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Slc39a1 to 3 (Subfamily II) *Zip* Genes in Mice have Unique Cell-Specific Functions during Adaptation to Zinc Deficiency

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Abstract

Subfamily II of the solute-carrier (Slc)39a family contains three highly conserved members (ZIPs 1 to 3) that share a twelve amino acid signature sequence present in the putative fourth transmembrane domain and function as zinc transporters in transfected cells. The physiological significance of this genetic redundancy is unknown. Herein, we report that the complete elimination of all three of these *Zip* genes, by targeted mutagenesis and cross-breeding mice, causes no overt phenotypic effect. When fed a zinc-adequate diet, several indicators of zinc status were indistinguishable between wild-type and triple-knockout mice; including embryonic morphogenesis and growth, alkaline phosphatase activity in the embryo, and ZIP4 protein in the visceral yolk sac and initial rates (30 min) of accumulation/retention of ^{67}Zn in liver and pancreas. When fed a zinc-deficient diet, embryonic membrane-bound alkaline phosphatase activity was reduced to a much greater extent and 80% of the embryos in the triple-knock mice developed abnormally compared to 12% of the embryos in wild-type mice. During zinc deficiency, the accumulation/retention (3 hr) of ^{67}Zn in the liver and pancreas of weanlings was significantly impaired in the triple-knockout mice compared to wild-type mice. Thus, none of these three mammalian Zip genes apparently plays a critical role in zinc homeostasis when zinc is replete, but they play important, non-compensatory roles when this metal is deficient.

Keywords

pregnancy; stable zinc isotope; triple knockout mice; zinc deficiency; zinc homeostasis

Introduction

The maintenance of zinc homeostasis is critical, and multiple genes have evolved to modulate the storage, efflux and uptake of this essential metal in response to its availability. In mice it is estimated that 28 different genes may contribute to zinc homeostasis. Two superfamilies of mammalian zinc transporters have been identified that belong to the solute carrier (Slc)30a and the Slc39a families (16;32;41). Slc30a members, named ZnTs, function in zinc efflux and compartmentalization and are cation diffusion proteins (32). Members of the Slc39a family, named ZIPs, function in the uptake of zinc and other metals (12;16;41). In mice and humans there are 14 members of the ZIP family most of which can be grouped into one of two subfamilies named subfamily II (3 members) and LIV-1 (9 members). Many of these zinc

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transporters are expressed in a tissue-specific manner and in specific cellular localizations. In addition, they can display specific changes in cellular localization and stability in response to zinc deficiency or excess (4;11;22;44).

Mutations in many of the members of the ZnT family and a few of the ZIP family members have begun to suggest their physiological functions. The mouse *Znt1* gene is essential during early development and may be involved in the transfer of zinc into the conceptus (1), whereas the *ZnT3* gene is expressed in neurons and is important for the acquisition of zinc in synaptic vesicles (3). Mutations in the mouse *Znt4* gene and human *Znt2* gene have been associated with a loss or reduction, respectively, of zinc in milk (2;18). *Znt5*-knockout mice survive but have multiple abnormalities in muscle and bone (20), probably due to impaired transport of zinc into the secretory compartment (36). *Znt7*-knockout mice also fail to thrive, have low body fat and show diminished acquisition and distribution of dietary zinc (17) perhaps also reflecting impaired zinc transport into the Golgi apparatus (25). Less is known about the genetics of mammalian *Zip* genes. Mutations in human *Zip4* cause the rare and fatal recessive disease acrodermatitis enteropathica (27;43) and the mouse *Zip4* gene is essential for early embryonic development and may be involved in the transfer of zinc via the visceral yolk sac into the embryo and later in uptake of dietary zinc (10).

Our previous studies of three members of the ZIP subfamily II (8) revealed that targeted deletion of mouse *ZIP1, 2* or *3* did not cause overt signs of zinc deficiency but did lead to an impaired ability to adapt to zinc deficiency (5;6;33). Analyses of single and double-knockout mice with no functional *Zip1* and/or *Zip3* suggested that they contribute essentially additively to the degree of sensitivity to zinc deficiency during pregnancy (6). Analysis of the patterns of expression of these genes in mice revealed co-expression in intestinal stromal cells, nephric-tubular epithelial cells, pancreatic ductal epithelial cells, and hepatocytes surrounding the central vein, as well as some unique cell-specific expression patterns (5;6). This suggested that these zinc transporters might exert some compensatory functions in the redistribution and/or retention of zinc rather than its acquisition from the diet (6;8). In contrast, the expression patterns of *Zip2* were found to be remarkably cell-type specific in peri-central hepatocytes, keratinocytes and immature dendritic cells (33), but the loss of function of this gene also rendered mice more sensitive to zinc deficiency during pregnancy.

Herein, we examined the effects of a complete loss of function of the ZIP subfamily II genes in mice. Remarkably, although these genes are highly conserved and related to one another, the entire subfamily II is dispensable when dietary zinc is replete. Consistent with the conclusions from previous studies, the functions of these genes is apparently required only during periods of zinc deficiency where they function in the accumulation/retention and distribution of dietary zinc. Compensatory functions among members of the Slc39a subfamily II are minimal, consistent with the concept that they evolved independent cell-specific functions in zinc homeostasis in mice.

Materials and Methods

Animal Care and Use

Experiments with mice were performed in accordance with the guidelines from the National Institutes of Health for the care and use of animals and were approved by the Institutional Animal Care and Use Committee. Mouse diets were purchased from Harlan Teklad (Teklad.com) and were identical except for zinc levels, which were as follows: zinc-deficient, 1 ppm zinc; zinc-adequate, 50 ppm zinc.

