expression of ZIP4 is associated with cancers of the liver, pancreas and brain [17-19]. Expression of ZIP10 mRNA is associated with aggressiveness of renal cell carcinoma [20] while a common polymorphic variant of ZIP6 is associated with survival-time in patients with oesophageal squamous-cell carcinoma [21]. Furthermore, expression of the closely related ZIP6 and ZIP10 is high in some breast cancers and contributes to their aggressive behaviour [22-27]. ZIP10 has also been found to be over-expressed breast cancer cells that metastasise to the lymph nodes [28]. Knockdown of ZIP10 in invasive and metastatic breast cancer cell lines (MDA-MB-231 and MDA-MB-435S) or treatment of the cells with a cell-permeable zinc chelator suppressed cell migration suggesting that ZIP10 stimulated migratory behaviour through its zinc transporting activity [28]. More recently it was shown that ZIP10 is transcriptionally regulated by STAT3 and STAT5, and suppresses apoptosis in human B-cell lymphoma [6]. Thus, both ZIP6 and ZIP10 are associated with aggressive behaviour in cancerous cells and are regulated by STAT3/5 acting on cognate cis-elements in their respective promoters.

Epithelial-mesenchymal transition (EMT) is a central event during cancer metastasis a process that has been shown to be regulated, in human breast cancer MCF-7 cells, by ZIP6 down-regulation of E-cadherin (CDH1) [23]. EMT is also a normal biological processes being involved in cellular events such as gastrulation and tissue regeneration [29]. During EMT programs, typical cell-cell adhesive interactions are altered such that connections between cells and with their local environment become weaker; the cells also acquire a more mesenchymal, spindle-shaped morphology as a result of cytoskeletal rearrangements. EMT is initiated by a number of factors, one of which is Signal Transducer and Activator of Transcription 3 (STAT3) [30]. Stat3 promotes expression of zip6 in the zebrafish gastrula organiser, which in turn is essential for the cell autonomous role of Stat3 in EMT of these cells. Zip6 was shown to cause nuclear localisation of Snail1, which is a master regulator of EMT [30], leading to repression of cdh1 expression [4].

In the present study, we show that ZIP10 stimulates EMT and cell migration in human MCF-7 breast cancer cells as well as in the zebrafish embryo in a comparable manner to that previously shown for ZIP6 [4, 23, 31]. During gastrulation of zebrafish it appears that both Zip6 [4] and Zip10 are necessary for cells to undergo EMT, suggesting that they operate as a unit. In support of this hypothesis we demonstrate that ZIP10 forms a heteromer with ZIP6 explaining their non-redundant requirement for these processes.

MATERIALS AND METHODS
Sequence Analysis
A multiple sequence alignment was generated with ClustalW incorporating phylogenetically related amino acid sequences of metal transporters, including the 14 human ZIPS, zebrafish Zip10, and iron(II) transport protein 1 (IRT1) from Arabidopsis thaliana. Their evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model [32]. Initial tree(s)
for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 16 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 198 positions in the final dataset. Alignment and evolutionary analyses were conducted in MEGA6 employing 500 bootstrap replicates with the final tree displayed rooted on AtlIRT1 [33]. Protein sequences were obtained from NCBI and are notated as ZIP1 (CAG33498.1), ZIP2 (NP_055394.2), ZIP3 (NP_653165.2), ZIP4 (NP_060237.2), ZIP5 (NP_001128667.1), ZIP6 (NP_036451.3), ZIP7 (NP_001070984.1), ZIP8 (NP_071437.3), ZIP9 (NP_060845.2), ZIP10 (NP_001120729.1), ZIP11(NP_001153242.1), ZIP12 (NP_001138667.1), ZIP13 (NP_001121697.1), ZIP14(NP_001121903.1), DrZip10 (NP_956965.1), and AtIRT1 (AEE84216.1). Potential PEST proteolytic cleavage sites [34] were identified using the epestfind tool within the European Molecular Biology Open Software Suite (EMBOSS) [35].

Antibodies
ZIP6 and ZIP10 antibodies were generated in-house; anti-rabbit phospho-GSK-3α/β (Ser21/9) Antibody #9331 was from Cell Signalling Technology; anti-rabbit E-cadherin (H-108) was from Santa Cruz Biotechnology; mouse anti-V5 antibody was from Invitrogen; rabbit anti-V5 antibody was from Bethyl Laboratories; rabbit anti-paxillin monoclonal antibody Y113 and Cdh1 rabbit polyclonal antibodies to Cdh1 in zebrafish were sourced from Abcam® (ab32084; ab53033) and β-actin in zebrafish was probed with a rabbit polyclonal antibody (Sigma®, A2066). The secondary antibodies used in zebrafish experiments were goat anti-rabbit IgG conjugated to either Alexa Fluor 488 (Abcam®, ab15007) or horseradish peroxidase (GE Healthcare Life Sciences, RPN2108). Animals and husbandry
The animal experiments were carried out under a project licence (PPL70/6379) in accordance with the UK Home Office Animals (Scientific Procedures) Act, 1986. Zebrafish embryos were obtained from breeding strains of King’s Wild-Type (KWT2) adult fish. Brood stock fish were maintained in a stand-alone tank system containing system water, which was made from reverse osmosis deionized water supplemented with 60 mg/L of sea salt (Tropical Marine®) and 200 µM CaCl₂ (VWR), giving a conductivity of 250-350 µS. The fish were reared at a temperature of 28.5 ± 0.5°C under a photoperiod regime of 14 h of light and 10 h of darkness. The system water was partially renewed every other days to maintain nitrogenous waste products at minimal levels (ammonia; <0.02 mg/L, nitrite; <1 mg/L, nitrate <50 mg/L). Fish were fed twice a day with tropical fish flakes (Aquarian). In preparation for breeding, fish were fed either live nauplii brine shrimp larvae (Artemia salina) or frozen brine shrimp to improve spawning. Fertilised eggs were produced either by pair-wise breeding method using a breeding tank containing a male and a female or by colony breeding method placing a box of marbles inside a tank with up to 25 fish. The tanks with breeding fish were inspected the following morning, 0.5 – 1 hours after light was switched on, for embryos that were collected and used for experimentation.