The effects of zinc deficiency during pregnancy on morphogenesis and growth of the embryo were determined as described in detail previously (5;6;9;33). Female mice were mated (d1 =

vaginal plug) and provided free access to zinc-adequate feed and deionized distilled water until d8 of pregnancy. Mice were then placed in pairs in cages with stainless steel false bottoms, and the diet was changed to the zinc-deficient feed. On day 14 of pregnancy, the embryos were collected and examined for gross morphological defects as described previously (5;10), and as presented in the legend to Figure 2 herein.

The effect of zinc deficiency on the accumulation/retention of zinc was examined in weaned mice. Newly weaned mice (< 5 days post weaning, 6 mice per group) were fed the zinc-adequate or the zinc-deficient diet for one week. Food was withheld overnight and mice were then given an oral gavage containing the stable isotope ^{67}Zn (26;30). The gavage solution was prepared as follows: Zinc-deficient feed was dissolved in deionized water (1g feed in 2 ml water) and the slurry was adjusted to 100 ppm ^{67}Zn. ^{67}Zn (>94% enriched, ^{67} zinc oxide) from Trace Sciences International Corporation (isotopetrace.com) was dissolved in 3 drops of concentrated HCl and then diluted to a final concentration of 5 mg/ml in distilled water. This stock solution was diluted 1:50 into the food slurry just before use. The pH of the gavage solution was neutral. Mice were given an oral gavage (100 μl food slurry containing 10 μg ^{67}Zn) and tissues (liver, pancreas) were harvested at the indicated times after the gavage, rinsed in PBS and prepared for elemental analysis as described below.

Zip1, Zip2 and Zip3 Targeting Vector Construction

Bacterial artificial chromosomes that contain these mouse *Zip* genes from the 129/SvJ mouse strain were obtained from Incyte Genomics (incyte.com) and sequenced by Bruce Roe (University of Oklahoma).

The methods of construction and final structures of each targeting vector have been described in detail previously (5;6;33). Each targeting vector had the coding region of the *EGFP* cDNA, from the vector pEGFPKT1loxneo was introduced immediately downstream of the start codon. A loxP-flanked *MC1-Neo* cassette was immediately downstream of the *EGFP* cDNA and the targeting vector was flanked by herpes simplex virus thymidine kinase and diphtheria toxin negative selectable markers.

Targeted Disruption of Zip Genes in Embryonic Stem Cells

Targeting vectors were linearized within the vector backbone and electroporated into RW4 embryonic stem (ES) cells. Colonies were selected with 300 μg/ml G418 (cellgro.com) and 2 μM gancyclovir (roche-applied-science.com). Homologous recombinants were screened for by Southern blot hybridization as described in detail previously for each knockout allele (5; 6;33). Positive clones were subsequently screened with a Y-chromosome-specific probe derived from pY2 (28), and a *Neo* cDNA to detect the presence of a single -targeted *MC1-Neo* cassette.

Generation of Zip-Knockout Mice

Chimeric mice were generated by microinjection of ES cell clones into 3.5-day-old C57BL/6 blastocysts, followed by transfer to pseudopregnant CD-1 foster mothers. Resulting chimeric mice were crossed with C57BL/6 females (harlan.com). Germline transmission was confirmed by PCR from tail DNA of agouti offspring.

The PCR screens for the targeted *Zip* alleles each utilized a set of three primers that directed amplification of the wild-type and the targeted alleles. The details of those primer sequences and where they anneal have been described previously (5;6;33). For the *Zip1* targeted allele the PCR product for the mutant allele was 433 bp, and the product for the wild-type allele was 328 bp. For the *Zip2* targeted allele the PCR product for the mutant allele was 319 bp, and that

for the wild-type allele was 403 bp. For the *Zip3* targeted allele the PCR product for the mutant allele was 326 bp whereas the product for the wild-type allele was 461 bp.

Agouti offspring heterozygous for the knockout allele were crossed to generate homozygous knockout mice. To remove the loxP-flanked MC1-Neo cassette, knockout mice were mated with commercially available Cre-expressing transgenic mice (strain name: B6.FVB-TgN (EIIa-cre) C5379 Lmgd from JAX.org) which express Cre in all tissues. *Zip*-knockout mice that lacked the Neo cassette were crossed to generate homozygous knockout mice and wild-type mice.

Generation of Zip1, Zip2, Zip3 Triple-Knockout Mice

Homozygous *ZipI* and *Zip3* mice were cross-bred, and then offspring were back-crossed to generate homozygous double-knockout mice and wild-type mice as described (6). These double-knockout mice and wild-type mice were used to create working colonies. The double-knockout mice were then crossed with homozygous Zip2-knockout mice yielding offspring heterozygous for each knockout allele. These mice were then crossed and offspring homozygous for two of the knockout alleles and heterozygous for the third knockout allele were crossed. Offspring homozygous for all three knockout alleles were identified and crossed to create a working colony. Triple-knockout mice were compared with wild-type mice generated during creation of the double-knockout strain. All of the knockout lines and the wild-type line were therefore on a similar mixed genetic background.

RT-PCR Amplification of Zip Transcripts

Total RNA (5 μg) isolated from mouse liver using TRIzol according to the manufacturer's instructions (invitrogen.com), was reverse transcribed using Superscript III Reverse Transcriptase (RT) (invitrogen.com) and then amplified using Taq polymerase or LA-Taq polymerase (TAKARABio.com). *Zip1* and *Zip2* mRNAs were amplified for 30 cycles to yield 1000 bp and 406 bp products, respectively, under reaction conditions described in detail previously (8). *Zip3* mRNA was amplified using Zip3 (S) primer (5′-GTTCTTCTTCATGCTGCTGGGCTCCCTGCT-3′) and Zip3 (AS) primer (5′-GCACCAGGAACAGCACCTTCAGC-3′) for 35 cycles to generate an 884 bp product. *GAPDH* was amplified using GAPDH (S) primer (5′-TCACGGCAAATTCAACGGCACAGTCAAGGC-3′) and GAPDH (AS) primer (5′-CAGCACCAGTGGATGCAGGGATGATGTTCT-3′). Negative control reactions, in which the RT was omitted, were performed in parallel.

Membrane Bound Alkaline Phosphatase Assay

The method of membrane preparation and the alkaline phosphatase assay have been described in detail previously (35;36). In brief, VYS and embryos (6 each) were collected on day 14 of pregnancy from wild-type and triple-knockout mice fed a zinc-adequate or zinc-deficient diet beginning of day 8. Samples were homogenized and membranes were recovered from the post-nuclear supernatant by centrifugation (100,000 × *g* for 15 min at 4°C). The membrane pellet was suspended in buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, and 0.1% Triton X-100) and frozen at −70 °C until assayed. Membrane proteins (5 μg) were assayed for alkaline phosphatase activity using 2 mg/ml *p*-nitrophenyl phosphate in 1 M diethanolamine buffer, pH 9.8, containing 0.5 mM $MgCl_2$. *p*-Nitrophenol release was measured by the absorbance at 405 nm. Antarctic phosphatase was used as a standard.

Western Blot Analysis

Membrane proteins from the VYS, prepared for the alkaline phosphatase assay described above, were also examined by Western blotting. Membrane proteins (40 μg) were heated in

1X SDS-sample buffer at 37°C for 10 min, resolved on a 10% SDS-polyacrylamide gel and transferred to polyvinylidine difluoride membranes. Membranes were blocked overnight and incubated with primary antibody as described previously (7;19;44). Immunoreactive bands were visualized using ECL Plus Western Blotting Detection System with Hyperfilm ECL (amershambiosciences.com). Anti-peptide antibodies against ZIP4 and ZIP1 have been generated and characterized as described previously (9;19).

Trace metal determination

Elemental profiling via inductively-coupled plasma mass-spectrometry (ICP-MS) was performed for Na, Mg, P, K, Ca, Fe, Co, Cu, Zn, As, Se, and Mo as described in detail previously (33). In addition, the stable isotopes of zinc, ^{67}Zn and ^{66} Zn were measured in each sample, as indicated. The natural ratio of these zinc isotopes is 0.146. Mouse pancreata and livers (n = 4 to 6 per group) were dried in a vacuum oven, digested in concentrated HNO_3 and analyzed on an Elan DRCe ICP-MS (PerkinElmer). Methane was used as a collision cell gas to measure iron. Gallium and Indium were used as internal standards. National Institute of Standards and Technology traceable single element ICP standards (ultrasci.com) were used to make up the calibration standards.

Statistics

Experimental data were evaluated by Student's t-test. Data are presented as means ± standard deviation (S.D.) and differences between groups are presented in the appropriate figure legends. Differences were considered significant at $P < 0.05$.

Results

Creation of mice with no functional Slc39a subfamily II genes (Zip1, 2 and 3 triple-knockout mice)

Zip1 and/or *Zip3*-knockout mice as well as *Zip2*-knockout mice were created as described previously (5;6;33). In each case, the targeting construct fused the initiator methionine codon with the open reading frame of the enhanced green fluorescent protein (EGFP) reporter.

Homozygous *Zip1, Zip3* double-knockout mice were crossed with homozygous *ZIP2*-knockout mice and the offspring were genotyped and then back-crossed to ultimately yield homozygous triple-knock mice. Wild-type mice, generated while creating *Zip1, Zip3* double-knockout mice and *Zip2*-knockout mice, were crossed to yield a working colony. Successful creation of homozygous triple-knockout mice was verified by genotyping PCR which showed amplification of only the knockout alleles in these mice (Fig. 1A) and by RT-PCR showing that these gene transcripts are absent in the adult liver of triple-knockout mice (Fig. 1B). These three *Zip* genes are expressed in a cell-specific pattern in the adult liver (6;33).

Mice with no functional *Zip1*, *2* or *3* genes appeared normal when fed a zinc-adequate diet. There was no difference between wild-type and triple-knockout mice with regard to growth rate, pregnancy rate, litter size or embryo growth and development (Fig. 2 and data not shown).

The triple-knockout mice showed no overt signs of zinc deficiency. On day 14 of pregnancy, the amount of membrane bound alkaline phosphatase activity in the embryo (Fig 3A), a zinc-dependent enzyme that is sensitive to zinc deficiency (36), and the abundance of ZIP4 protein in the visceral yolk sac (Fig 3C), a sensitive indicator of zinc deficiency (7;9;44), were indistinguishable between the triple-knockout and wild-type mice.

The initial accumulation/retention of exogenous zinc in the liver and pancreas were examined by giving mice an oral gavage containing the stable zinc isotope ^{67}Zn mixed into a slurry of

zinc-deficient feed (100 ppm final Zn content). Liver and pancreas were harvested 30 min after the gavage and the ratio of ^{67}Zn to ^{66}Zn was determined using inductively coupled plasma mass spectrometry (ICP-MS) (33). The natural ratio of these isotopes is 0.146. As shown in Figure 4A, the initial rate of accumulation/retention of zinc was the same in triple-knockout and wild-type mice.