Generation of Zip10 (Slc39a10) morphants
Anti-sense morpholino modified oligonucleotides (morpholinos) for zebrafish zip10 were designed and procured from GENE TOOLS Inc (Philomath, USA). The nucleotide sequences of the morpholinos used are shown in the Table 1. Because of the potential problem with off-target effects produced by some morpholinos [36], both translational blocking and splice blocking morpholinos were designed for zip10 in addition to a p53 translational blocking morpholino used for co-injection with zip10 morpholinos to suppress potential p53-mediated apoptosis [37, 38]. Zebrafish embryos were micro-injected at the 1-4 cell developmental stage with 1 – 2nl of 2-4 ng of each morpholino as previously described [39] and the development of embryos were monitored by inverted light microscope. The embryo morphants (MO) were collected and incubated in ‘fish water’ (deionized water containing 60 mg/L of sea salt; 200 µM CaCl₂; 0.01% methylene blue) at 28.5°C and the stages of development
compared to those of un-injected wild type controls and mismatch morpholino injected controls (i.e. scrambled sequence) (Table 1). In some experiments, the p53 translational blocking morpholino was co-injected with either of the two types of zip10 morpholinos at a ratio of 1.5 : 1.0 and the effect on embryonic development was compared to that resulting from injection of either of the zip10 morpholinos alone without p53 knockdown.

**Cell line culture**

Wild type MCF-7 breast cancer cells, a gift from AstraZeneca (Macclesfield, UK), were cultured in phenol-red-free RPMI 1640 with 5% (v/v) foetal calf serum plus 200 mM L-glutamine, 10 IU/ml penicillin, 10 μg/ml streptomycin and 2.5 μg/ml fungizone at 37°C in a humidified 5% CO₂ atmosphere. Tissue culture media and constituents were obtained from Life Technologies Europe Ltd (Paisley, UK), plasticware from Nunc (Roskilde, Denmark). CHO cells were maintained in minimal essential medium, α-modification (Sigma) with 10% (v/v) foetal calf serum, 4 mM glutamine, 10 μg/ml penicillin, 10 μg/ml streptomycin and 2.5 μg/ml fungizone under 5% CO₂ at 37°C as previously described [40]. Epithelial mouse NMuMG cells (CRL-1636, American Tissue Culture Collection) cells were maintained as per the ATCC distributor’s recommendations.

**CRISPR/Cas9 mediated ZIP6 knock-out**

The CRISPR/Cas9-based ZIP6 knock-out (ko) clones (ZIP6 0/0) were generated in NMuMG cells by introducing single-strand genomic cuts within opposite strands of the first coding exon of the mouse Zip6 gene using a Cas9 nickase. Cell autonomous non-homologous end-joining then led to frame shifts that generated premature nonsense codons giving rise to non-productive Zip6 mRNAs subjected to nonsense-mediated decay. The NMuMG ZIP6 ko clone was characterized by western blot analysis and genomic sequencing.

**Affinity capture and quantitative mass spectrometry**

Wild-type mouse NMuMG cells or ZIP6 0/0 cells derived from them by CRISPR/Cas9-based technology cells were grown to near-confluency, in vivo crosslinked in the presence of 1% formaldehyde in PBS and lysed in the presence of Lysis buffer, which consisted of 1% NP-40, 1 mM EGTA, 10% glycerol, 20 mM NaF, 100 mM NaCl, 1x protease inhibitor cocktail (Complete; Roche), 1x phosphatase inhibitor cocktail (PhosSTOP; Roche) in 150 mM Hepes, pH 7.3. The capture of the ZIP6 bait and its co-isolating binders was based on affinity matrices generated by the non-convalent saturation of Protein A agarose with in-house generated rabbit polyclonal anti-ZIP6 antibodies. Following extensive washes with Lysis buffer, binders to the affinity matrix were eluted in 0.2% trifluoroacetic acid and 20% acetonitrile. Subsequently, samples were denatured in 9 M urea, reduced in 5 mM tris-(2-carboxyethyl) phosphine (Sigma-Aldrich), alkylated with 4-vinylpyridine (Sigma-Aldrich) and trypsinized with sequence-grade modified porcine trypsin (Promega). Digests were isobarically labelled with six-plex tandem mass tag (TMT) reagents before in-depth nano-ESI analysis on an Orbitrap Fusion Tribrid mass spectrometer.

Raw mass spectrometry data files were analysed as described previously (PMID: 25490046). Briefly, Mascot (Version 2.4; Matrix Science Ltd, London, UK) and Sequest HT search engines within Proteome Discoverer software (Version 1.4; Thermo Fisher Scientific) were employed to match raw spectra to peptides in the mouse international protein index (IPI) (release 3.87). Search parameters were set to tolerate up to two missed tryptic cleavages. With 4-vinylpyridine used as the alkylating agent, searches assumed all cysteine side-chains were pyridylethyl-derivatized. Variable modifications considered were TMT reagent modifications of primary amines, oxidations of methionines, deamidations of asparagine and glutamine, as well as phosphorylations of serines, theonines and tyrosines. For relative quantitation, low mass TMT signature ion distributions were analysed by Proteome Discoverer software using an embedded functionality, which also generated the graphs depicting the enrichment ratios of selected proteins. Note that the full interactome dataset will be made available and presented under separate cover.
Invasion assays
Invasion assays of MCF-7 cells across Matrigel have been described previously [34]. In this case, transiently transfected cells were transferred to invasion chambers 8 hours post transfection and harvested 16 hours later.

The generation of recombinant expression constructs for ZIP6 [41], ZIP7 [40] and ZIP14 [41, 42], ZIP7 [40] and ZIP14 [42] with C-terminal V5 tags using vector pcDNA3.1/V5-His-TOPO (Invitrogen) and the LacZ control has already been described. Cells were transfected with the relevant constructs using Lipofectamine 2000 (Life Technologies), according to the manufacturer’s instructions. Briefly, 9 x 10^5 cells, grown on 60mm dishes for 24 hours, were transfected with 8.8µg of DNA and 27.5 µl of reagent free of serum, antibiotics. Serum was added to the cells during transfection and 3 mM sodium butyrate was added to cells transfected with constructs encoding ZIP6 and ZIP10 for 14 hours prior to harvest.

Quantitative real-time polymerase chain reaction (qPCR)
Injected zebrafish embryos were observed for epiboly and gastrulation cell movements up to 10 hours post fertilisation (hpf), the tail bud stage that signifies the end of gastrulation stage. Total RNA was isolated from 10 hpf embryos using TRIzol® (Invitrogen™) according to the manufacturer’s protocol. Following first strand cDNA synthesis using the High Capacity Reverse Transcription kit (Applied Biosystems™), gene expression levels were analysed by real-time quantitative polymerase chain reaction (qPCR) on ABI Prism 7700HT Sequence Detection System using a hydrolysis probe assay. The probe and primer sets for genes of interest were designed using Roche Universal Probe Library software (www.universalprobelibrary.com) and the sequences are shown Table 2. 18s rRNA was used as housekeeping gene as described before [43]. Cycle thresholds (Ct) were obtained for each of the test genes and for the housekeeping gene and the differences between these (ΔCt) calculated. The ΔCt values were transformed into absolute values according to the 2^(-ΔΔCt) calculation [44-46]. Primer pairs for each gene were amplified with equal efficiency, as verified using serial dilutions of the respective cDNA. Average expression data per gene was calculated and expressed as log2 fold change relative to WT fish.