Effects of zinc deficiency during pregnancy on morphogenesis, membrane bound alkaline phosphatase activity and ZIP4 protein abundance in triple-knockout and wild-type mice

Our previous studies showed that the functions of ZIPs1, 2 and 3 individually are not evident when dietary zinc is replete, but become apparent when zinc is limiting. Therefore, we examined the effects of dietary zinc deficiency during pregnancy in triple-knockout and wild-type mice (Fig 2A). Mice were fed a zinc-adequate or zinc-deficient diet beginning of day 8 of pregnancy and embryos were examined on d14 for morphological defects (Fig 2B). Embryos in wild-type mice were relatively resistant to these conditions of zinc-deficiency and about 14% showed developmental defects when examined. These included growth retardation, abnormal limb development and cranio-facial abnormalities. Only a small percentage of wild-type embryos were severely affected (see the legend to Fig 4). In contrast about 80% of the embryos in the triple-knockout mice were abnormal on day 14 and about 60% were severely affected (Fig 2B).

Measurements of membrane bound alkaline phosphatase activity in the embryo revealed that zinc deficiency caused a large reduction in the wild-type and triple-knockout embryos (Fig 3A). However, triple-knockout embryos retained less than half the amount of enzyme activity (2.5 mU/μg membrane protein) relative to that in wild-type embryos (5.2 mU/μg membrane protein). The visceral yolk sac contains only low amounts of membrane bound alkaline phosphatase activity that were not dramatically reduced by zinc deficiency (Fig 3B). Taken together these results show that triple-knockout mice are very sensitive to dietary zinc deficiency during pregnancy.

Mouse ZIP4 plays a critical role in zinc homeostasis during development (10), and this protein is induced during periods of zinc deficiency in the visceral yolk sac. Western blot analysis of membranes from the visceral yolk sac demonstrated that ZIP4 is dramatically induced to the same extent during zinc deficiency in triple-knockout and wild-type mice (Fig 3C). Thus, this response to zinc deficiency is not impaired in the triple-knockout and ZIP4 cannot compensate for the loss of function of ZIPs 1, 2 and 3.

Triple-knockout mice show impaired accumulation/retention of oral ^{67}Zn in the liver and pancreas only when fed a zinc-deficient diet

The above results suggest that triple-knockout mice may be impaired in the ability to accumulate zinc during periods of zinc deficiency. To directly test this possibility, weanling mice were fed a zinc-deficient diet for 7 days and then given an oral gavage (100 μl) containing 100 ppm ^{67}Zn mixed into a slurry of zinc-deficient feed. Liver and pancreas were harvested 3 hr after the gavage and the ratio of ^{67}Zn to ^{66}Zn was determined using ICP-MS (Fig 4B). Wild-type mice accumulated/retained twice as much ^{67}Zn in the liver and pancreas than triple-knockout mice within 3 hr. By 6 hrs the accumulation/retention of ^{67}Zn in the liver and pancreas had increased in the triple-knockout mice but was highly variable between individuals (data not shown). These results are consistent with the concept that ZIP1, 2 and 3 function during periods of zinc deficiency to facilitate the accumulation/retention of zinc in the liver and pancreas.

Triple-knockout mice do not accumulate hepatic iron during zinc deficiency

Our previous studies demonstrated that *Zip2*-knockout mice fail to accumulate iron in the liver during periods of zinc deficiency. Whether ZIP1 and ZIP3 also play a role in iron metabolism in the liver is unknown. To explore this possibility, liver was collected from day 14 pregnant mice that had been fed the zinc-adequate or zinc-deficient diet beginning of day 8 of pregnancy. Livers were dehydrated and subjected to elemental analysis by ICP-MS (Fig 5). Iron content in the liver was indistinguishable in triple-knockout and wild-type mice fed the zinc-adequate diet, and during zinc deficiency, iron content doubled in the liver of wild-type mice but remained unchanged in the triple-knockout mice (Fig 5A & C). These findings are similar to those reported in *Zip2*-knockout mice (33). Interestingly, zinc content in the liver was not clearly different between wild-type and triple-knockout mice and the loss of zinc from the liver was not apparently exacerbated in the triple-knockout mice during zinc deficiency. However, it should be noted that only a small proportion (< 20%) of total cellular zinc appears to be labile and a further loss of total zinc content leads to lethality.

Discussion

The evolution of two large gene families of zinc transporters in mammals is consistent with the complexity of essential metal homeostasis between different tissues and organs as well as within specific microenvironments both intra- and extracellular. There is a need to acquire the metal from varied diets, distribute the metal within the body and accumulate, store or eliminate the metal in a dynamic and often cell-specific manner. Mutations in many (7/10) of the *ZnT* gene family (Slc30a) of zinc transporters have been shown to cause overt phenotypes indicative of tissue-specific defects in zinc homeostasis in mice or humans (21;22;32). In contrast, to date only mutations in *Zip4*, among the nine Slc39a LIV-1 subfamily members, have been associated with zinc deficient phenotypes in mice and humans (10;43). The effects of mutations in other members of the LIV-1 family of zinc transporters in animals have not been reported, although many studies suggest that these genes play important roles in many fundamental cellular processes (38;40).

Studies reported herein focused on the effects of mutating three members of Slc39a subfamily II called *Zip 1, 2* and *3*. These genes have been well conserved during evolution. Mouse and human ZIP1 share 93% amino acid sequence identity (8). Structural (amino acid sequence) relatedness groups these three proteins into subfamily II (12). Remarkably, a complete loss of function of the entire subfamily II of genes in mice did not lead to overt signs of zinc deficiency based on several criteria. However, subfamily II genes were found to be important during periods of zinc deficiency in mice. The high degree of conservation of these genes suggests that their roles in protecting against zinc deficiency are also important in humans, particularly during pregnancy.

Our previous studies showed that knocking out the *Zip1*, *Zip2* or *Zip3* genes rendered mice more sensitive to the effects of zinc deficiency during pregnancy (5;6;33) Under the same experimental conditions employed herein about 45% of the *Zip1*-knockout embryos and 36% of the *Zip3*-knockout embryos developed abnormally. In contrast, 91% of the embryos were abnormal in the double-knockout mice (*Zip1* and *Zip3*) under these conditions. *Zip1* and *3* genes appear to function in an additive manner in this model of zinc deficiency (6). Studies of *Zip2*-knockout mice under identical experimental conditions of zinc deficiency revealed that 57% of the *Zip2*-knockout embryos developed abnormally (33). This suggested that ZIP2 might have a more profound influence on zinc homeostasis than either ZIP1 or ZIP3 alone. Surprisingly, mice lacking all three of these *Zip* genes were not more sensitive to zinc deficiency than the *Zip1-3*-double-knockout mice. Thus, members of Slc39a subfamily II appear to have evolved unique non-compensatory functions during zinc deficiency. We previously reported that ZIP2 plays a role in iron accumulation/retention in the liver during

zinc deficiency; *Zip2*- knockout mice do not accumulate hepatic iron under these conditions (33). Whether ZIP1 and ZIP3 play roles in hepatic iron accumulation is unknown, but as shown herein deletion of *Zip1* and *Zip3* did not modify this *Zip2*-knockout phenotype. These results are consistent with the concept that these three zinc transporters have evolved unique tissue- and cell-specific functions.

Results obtained studying triple-knockout mice fed a zinc-adequate diet revealed that these genes are not essential for the rapid uptake and/or distribution of zinc in the body. In contrast, studies of these mice fed a zinc-deficient diet suggested a significant attenuation in the accumulation/retention of zinc in liver and pancreas. The amount of ^{67}Zn accumulated in the pancreas and liver within 30 minutes in zinc-replete mice was twice that of the natural abundance of this isotope in these tissues. ^{67}Zn represents 4.2% of the total natural Zn isotopes (^{64}Zn> ^{66}Zn = ^{68}Zn $\gg$ ^{67}Zn in relative abundance). In these zinc-replete mice an increase of 4.2% in total cellular zinc was detected within 30 min of the gavage. This is a reasonable approximation of a physiologically relevant amount. In the zinc-deficient mice, by three hours after the gavage triple-knockout mice had accumulated only a small increase in total zinc in the liver (1.6% increase) and pancreas (1.1% increase) whereas the wild-type mice had accumulated 6.8% more zinc in the liver and 7.6% more zinc in the pancreas. Although these studies do not reveal the exact reason for these differences (uptake, distribution, retention) in zinc metabolism, they clearly reveal that these *Zip* genes play an important role(s) in zinc homeostasis during periods of zinc deficiency.

The physiological mechanisms of action of these, and most other ZIPs remain to be determined. We hypothesize that on an organismal level, subfamily II members function in zinc homeostasis by controlling the distribution and/or retention of zinc in specific cells and organs. *Zip1* and *Zip3* are actively expressed in the lamina propria of the intestine and are thus unlikely to be important in the uptake of dietary zinc from the intestinal lumen (6). They may instead function in the distribution of zinc once it is acquired from the diet and transported through enterocytes. All three of these *Zip* genes are expressed in liver surrounding the central vein in a remarkable gradient pattern. *Zip2* is expressed at high levels exclusively in a single layer of peri-central hepatocytes (33). *Zip1* and *Zip3* are also expressed in these cells as well as in a gradient that diminishes away from the central vein in deeper layers of hepatocytes (5;6). This pattern of expression suggests that these proteins function in the retention of zinc in the liver by preventing its escape into the central vein or that they function to collect zinc from the venous blood back into the liver. The cellular localization of these proteins in hepatocytes remains to be determined. These genetic studies indicate that these three zinc transporters are not essential for the rapid accumulation/retention of zinc in the liver when zinc is replete, that their functions are not interdependent, and that they function in the accumulation/retention of zinc in the liver (and pancreas) during periods of zinc deficiency. *Zip2* appears to have a unique role in iron metabolism in the liver although the mechanism is not understood (33).

Studies of ZIP1 and ZIP3 in transfected cells suggest that these proteins are recruited to the cell surface under zinc-deficient culture conditions (42) and *in vivo* studies of *Zip1* expression and protein abundance do not suggest significant changes during zinc deficiency in the liver, intestine and visceral yolk sac (Fig. 3C) (44), but studies of the cellular localization of these proteins *in vivo* have yielded conflicting results. ZIP1 can display different localization patterns in adherent versus non-adherent cells, being vesicular in epithelial cell types and apical in K562 cells (31). Perhaps *in vivo* the localization of this protein reflects the microenvironment of each cell-type. When zinc levels are low these proteins may be moved to the cell surface. However, in the human prostate, which is very rich in zinc, ZIP1 plays a role in zinc uptake by normal cells and diminished expression of this gene has been associated with prostate cancer (13). In contrast, over-expression of *Zip1* inhibits growth of malignant PC-3 cells (14). There is little information on the role of zinc in the mouse prostate gland but as yet we have seen no evidence

for prostate or other tumors in the triple-knockout mouse colony. Whether these mice may be more prone to the induction of cancer remains to be examined. ZIP1 has also been reported to induce an osteogenic phenotype in transfected mesenchymal stem cells (37). We have noted no difference in the bone zinc content of triple-knockout versus wild-type mice and no gross abnormalities in bone formation in the triple-knockout mice (J. Geiser and G.K. Andrews, unpublished observations).