In-Situ Hybridization (ISH)
Assay of mRNA expression for E-cadherin (cdh1 gene) was performed on whole mount embryos at 10 hpf by in situ hybridisation (ISH) as described before [47].

Immunocytochemistry and Immunohistochemistry
For immunofluorescence, 2.2 x 10^5 cells were seeded on 0.17 mm thick coverslips for 24 hours prior to transfection. Cells were fixed with 4% formaldehyde for 15 mins, permeabilised or not with 0.4% saponin in 1% BSA in 1xPBS for 15 mins, blocked with 10% normal goat serum, incubated with primary antibodies at room temperature, Alexa 488 or 594 anti-mouse or rabbit (1/2000, Molecular Probes) for 30 minutes, and assembled onto slides using Vectorshield with DAPI (Vector Laboratories). Coverslips were viewed on a Leica RPE automatic microscope using a 63x oil immersion lens. The fluorescent superimposed images were acquired using a multiple bandpass filter set appropriate for 4’,6-diamidino-2-phenylindole (DAPI) and fluorescein as well as bright field for Differential Interference Contrast. All images were processed with one level of deconvolution using Openlab software (Improvision) to aid clarity. The brightfield images were obtained using the differential interference contrast capability.

For zebrafish immunofluorescence histochemistry, embryos were collected at 10 hpf (tail-bud stage), dechorionated and fixed overnight in 4% paraformaldehyde (PFA) at 4°C. Fixed embryos were rinsed 3 times at 5 min intervals using ice cold 1xPBS and subsequently permeabilised by emersion in 100% methanol at -20°C for 10 min before rinsing in 1xPBS for a further 5 min. The embryos were then blocked in 1% Bovin Serum Albumin (BSA) diluted in Phosphate Buffered Saline with 0.1% (vol/vol) Tween 20 (PBST) for 30 min and incubated overnight at 4°C in diluted primary antibody
with 1% BSA. The antibody solution was removed and the embryos washed 3 times in 1xPBS at 5 min intervals. Thereafter, the embryos were incubated in the dark for 1 hour at room temperature in diluted secondary antibody conjugated with Alexa Fluor 488 with 1% BSA. The primary antibody to E-cadherin (Anti-Cdh1 rabbit polyclonal; Abcam®, ab53033) was used at 1:100 while the secondary antibody (goat anti-rabbit conjugated to Alexa Fluor 488; Abcam®, ab15007) was used at 1:300. Expression and cellular location of Cdh1 protein in stained embryos was observed under laser scanning confocal microscope (Leica, DMIRE2), 2-photon laser scanning confocal microscope (Nikon; Eclipse Ni-EFN Upright/ A1R Si MP Confocal) and epi-fluorescence microscope (Nikon eclipse 400).

**SDS-PAGE and Western Analysis**

Cells were harvested, washed with PBS, lysed for 1 hour at 4°C with 5.5 mM EDTA/ 0.6% Nonidet P40/ 10% mammalian protease inhibitor cocktail (Sigma Aldrich, UK) in Krebs-Ringer HEPES buffer [40] and centrifuged at 13,000 rpm for 15 min at 4°C. Protein was measured using the DC assay kit (Bio-Rad Laboratoires Ltd., Hemel Hemstead, UK). Transfected CHO cells were harvested between 16-24 hours after transfection as described previously [40]. Primary antibodies used were diluted ZIP6-SC, ZIP6-M and ZIP6-Y 1/1000, V5 1/2000, β-actin 1/10,000 from Sigma-Aldrich Company Ltd., UK. Quantification of Western Blot results was performed by normalization of three separate experiments to ß-actin values [40].

For analysis of zebrafish proteins by western blot, embryos were collected at 10 hpf (tail-bud stage), washed in PBS, dechorionated and deyolked in a solution containing Ringer solution with the addition of 10% EDTA (10mM) and PMSF protease inhibitor at 0.3 mM final concentration. Thereafter de-yolked embryos were collected in Eppendorf tube, rinsed twice in cold Ringer’s solution and homogenized at 4°C in lysis buffer containing 1x Phosphate Buffered Saline and PMSF protease inhibitor cocktail (Sigma) in the ratios of 90:10 µl. Tissue lysates were then centrifuged at 1000 rpm at 4°C for 5 min and the supernatant was transferred to a fresh tube. Total protein was quantified by Pierce assay kit-rapid (51254-1KT, Sigma, Dorset, UK) and subjected to SDS-PAGE Western Blot using standard procedures. Primary anti-Cdh1 antibody (rabbit polyclonal, Abcam®, ab53033) was used at 1:1000 and visualised using Enhanced Chemiluminescence (ECL) with goat anti-rabbit IgG antibody conjugated to horseradish peroxidase used at 1:7500 (GE Healthcare Life Sciences, RPN2108). β-actin (rabbit monoclonal; Sigma®, A2066) was also at 1:1000 with a secondary antibody (goat anti-rabbit horseradish peroxidase, GE Healthcare Life Sciences, RPN2108) at 1:2000. Quantification of immunoblot results was performed by normalization of three separate experiments to ß-actin values.

**FACS analysis and zinc uptake assay**

For cell cycle analysis, adherent cells, harvested carefully by pipette, or non-adherent cells were collected and the analysis was performed using CycleTEST PLUS DNA reagent kit (Becton Dickinson) and following manufacturer’s instructions.

For zinc uptake assay, cells were loaded with 5 µM of the zinc indicator, Newport Green diacetate DCF (Invitrogen), treated with 10 µM, 25 µM or 50 µM zinc plus 10 µM zinc ionophore sodium pyrithione (Sigma-Aldrich) at 37°C for 20 minutes before reading on a Becton-Dickinson FACS III flow cytometer.

**Proximity Ligation Assay**

MCF-7 cells on 8-well chamber slide (Lab-Tek, Fisher) were fixed with 3.7% formaldehyde in PBS for 15 min followed by proximity ligation assay (PLA) using Duolink red kit (Sigma-Aldrich) as described previously [5]. Antibodies against ZIP6 (ZIP6-Y, mouse monoclonal, in-house) and ZIP10 (ZIP10-R, rabbit polyclonal, in-house) were applied at 1:100 and 1:100 dilutions, respectively. Slide was mounted with Vectashield with DAPI, and viewed on a Leica RPE microscope with a 63x oil immersion lens. Images were acquired with a multiple band-pass filter set appropriate for DAPI and Texas Red and presented as maximal projections of 25 stacks taken 0.3 µm apart. Dots per cell were
determined with ImageTool software (Olink) using at least 12 cells from at least three experiments and presented as average values ± standard errors.