ZIP3 has been proposed to play a major role in zinc uptake by mammary epithelial cells (23; 24) and even in the maintenance of viable mammary cells (24), but neonatal pups nursing from triple-knockout females showed no signs of zinc deficiency, unlike the lethal milk mutant phenotypes (34) or the phenotypes noted during dietary zinc deficiency while nursing. Perhaps ZIP3 functions transiently during lactation but its absence and the absence of the all subfamily II members does not lead to obvious phenotypic abnormalities in mice when zinc-replete. ZIP3 does not appear to play a pivotal physiological role in the survival of functional secretory mammary epithelial cells in mice.

The functions of ZIP2 are clearly unique from those of ZIP1 and ZIP3 as is its pattern of expression. ZIP2 influences calcium and iron metabolism in zinc deficient embryos and liver, respectively (33). ZIP14 (39) has recently been reported mediate transport of non- transferrin bound iron into hepatocytes (29) although this finding has recently been challenged (15). Our studies reveal that ZIP2 plays a unique role in iron accumulation in the liver. Whether ZIP2 can transport iron is under investigation.

Perspectives and Significance

The molecular mechanisms regulating zinc homeostasis in mammals are important to understand because zinc deficiency is a world-wide problem that affects hundreds of thousands of children, in particular. A deficiency of zinc causes a multitude of negative physiological effects during periods of rapid growth and differentiation. It is thought that the 14 members of the solute-carrier (Slc)39a family are critical for the uptake of zinc into cells, but the physiological functions of most remain obscure. Herein we report studies of three highly conserved members (ZIPs 1 to 3) that compose subfamily II. These studies show that this family of genes has evolved to function during periods of dietary zinc deficiency but are dispensable when dietary zinc is replete. They play important, non-compensatory roles consistent with their tissue-specific patterns of expression. Although much remains to be learned about the structure, function and regulation of the mammalian ZIP proteins, our studies show that mutations in these *Zip* genes can modify sensitivity to the stress of dietary zinc deficiency and could therefore be important in human health.

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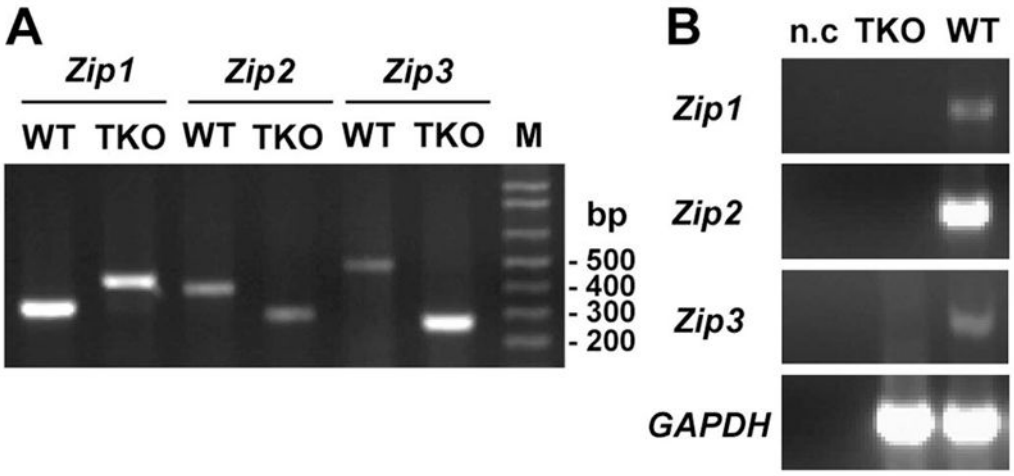

Figure 1. Confirmation of the genotype of triple-knockout mice with targeted deletions of the *Zip1*, *Zip2* and *Zip3* genes. Part A

PCR genotyping of wild-type (**WT**) and knockout alleles for *Zip1*, *Zip2* and *Zip3* genes in triple-knockout (**TKO**) mice. PCR reactions contained three primers each that directed the amplification of both wild-type and knockout alleles of these *Zip* genes as described in Methods. Details of the targeting of each of theses genes have been published previously (5; 6;33). **M:** DNA ladder. **Part B**. RT-PCR detection of *Zip1*, *Zip2* and *Zip3* mRNAs in total liver RNA from wild-type and triple-knockout mice. *GAPDH* served as an internal control for the RT-PCR, and **n.c**. indicates samples in which the reverse transcriptase was omitted from the reaction.

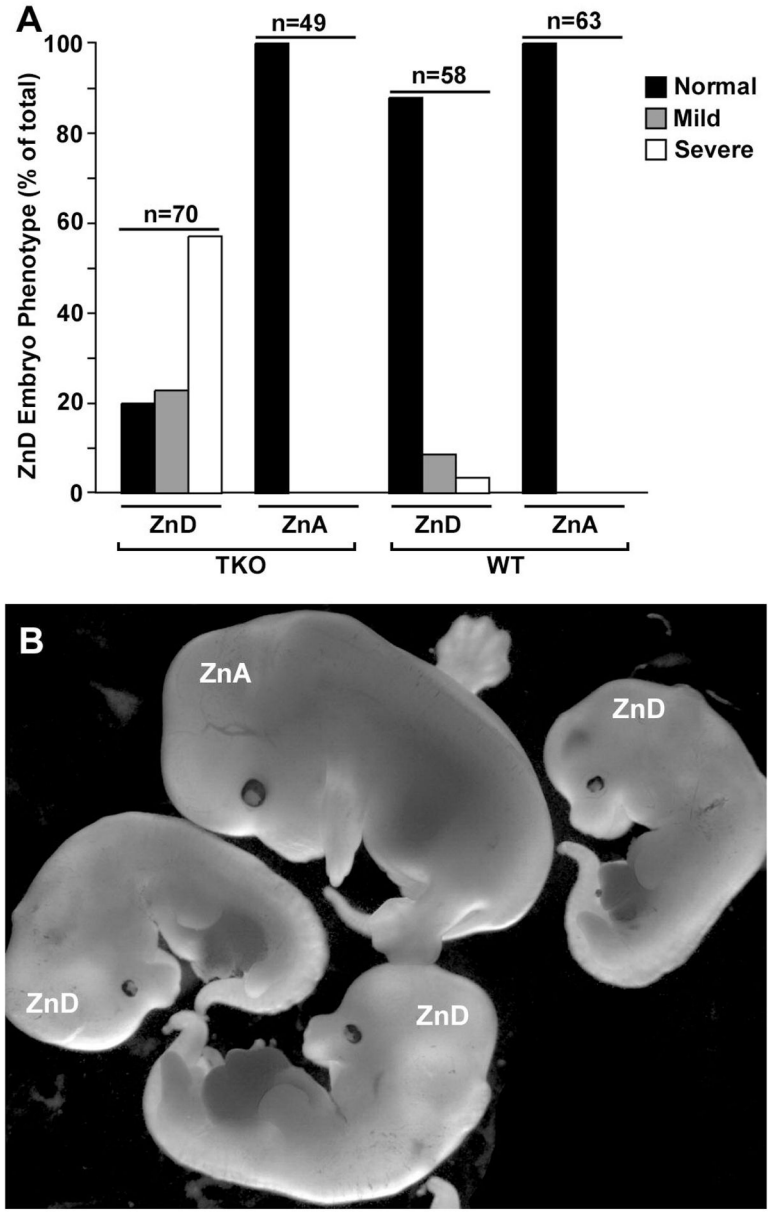

Figure 2. Effects of dietary zinc deficiency during pregnancy on embryonic growth and morphogenesis in triple-knock and wild-type mice

Mice were fed a zinc-adequate (**ZnA**) or zinc-deficient (**ZnD**) diet beginning on day 8 of pregnancy and the morphology of embryos was examined on day 14. **Part A**: Embryos were divided into three groups based on morphology; normal, mildly affected and severely affected. Embryos of normal size, with well-formed forelimb digits and normal gross features were called **Normal**. Embryos that were smaller and had delayed formation of the forelimb digits but were otherwise apparently normal were called **Mild,** whereas those that were very small, had abnormal forelimbs and hind limbs as well as craniofacial abnormalities were called **Severe**. An example of a normal zinc-adequate triple-knockout embryo (ZnA) and several severely zinc-deficient (ZnD) triple-knockout embryos are shown in **Part B**.

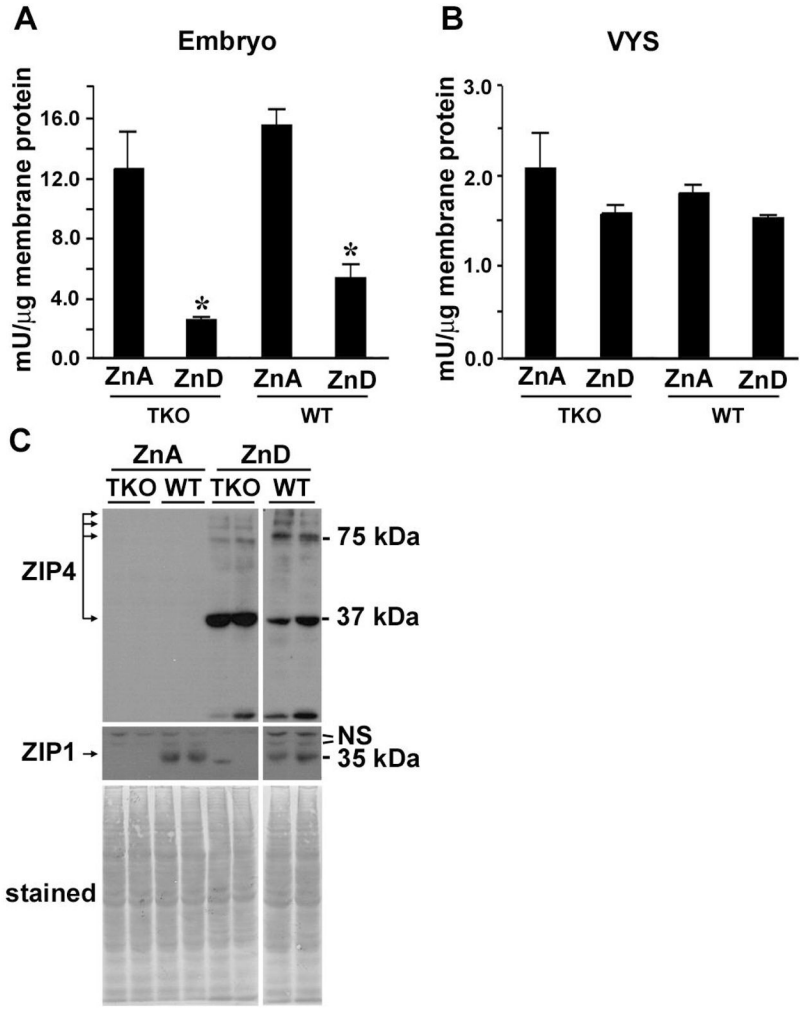

Figure 3. Effects of dietary zinc deficiency during pregnancy on embryonic membrane bound alkaline phosphatase activity and ZIP4 protein abundance in triple-knock and wild-type mice

Wild-type (**WT**) and triple-knockout (**TKO**) embryos (**Part A**) and their visceral yolk sacs (**VYS: Part B**) were collected (6 per group) on day 14 of pregnancy from mice fed a zinc-adequate (**ZnA**) or zinc-deficient (**ZnD**) diet beginning of day 8. Membranes were isolated from each tissue sample and assayed for alkaline phosphatase activity. The data are expressed as the mean ± S.D. from six determinations each. *, Signifies a significant difference between ZnD TKO and WT ($P < 0.001$). **Part C**: Western blot detection of ZIP4 in membrane proteins from the day 14 visceral yolk sacs of wild-type and triple-knockout embryos from samples used in Part A. Several ZIP4 peptides are detected with a prominent band at ~37 kDa and another at ~75 kDa, as reported previously (44). ZIP1 was also detected by Western blotting of these same membrane preparations (middle panel), as described previously (44), and the proteins transferred to the membrane were detected by staining with Coomassie Blue (bottom panel). N.S. indicates non-specific bands. The minor band in lane 5 (TKO ZnD) of the ZIP1 blot is a spurious artifact that was not reproducible.