**Statistical analysis**

Statistical analysis of cell data was performed using ANOVA with Post-Hoc Dunnett and Tamhane tests. Significance was assumed with P<0.05. Values are expressed as means of at least three experiments (n≥3) with error bars showing the standard error of the mean (SEM). For zebrafish analysis, data were analysed using one way ANOVA or Student’s unpaired t-tests with significance at P<0.05. Values are presented as the mean ± SEM (N=12).

**RESULTS**

ZIP6 and ZIP10 are closely related paralogues

Phylogenetic analysis of the amino acid sequences of proteins within the SLC39A (ZIP) family reveals that ZIP6 and ZIP10 are very closely related members of the LIV-1 sub-family (Fig. 1A). Furthermore, zebrafish Zip10 is more similar to human ZIP10 than to any other human protein and these two proteins are considered orthologous (Fig. 1A)\[48\]. In addition to the motifs in common to the whole mammalian ZIP protein family, ZIP6 and ZIP10 both have long histidine-rich (20 – 28 His) N-terminal domains preceding the first transmembrane domain (Fig. 1B). Whilst ZIP7 also has multiple histidines in its N-terminal domain, this region in ZIP7 is considerably shorter than ZIP6 and ZIP10. Another feature of ZIP6 and ZIP10 is the presence of a potential PEST cleavage site before the CPALLY motif, which is found in all LIV-1 sub-family of ZIP proteins except ZIP7 and ZIP13. The potential PEST motif in ZIP10 is located between amino acid residues 206 and 219 (RGEPSNEPSTETNK). Whilst it is not known if this is an actual site for proteolytic cleavage in ZIP10, we have previously reported experimental evidence that ZIP6 is cleaved at the corresponding predicted PEST site (residues 210 – 223) in the endoplasmic reticulum (ER) before locating to the plasma membrane (PM) and mediating zinc influx \[23\].

ZIP10 mediated zinc influx causes loss of adhesion and stimulates cell proliferation

Exogenous expression of ZIP10 in cells consistently resulted in a low percentage of cells expressing the protein. Using an antibody directed to the N-terminus, immunofluorescence of ZIP10 in MCF-7 breast cancer cells overexpressing the protein demonstrated PM staining (Fig. 2A). Influx of zinc in CHO cells transfected with ZIP6, ZIP10, or ZIP14, was analysed by FACS using the cell-permeant fluorescent zinc probe Newport Green™ (Kₐ = 1 µM). Cells overexpressing either of these transporters showed increased accumulation of chelatable zinc with increasing concentrations of zinc added to the medium, compared to the untransfected CHO cells, over the 20 min transport assay (Fig. 2B). Zebrafish Zip10 has previously been shown to be a zinc importer using the Xenopus oocyte expression system \[43\] and these experiments confirm that human ZIP10 performs the same transporter function.

Exogenous expression of ZIP6 or ZIP10 in MCF-7 cells resulted in a large proportion of the cells rounding up and floating to the surface. This change in morphology was accompanied by loss of paxillin (PXN) focal adhesions, reducing binding to the surface (Fig. 2C; Supplementary Fig. 1). When these floating cells were collected and re-seeded on coverslips they re-attached and continued to grow (Fig. 2D). In contrast, control untransfected or LacZ transfection control cells that detached from the substrate did not easily re-adhere and neither did cells transfected with ZIP7. Intriguingly, in contrast to the ZIP6 / ZIP10 which migrate to the plasma membrane ZIP7 is only observed located in the ER and never observed in the PM \[5\]. These results indicate that the PM located zinc importers, ZIP6 and ZIP10, are stimulating the cells to round-up and detach and that this population of floating cells is still viable. Furthermore, FACS analysis of MCF-7 cell cycle transfected with the same constructs showed dramatic effects of ZIP6 and ZIP10 on cell proliferation, increasing the number of cells in G2/M phase from 14 to 40% (Fig. 2D).
ZIP10 promotes epithelial-mesenchymal transition in MCF-7 cells
To investigate whether the rounding up and detachment of cells overexpressing ZIP10 may be due to initiation of EMT we first quantified the migratory potential of MCF-7 cells transfected with ZIP10 over fibronectin substrate and observed that this tripled over a 16 hours compared to mock-transfected controls (Fig. 3A). Previous studies had shown that during EMT expression of E-cadherin (CDH1) was downregulated [49]. To confirm whether the downregulation of CDH1 was involved in causing the observed increase in cell migration in response to over-expression of ZIP10, we performed immunofluorescence analysis of V5-tagged ZIP10 and native CDH1 in MCF-7 cells transfected with a ZIP10-V5 construct. Cells that stained positively for ZIP10-V5 showed little or no staining for CDH1, in contrast to ZIP10-V5 negative cells, which abundantly expressed CDH1 (Fig. 3B). During EMT GSK-3α and GSK-3β are inactivated through phosphorylation at Ser21 and Ser9, respectively. ZIP10 transfected cells showed increased abundance of serine phosphorylated GSK-3α (Ser21) and of GSK-3β (Ser9, Figure 3C). Interestingly, transfection of cells with the ER located ZIP7 had no effect on GSK-3 phosphorylation.

Zip10 regulates embryonic development of zebrafish
Since all evidence suggested that ZIP10 induced EMT in the human MCF-7 breast cancer cell line, it was of interest to know if this zinc transporter has a similar function in living organisms. Earlier work in zebrafish has demonstrated that Zip6, the closest paralogue of Zip10, regulates EMT in the gastrula organiser [4]. Thus, we investigated the effect of anti-sense morpholino knock-down of zip10 mRNA on zebrafish development. Zip10 morphants (Zip10 MO) showed dose-dependent developmental morbidity and few embryos injected with 10 ng of either zip10 translation-blocking or splice-blocking morpholino developed beyond the early stages of epiboly and, hence, did not gastrulate. At a concentration of 2 ng, embryos were viable but showed distinct phenotype (Fig. 4A; Supplementary Fig. 2). Similar effects were observed for the zip10 translation-blocking morpholino or splice-blocking morpholino, whether injected alone (Fig. 4A, B; Supplementary Fig. 2) or co-injected with p53 morpholino to suppress potential off-target induced apoptosis (Fig. 4C). Typical developmental abnormalities observed in Zip10MO embryos injected with 2 ng of the translation-blocking morpholino included delayed epiboly and EMT movement (Fig. 2B), abnormally small head, small eyes with delayed pigmentation, general under-development with shorter anterior-posterior axis (i.e. runted embryo), coiled or twisted tail, oedema of the pericardial sac with abnormal string-heart, and wide-spread oedema of the embryo post hatching (Fig. 4C; Supplementary Fig. 2). Some of the Zip10 MO were missing the tail altogether. Such effects were uncommon in either un-injected embryos or those injected with a scrambled morpholino sequence (Fig. 4A).