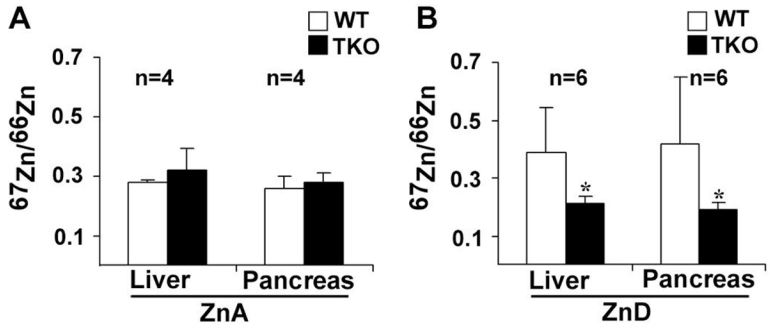

Figure 4. Effects of dietary zinc status on the accumulation/retention of an oral gavage of ^{67}Zn in the liver and pancreas of triple-knockout and wild-type mice

Newly Weaned wild-type (**WT)** and triple-knockout (**TKO**) mice were fed a zinc-adequate (**ZnA**) or zinc-deficient (**ZnD**) diet for 7 days. Food was withheld overnight and the next morning the mice (6 per group) were given an oral gavage containing ^{67}Zn mixed into a slurry of zinc deficient feed (10 μg zinc in 100 μl feed). This was considered a physiologically relevant dose of zinc. **Part A**: The liver and pancreas were harvested 30 minutes after the gavage to detect the initial accumulation/retention of ^{67}Zn in mice fed the zinc-adequate diet. **Part B**: The liver and pancreas were harvested 3 hr after the gavage to monitor the accumulation/ retention of zinc in mice fed the zinc-deficient diet. Tissues were dehydrated, and multiple trace metals were measured by ICP-MS. The ratio of the stable isotopes ^{67}Zn/^{66}Zn in each sample was determined. The natural ratio of these isotopes of zinc is 0.146. Data are expressed as the mean ± S.D. from 6 determinations. *, Signifies a significant difference between ZnD TKO and WT (liver, P = 0.014; pancreas, P = 0.029).

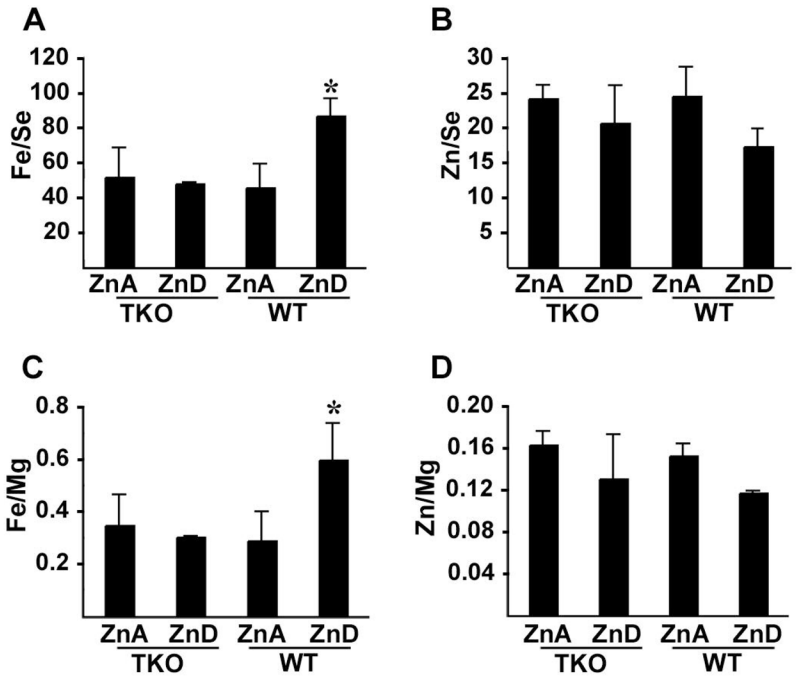

Figure 5. Accumulation of hepatic iron during zinc deficiency in wild-type but not in triple-knockout mice

Pregnant wild-type (**WT**) and triple-knockout (**TKO**) mice were fed a zinc deficient (**ZnD**) or zinc adequate (**ZnA**) diet beginning on day 8 of pregnancy and the maternal liver was harvested on day 14. The liver (6 per group) was dehydrated, and multiple trace metals were measured by ICP-MS. Data for iron (**Parts A & C**) and zinc (**Parts B &** D) are shown and are expressed as the mean of the ratios to either selenium (**A & B**) or magnesium (**C & D**) ± S.D. from 6 determinations. No elements assayed in addition to zinc and iron showed reproducibly significant changes in these samples. *, Signifies a significant difference between ZnD TKO and WT (Fe/Se, $P < 0.001$; Fe/Mg, $P < 0.01$).