The delayed epiboly movement of cells over the yolk and/or EMT migration could be observed at 6hpf and 10hpf in embryos injected with zip10 morpholino (Fig. 4B). At 10 hpf Zip10MO showed increased expression of cdh1 mRNA as analysed by in situ hybridisation (Fig. 5A) and qPCR (about 2.4-fold increase; Fig. 5B). This translated into increased Cdh1 protein expression as indicated by immunohistochemistry techniques (Fig. 5C) and confirmed by western blot (Fig. 5D, E). Interestingly, morpholino knockdown of zip10 also resulted in increased abundance of stat3 and zip6 mRNA in embryos (Fig. 5F, G).

ZIP10 forms a heteromeric complex with ZIP6
Results from knockdown experiments in zebrafish embryos suggest collectively that Zip10 is required for EMT and cell motility in the early zebrafish embryo. The same function has been attributed to Zip6 [4] and it, thus, appears that both these proteins are required for induction of EMT during epiboly in zebrafish. Furthermore, we have shown before that ZIP6 stimulates migratory behaviour and EMT also in human cells [23, 50]. Therefore, we asked if ZIP6 and ZIP10 might operate as a heteromeric complex and investigated this first through the proximity ligation assay (PLA), which utilises ‘rolling circle amplification’ of complementary DNA oligonucleotides linked to two separate
secondary antibodies raised against primary antibodies from different species [51]. With primary antibodies raised in the corresponding two species against two potentially interacting proteins the two complementary oligonucleotides will hybridise forming a circular DNA if the two proteins reside <40 nm apart. The proximity of the two proteins is visualised by biotin labelling as a dot. Application of either ZIP6-Y or ZIP10-R antibody alone to fixed MCF-7 cells generated less than two dots per cell (Fig. 6). In contrast, when both antibodies were applied together the PLA showed on average six dots per cell, indicative of ZIP6:ZIP10 heteromers being present (Fig. 6).

In search of further evidence for the formation of a ZIP6:ZIP10 complex and in order to establish the presence of this complex in other cell types we generated ZIP6 knockout (ZIP6 0/0) mouse NMuMG mammary gland cells using the CRISPR/Cas9 system (Fig.7A). Proteins in wild type (wt) or ZIP60/0 cells were in vivo cross-linked, lysed, and subjected to affinity capture using a ZIP6 antibody non-covalently linked to Protein A agarose (Fig.7). ZIP6 and its interacting partners were eluted, trypsinised and peptides quantified in three technical replicates by Tandem Mass Tag (TMT) analysis using six-plex isobaric mass tags to separate the six biological samples. Consistent with expectations, the ZIP6 bait protein was reproducibly observed as the most highly enriched protein in the three biological replicates and this observation was selective for co-immunoprecipitations from wild-type NMuMG cells (Fig. 7B, C). The identification of ZIP6 was made with a high degree of confidence being supported by the presence of 16 tandem MS spectra, with 11 high-energy collision-induced dissociation fragment spectra passing stringent filtering criteria for relative quantitation of their TMT signature ion profiles. ZIP10 represented the second most abundant protein in the dataset, exhibiting enrichment ratios that indicated its reliance on ZIP6 for co-purification. In contrast, the protein Titin (aka connectin) was also confidently identified but co-isolated non-specifically with affinity matrices, as evidenced by TMT ratios near 1.0 (log2) in all TMT ratio calculations (Fig. 7B; Fig. 8).

**DISCUSSION**

In the present study we show that ZIP10 can stimulate cells to undergo EMT through inactivation of GSK-3 and downregulation of CDH1. More importantly, we show that this occurs not only in a breast cancer cell line, but also in zebrafish embryos in which Zip10 stimulates Cdh1 downregulation and cell autonomous migration responsible for gastrulation and anterior-posterior axis formation. All these ZIP10-related observations are very similar to data previously reported for ZIP6 [4, 23, 50]. Evidently, both Zip6 and Zip10 need to be present for proper EMT during zebrafish epiboly and the conclusion from this is that Zip6 and Zip10 must operate as a unit in this process. Indeed, we demonstrate that Zip6 and Zip10 exist as a heteromer and can likely function as a native complex. It has been indicated before by immunoblots that endogenous ZIP6, ZIP7, and ZIP10 can form homodimers [5, 23, 52] and recombinant ZIP5 and ZIP13 have been characterized to homodimerise [53, 54], but to our knowledge this is the first study to show that two different protein members of the ZIP family of zinc transporters can operate as a heteromeric complex.

ZIP6 and ZIP10 being able to form a functional zinc-transporting unit explains a number of observations. The perhaps most compelling of these is that the morpholino-based knockdown of either zip6 [4] or zip10 in the zebrafish embryo inhibits EMT during the gastrulation process and results in very similar phenotypes, suggesting that they are not functionally redundant. To operate as a complex their expression is coordinated through Stat3 transactivation [4, 6, 23]. Knockdown of zip10 in zebrafish embryos resulted in upregulation of mRNA for both stat3 and zip6. It may be speculated that this is a compensatory response to restore Zip6:Zip10 mediated EMT during zebrafish gastrulation. Recently published results give evidence for an even more diverse role for ZIP6 and ZIP10 as a functional moiety. The mouse oocyte rapidly accumulates zinc during the final phases of maturation [55]. Intriguingly, this zinc influx is mediated by maternally derived ZIP6 and ZIP10, and is required for progress through telophase I and subsequent arrest in metaphase II [55, 56]. Thus, in a variety of cell types and physiological states ZIP6 and ZIP10 are co-expressed and participate in the same molecular and cellular processes.
ZIP6 and ZIP10 stimulate motility in cells is at least in part by down-regulation of CHD, through a signalling pathway which may involve the inactivation of GSK-3 through serine phosphorylation and transcriptional repression by SNAIL [4, 23]. In the present study, forced expression of ZIP10 in MCF-7 cells resulted in increased abundance of the inactive serine phosphorylated GSK-3α and GSK-3β. According to the canonical pathway, active GSK-3β is phosphorylating SNAIL resulting in export from the nucleus, ubiquitination by β-TRCP and degradation through the proteasome pathway [57]. However, GSK-3 also inhibits SNAIL transcription in human cells and it has been reported that inhibition of both GSK-3α and GSK-3β isoforms are required for increased SNAIL expression [58]. Interestingly, we have recently discovered that both GSK-3α and GSK-3β co-immunoprecipitate with the ZIP6:ZIP10 complex (PMD: 12083774), consistent with the interpretation that their biology might be integrated and might involve the zinc-dependent regulation of these kinases.

Zip10 is required for zebrafish development because morpholino-based knockdown of this gene produced a severe developmental morphant phenotype typified by shorter anterio-posterior axis at 24hpf. The embryo at 24hpf was runted, generally under-developed, and was very similar to the zip6 morphant zebrafish described before [4]. This observation is consistent with the reported expression of the zip10 gene very early in life in the zebrafish embryo [59]. Central nervous system abnormalities, including a severely dysmorphic head and missing or small eyes, were observed in most affected embryos with shorter and thickened anterio-posterior hypoblast. These deformities were similar to the ones obtained by zip6 or stat3 knockdowns [4, 60] and, clearly, Zip6 and Zip10 are both required for cell migration responsible for anterior-posterior axis formation. Some of the defects observed in the Zip10KO zebrafish were also found following morpholino knockdown in zebrafish of Zip7 [61], which mediates gated zinc(II) flux from the endoplasmic reticulum [5] and the Golgi apparatus [62]. Specifically, knockdown of Zip7 also resulted in embryos with decreased size of the head and eye, and shorter and curved spinal chord [61], showing that several zinc transporters contribute to formation of these structures.

Although loss of Cdh1 is associated with EMT during zebrafish gastrulation, it has also been shown that Cdh1 is required for epiboly, as well as convergence and extension cell movements [63, 64], where it connects deep cell and enveloping cell layers. During epiboly, a program executed during zebrafish development between 4-10 hpf, a multi-layered sheet of ectodermal cells spreads over and encloses deeper cell layers, becoming thinner as a result [65]. It is interesting to note that the zip10 morphants in the present study looked similar to Cdh1 mutants described previously (i.e. delayed epiboly, runted body and sometimes no tail) [65]. Considering the numerous signalling pathways involved in gastrulation, disrupting any of these may converge to a similar shortened anterior-posterior and broadened mediolateral body axes. Thus, these similar phenotypes could be the result of distinct mechanisms [66]. Our findings indicate a fundamental role of Zip10 in zebrafish embryogenesis, particularly in anterior-posterior body axis formation and in development of the heart and central nervous system. Because of the non-redundant nature of cellular functions carried out by Zip6 and Zip10 and their formation of a heteromer, it is possible that the observed upregulation of zip6 and stat3 expression in Zip10MO embryos was a compensatory response to counteract the loss of Zip10.

In addition to the phenotypic effects in common with those published for the zip6 morphant [4], knockdown of zip10 caused pericardial oedema and deformed string-shaped heart. Similar deformities in zebrafish were observed through manipulations of the zinc finger transcription factor, Tbx5 [67, 68]. It was also shown in rats that zinc deficiency in the dam results in foetuses with heart anomalies because of the alterations in the expression and distribution of several proteins involved in its development, including HNK-1, Cx43, SMA, GATA-4 and FOG-2 [69].

The evolutionary and general relevance of the findings in the present study are underlined when considering that, based on sequence homology, Drosophila has only a single orthologue representing the vertebrate zip6 and zip10 genes, known as fear-of-intimacy (FOI). Like its vertebrate orthologues, FOI is essential for proper cell migration during embryogenesis [70]. More specifically, FOI has been shown to be required for morphogenesis of gonads and trachea, and
controls glial cell migration [71-73]. Thus, the role of ZIP6/ZIP10 in executing EMT is evolutionary conserved from fly to man. The effects of a particular zinc flux on cellular signalling can be the result of its movement of zinc(II) across cellular membranes or may reflect its ability to interact with other proteins. For instance, members of the ZIP6/10-containing sub-branch of ZIP zinc transporters have been shown to interact with the prion protein (PrP), and were subsequently shown to have a common ancestry with the prion gene family [74, 75]. Consistent with their evolutionary relationship, members of this ZIP branch comprise at their N-terminus a PrP-like ectodomain that appears to contain elements required for dimerisation, a feature that might also explain the ability of these proteins to interact with PrP [54]. It has been proposed that PrP plays a role in cell adhesion by regulating CDH1 delivery to the cell membrane, which in turn controls the formation of adherens junctions. Moreover, the knockdown of PrP in a zebrafish gastrulation model or its knockout in a mammalian model of epithelial-to-mesenchymal transition interfere with autonomous cell movements and the execution of EMT [76-78]. This observation is consistent with the independent findings that ZIP6 and ZIP10 are important for cell autonomous anterior mesendodermal cell movement. Thus, this study adds to an emerging body of data, which increasingly supports the conclusion that ZIPs carrying PrP-like ectodomains and their PrP molecular cousin [78] are integral to and essential for cellular plasticity programs, which are activated when cells are mobilised.

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The authors have no interests to declare

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AUTHOR CONTRIBUTION
CH, KMT, GSU and PK conceived and coordinated the study and CH drafted the paper. GSU designed and coordinated the work on NMuMG cells. IM carried out all zebrafish experimentation. The cell culture work was done by TN and DB. All authors contributed to the manuscript and read the final version.
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### Tables

#### Table 1. Sequences of morpholinos used for gene knockdown experiment.

| MO type       | Sequences (5’-3’) |
|---------------|-------------------|
| zip10 TB      | TGGTATGTGTGTAACCTCTCAT |
| zip10 SB      | ATCACAGCAGTGAACCTACCTTT |
| p53 TB        | GGGCCATTCGGTTTGAAGAATTG |
| Random-control-MASO scrambled |                     |

TB = translation blocker, SB = Splice junction blocker.

#### Table 2. Zinc transporter genes and genes of other molecules associated with EMT with their qPCR primer sequences, UPL probe number, product lengths and accession number.

| Gene   | qPCR primer/probe sequences (5’-3’) | Tm | Probe# | Amplicon size (bp) | Accession No |
|--------|-------------------------------------|----|--------|--------------------|--------------|
| cdh1   | F: tgtcagagttgagctgtcc              | 60 | 6      | 93                 | NM_131820.1  |
|        | R: ggaataatccaaacctttttactttt      | 59 |        |                    |              |
|        | P: ttctctcg                         |    |        |                    |              |
| stat3  | F: gtgtgtattgtcaaggtcagtt          | 59 | 46     | 64                 | NP_571554    |
|        | R: ggaattgttaagggacctgtaa          | 59 |        |                    |              |
|        | P: gcagccat                         |    |        |                    |              |
| zip6   | F: gaacgcgttaccttttcgact           | 59 | 49     | 94                 | NM_001001591.1 |
|        | R: acacagtgcgctagcact              | 59 |        |                    |              |
|        | P: ttgggcc                          |    |        |                    |              |
| zip10  | F: gtgtgtaattgtggctgttcatt         | 60 | 145    | 73                 | NM_200671.1  |
|        | R: caactgttcaagctacat              | 59 |        |                    |              |
|        | P: ttggcca                          |    |        |                    |              |
| 18s    | F: aacgttttccatcaaaccctg            | 59 | 48     | 67                 | FJ915075.1   |
|        | R: ggactaaatcaagcgaaccgcc          | 59 |        |                    |              |
|        | P: ttcccgt                          |    |        |                    |              |
| Snai 3 | F: gtgcaagchtggtagtagggcttgg        | 60 | 80     | 88                 | NM_001077385.1 |
|        | R: gcacgtgaatggtttctacctg           | 59 |        |                    |              |
|        | P: ttccagg                          |    |        |                    |              |

F, R and P denote forward primer, reverse primer and probe sequences.
FIGURE LEGENDS

Figure 1. Structural relationships of the human ZIP (SCL39A) family of zinc transporters
(A) Molecular phylogenetic analysis of human ZIP (SCL39A) family of zinc transporters and zebrafish Zip10 (DrZip10) as generated by the Maximum likelihood method. The tree with the highest log likelihood (-5949.0421) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Sequences belonging to the LIV-1 subfamily of ZIP proteins are denoted. (B) Depiction of motifs in selected ZIP proteins from the LIV-1 sub-family. Transmembrane domains are illustrated by rectangular boxes; His = histidine cluster; CPALLY = unique motif in plasma membrane located LIV-1 sub-family members; Arrow = proteolytic cleavage site at putative PEST motif; ZIP = ZIP family amino acid signature motif in transmembrane domain 4; HEXPHE = PDF metalloprotease motif in transmembrane domain 5; numerals 1, 2, 3, 6, 7 and 8 in rectangles denote the other transmembrane domains.

Figure 2. ZIP10 has plasma membrane localization, mediates zinc influx and stimulates cell proliferation
(A) Immunofluorescence of unpermeabilised MCF-7 cells transfected with a human ZIP10 construct and probed with an anti-ZIP10 antibody. (B) Uptake of zinc(II) in CHO cells over 20 minutes at different concentrations of total zinc(II) added to the culture medium (in absence of FBS). The values show a mean of three replicate assays and the error bars represent SEM. (C) Paxillin focal adhesion are greatly reduced in MCF-7 cells overexpressing exogenous ZIP10 and rounding up as consequence. MCF-7 cells were transfected with ZIP10 (V5 tag) and probed for V5-tagged ZIP10 (red) and paxillin (PXN, green); chromatin was stained with DAPI (blue). PXN focal adhesions are evident in adherent ZIP10 negative or positive cells. Overexpression of ZIP10 causes cells to round up (white arrow) and loose adhesion. These cells have limited or no PXN focal adhesions (white arrow). (D) Attachment potential of collected detached and re-seeded MCF-7 cells from cultures of either WT cells, or MCF-7 cells transfected with LacZ (transfection control) or human ZIP7, ZIP6, or ZIP10. (D) Cell cycle stage analysis of by FACS of either wild type MCF-7 cells (WT), or MCF-7 cells transfected with LacZ (transfection control), or human ZIP7, ZIP6, or ZIP10. Values are means from three experiments.

Figure 3. ZIP10 promotes motility of MCF-7 cells through down-regulation of CDH1 and inactivation of GSK-3
(A) MCF-7 cells transfected with ZIP6 or ZIP10 for 8 hour migrated across Matrigel™ on a Transwell plate over the next 16 hour more than controls. Images represent migrated DAPI-stained cells. In each of three experiments, 12 fields of view were assessed and data averaged. Values are presented as means ± SEM (N=3). The asterisk indicates statistical difference from control at p<0.05. (B) Loss of E-cadherin (red) only in recombinant ZIP6-V5 positive cells probed with anti-V5 antibody (green). (C) Representative immunoblot of Ser9-GSK-3α and Ser21-GSK-3β in wild type MCF-7 cells (WT) and in MCF-7 cells transfected with LacZ (transfection control), or human ZIP10 or ZIP7. Immunoblot for β-actin was used as loading control.

Figure 4. Phenotype of Zip10 knock-down zebrafish embryo
Zebrafish embryos were co-injected with 2 ng of translation blocking morpholino-modified oligonucleotides against zip10 and p53 (to block off-target effects causing apoptosis; Zip10MO) or with 2 ng of a scrambled control morpholino (CtrlMO) and compared to uninjected wild type (WT) embryos. (A) Percentage of embryos displaying morbidities (% phenotypes) at 10, 24, 72 and 120 hours post fertilization (hpf). (B) Epiboly cell migration in wild-type embryos and in embryos injected with a translation blocking morpholino against zip10 (Zip10MO) or with a scrambled control morpholino (CtrlMO). The extension of epipoly migration was delayed in Zip10MO embryos compared with time-matched controls (Arrows) at 6 and 10 hpf with resulting delay in tail bud formation in
morphants at 10 hpf compared to wild-type. Morphant was yet to complete epiboly (85% complete) at 10 hpf when tail bud has already formed in wild-type. (C) Common phenotypes of Zip10MO embryos at later stages of development (24 – 120 hpf). Note the Zip10MO embryo at 24 hpf with shorter anterio-posterior axis with abnormal head and eye formation (highlighted with white broken circle). Also note the oedema of the pericardial sac (black arrow-head) and the miss-formed heart (heart-string) feature (white arrow) in 72 hpf Zip10MO embryos. Severe oedema of pericardium and yolk sac was observed in many embryos at 120 hpf. Note also the curled or twisted tail at 72 & 120 hpf (white broken circles). The experiment was carried out five times with 20 - 50 embryos each time (N=5). Results were also replicated using a splicing blocking morpholino against zip10 (data not shown).

Figure 5. Effect of Zip10 knock-down on expression of genes involved in epithelial mesenchymal-transition (EMT) during gastrulation of the zebrafish embryo 10 hpf

(A) In situ hybridisation of E-cadherin (cdh1) mRNA in zebrafish wild-type embryos (WT), control embryos injected with a scrambled morpholino (CtrlMO), and in embryos injected at 1-4 cell stage with a translation blocking morpholino against zip10 (Zip10MO) at 10 hpf. Ten embryos from each group were assessed and the experiment was repeated with the same results. (B) Expression of cdh1 mRNA in WT, CtrlMO and Zip10MO zebrafish embryos as measured by qPCR. Data are presented as the mean ± SEM (N=6 and the experiment was repeated with the same results). (C) Expression of Cdh1 protein during epiboly in WT and Zip10MO embryos as assessed by immunohistochemistry (n=3) or (D) by immunoblots, which were (E) quantified by band intensity (mean±SEM, n=3). qPCR analysis of mRNA for the gastrulation-inducing gene products of (F) stat3 and (G) zip6. Data are expressed as log2 fold-change and bars represent mean±SEM (N=6 and the experiment was repeated with the same results). Data were statistically evaluated by ANOVA and significant differences (p<0.05) relative to the WT indicated by an asterisk.

Figure 6. Proximity ligand assay of ZIP6 and ZIP10 in MCF-7 cells

(A) Proximity ligation assay using ZIP6-Y and ZIP10-R antibodies in MCF-7 cells produces multiple red fluorescent dots indicative of binding, with presence of few dots in ZIP6-Y only and ZIP10-R only controls. (B) Quantification of numbers of ZIP6:ZIP10 interaction events per cell showing multi-fold greater numbers of dots when antibodies to both proteins are applied compared to controls where ZIP6-Y or ZIP10-R antibodies are applied separately. Results are demonstrated as mean ± SD. * p < 0.05, ** p < 0.01. Scale bar, 25 μm.

Figure 7. Co-isolation of ZIP6 and ZIP10 by affinity capture followed by quantitative mass spectrometry

(A) Workflow of quantitative mass spectrometry analysis. Wild-type (wt) mouse NMuMG cells or ZIP60/0 cells were grown to near confluency and in vivo cross-linked in the presence of 1% formaldehyde (in PBS) prior to harvest and lysis. The affinity capture was based on identical affinity chromatography matrices generated by the non-covalent capture of an anti-ZIP6 antibody on Protein A agarose. Following extensive washes, ZIP6 and its co-isolating binding partners were eluted by pH drop before samples were denatured in urea, reduced, alkylated and trypsinised. To minimize intra-assay variance and enable the relative quantitation of proteins, digests were isobarically labelled with six-plex tandem mass tag (TMT) reagents before in-depth nano-ESI analysis on an Orbitrap Fusion Tribrid mass spectrometer. (B) ZIP6 interacts with ZIP10 but not with Titin. Presented are benchmarks of enrichment for a subset of proteins identified in the ZIP6 interactome dataset. (C) Chart depicting distributions of TMT signature ion ratios within peptide-to-spectrum matches (PSMs) that supported the identification of ZIP10. Note the selective co-enrichment of ZIP10 only in co-immunoprecipitations from wild-type NMuMG cells.
Figure 8. Evidence for selective isolation of the ZIP6 bait protein and non-specific isolation of Titin. TMT signature ion ratios derived from peptide-to-spectrum matches (PSMs) that supported the identifications of (A) ZIP6, and (B) Titin. Note the expected affinity capture of ZIP6 only in immunoprecipitations from wild-type NMuMG cells, and the non-specific co-isolation of Titin in all affinity capture reactions. See Figure 7 for further details.

LEGENDS FOR SUPPLEMENTARY FIGURES

Supplementary Figure 1. Paxillin focal adhesion are absent in MCF-7 cells overexpressing exogenous ZIP6 and rounding up as consequence.
MCF-7 cells were transfected with ZIP6 (V5 tag) and probed for paxillin (green) and V5-tagged ZIP6 (red); chromatin was stained with DAPI (blue). Paxillin focal adhesions are evident in adherent ZIP6 negative or positive cells. Overexpression of ZIP6 causes cells to round up (white arrow) and loose adhesion. These cells have limited or no paxillin focal adhesions (white arrow).

Supplementary Figure 2. Phenotype of Zip10MO zebrafish.
Zebrafish embryos were injected with 2 ng of translation blocking morpholino-modified oligonucleotides against zip10 and compared to uninjected wild type (WT) embryos. Morphological defects observed from 6 to 72 hpf included delayed epiboly, underdeveloped and deformed head, brain and eyes, stunted body axis, bent or curled tail, oedema of the pericardial sack, deformed heart (heart-string), delayed hatching and reduced hatching success. Other details as in Figure 4.
Figure 1
Figure 3

A 16 hour motility on fibronectin

| Cells per field | WT | ZIP10 | ZIP6 |
|-----------------|----|-------|------|
| 100             |    |       |      |
| 80              |    |       |      |
| 60              |    |       |      |
| 40              |    |       |      |
| 20              |    |       |      |

B Merge  V5/DAPI  Alexa 594

C

| ZIP10  | WT  | LacZ | ZIP7 |
|--------|-----|------|------|

Ser9-GSK-3α

Ser21-GSK-3β

Actin
Figure 4

**A**

![Graph showing % of phenotype at different time points (10hpf, 24hpf, 72hpf, 120hpf). The graph compares WT, CtrlMO, Zip10MO, and Zip10MO+p53MO.]

**B**

**WT** and **Zip10MO** embryos at 6hpf and 10hpf, showing differences in phenotype.

**C**

**WT**, **CtrlMO**, and **Zip10MO+p53MO** embryos at 24hpf, 72hpf, and 120hpf, highlighting morphological changes.
Figure 5

A

WT    Ctrl MO    Zip10 MO

B

zip6 mRNA expression

0.0  0.5  1.0  1.5  2.0

WT   Control MO   Zip10 MO

C

WT    Zip10 MO

Brightfield

Cdh1 epifluorescence

Cdh1 confocal

Cdh1 2-photon

D

WT    Zip10 MO

Cdh1

ß-actin

E

Cdh1 protein expression

1.0  1.5  2.0  2.5  3.0

WT    Zip10 MO

F

stat3 mRNA expression

0.0  0.5  1.0  1.5  2.0

WT   Control MO   Zip10 MO

G

zip6 mRNA expression
Figure 6

A

PLA: ZIP6-Y + ZIP10-R

DAPI  PLA  Merge

PLA: ZIP10-R only

DAPI  PLA  Merge

PLA: ZIP6-Y only

DAPI  PLA  Merge

B

![Bar chart showing signals per cell for ZIP6-Y + ZIP10-R, ZIP6 Y only, and ZIP10-R only. The chart indicates significant differences between the groups.](https://via.placeholder.com/150)
Figure 7

A. CRISPR/CAS9-based knockout of ZIP6

- Biological replicates: 1, 2, 3
- Procedures:
  - In vivo formaldehyde crosslinking
  - Cell lysis in NP40 and anti-ZIP6 affinity capture
  - pH drop elution and trypsinization
  - TMT labeling: 126, 127, 128, 129, 130, 131
  - Strong cation exchange and reversed phase clean-up
  - Technical replicates: A, B, C
  - Nanospray MS³ on Orbitrap Fusion Tribrid instrument

B. Table of Peptides and TMT-based enrichment ratios

| Accession | Description       | Coverage | Unique | Total | PSMs | 126/131 | 127/131 | 128/131 | 129/131 | 130/131 | Count | Sequest score |
|-----------|-------------------|----------|--------|-------|------|---------|---------|---------|---------|---------|-------|--------------|
| IPI00469000.4 | Zinc transporter Z1P6 | 27.45%   | 16     | 21    | 97   | 9.63    | 1.045   | 9.727   | 1.36    | 6.919   | 11    | 92.04        |
| IPI00273801.3 | Zinc transporter ZIP10 | 22.45%   | 13     | 17    | 80   | 3.027   | 0.664   | 2.522   | 1.038   | 3.053   | 19    | 97.53        |
| IPI00756257.1 | Isoform 1 of Titin | 15.45%   | 2      | 369   | 501  | 0.996   | 0.992   | 1.02    | 1.026   | 0.995   | 121   | 297.02       |
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