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Regulation and function of *Zip4*, the acrodermatitis enteropathica gene

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Abstract

The SLC39A (solute carrier 39A) [ZIP (Zrt-Irt-like protein)] family consists of 14 members which are thought to control zinc uptake into the cytoplasm. Among these, *ZIP4* is known to be particularly important for zinc homoeostasis. Mutations in this gene cause acrodermatitis enteropathica, a rare recessive-lethal human genetic disorder. In the present paper, our studies of the regulation and function of the mouse *Zip4* gene are briefly reviewed. Mouse *Zip4* is expressed at highest levels in tissues involved in absorption of dietary or maternal zinc, and the gene and protein are dynamically regulated by multiple post-transcriptional mechanisms in response to zinc availability. ZIP4 accumulates at the apical surface of enterocytes and endoderm cells when zinc is deficient, because of increased stability of the mRNA and stabilization of the protein. In contrast, when zinc is replenished, the mRNA is destabilized and the protein is internalized and degraded rapidly. The critical importance of ZIP4 in zinc homoeostasis is revealed in mice with targeted deletions of this gene. Homozygous *Zip4*-knockout embryos die during early morphogenesis and heterozygous offspring are significantly underrepresented and display an array of developmental defects, including exencephalia, anophthalmia and severe growth retardation. Mice heterozygous for *Zip4*-knockout are hypersensitive to zinc deficiency, which suggests that humans heterozygous for this gene may also be very sensitive to zinc deficiency.

Keywords

acrodermatitis enteropathica; homoeostasis; solute carrier 39A4 (SLC39A4); teratogenesis; transporter; zinc

Introduction

Zinc deficiency can cause growth retardation, immune system dysfunction, male hypogonadism, skin lesions and neurological disorders in humans [1–3]. Maternal zinc deficiency impairs embryonic, fetal and postnatal development [4–6]. In humans, the rare autosomal recessive disorder AE (acrodermatitis enteropathica) is thought to be caused by the inability to absorb sufficient intestinal zinc [7]. Symptoms of severe nutritional zinc deficiency often develop soon after birth in bottle-fed infants or after weaning in breastfed infants [8]. However, zinc supplementation reverses many of the symptoms of this otherwise lethal disease.

In 2002, the gene responsible for AE was mapped to human chromosomal region 8q24.3 and was shown to be a member of the SLC39A (solute carrier 39A) superfamily [9,10], historically

[1]Owing to exceptional unforeseen circumstances, this speaker was unable to give this presentation at the meeting. This paper is included in the interests of completeness of the session.
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named the ZIP (Zrt-Irt-like protein) family [11]. ZIP family members have eight predicted transmembrane domains and function to transport zinc, and perhaps other metals, into the cytoplasm. To date, 14 members have been identified in mammals [11,12], which group into four subfamilies on the basis of sequence homology [12]. The AE gene is *Zip4* (*Slc39a4*) which is a member of the LIV-1 subfamily (nine members).Multiple amino acid substitutions due to point mutations have been identified in AE patients [9,10,13–15] (Figure 1). Almost all occur at residues that are also conserved in the mouse protein [16]. Other *Zip4* mutations in AE patients involve premature termination codons (Figure 1), insertions, deletions or rearrangements of the gene that must cause a complete loss of function.

Much of what we understand about *Zip4* regulation and function comes from recent studies of mice and transfected cells in culture [16–21]. The mouse and human ZIP4 proteins are well conserved. ZIP4 functions as a zinc transporter in transfected cells, and, although several of the amino acid substitutions found in AE patients appear to abolish its activity by causing retention in the endoplasmic reticulum (e.g. G526R), others apparently diminish its zinc transporter activity (e.g. P200L) [16,17] and must be hypomorphic alleles. The extracellular domain of ZIP4 represents approx. 50% of the protein and is rich in histidine and cysteine residues, suggesting that this domain may bind zinc. Several AE mutations are found in this region.

How ZIP4 transports zinc, the precise topology of this protein within the apical membrane and the structure and potential metal-binding activity of the extracellular domain all remain to be determined.

Regulation of *Zip4* by zinc

Zip4 gene expression is dynamically regulated by several unique and poorly understood post-transcriptional mechanisms in response to zinc availability. *Zip4* gene expression is most active in mouse tissues involved in nutrient uptake (e.g. intestine and embryonic visceral yolk sac). The abundance of *Zip4* mRNA and the cellular localization and turnover of this protein are regulated by zinc availability in these tissues [16,18–20,22]. RNA and protein synthesis inhibitor studies and run-on transcription assays revealed that zinc deficiency causes the stabilization of *Zip4* mRNA, but has little effect on transcription of this gene [22]. ZIP4 accumulates at the apical surfaces of enterocytes in the intestine and visceral endoderm cells in the embryonic visceral yolk sac when dietary zinc is deficient [16,19]. However, zinc repletion destabilizes this mRNA while causing the rapid endocytosis and degradation of ZIP4 *in vivo* [22] and *in vitro* [18,20]. The mechanisms underlying this control are not well understood. A recent study of human ZIP4 suggests that a histidine-rich region within the large intracellular loop between putative transmembrane domains III and IV plays a role in regulating endocytosis and ubiquitination of ZIP4 in response to zinc [20].Western blot analyses of ZIP4 in the intestine and visceral yolk sac during zinc deficiency revealed that the major form of ZIP4 under these conditions is a 37–40 kDa peptide [16,19,22]. This suggests that ZIP4 undergoes proteolytic processing in these cells during zinc deficiency. Studies of that phenomenon are in progress.

ZIP4 function

The dynamic post-transcriptional control of ZIP4 in response to zinc is consistent with its important role in zinc homoeostasis. To examine the functions of ZIP4 directly,we created mice in which the *Zip4* gene was mutated, leading to a complete loss of function [21]. Crossing *Zip4*$^{+/-}$ mice failed to yield homozygous offspring, and heterozygous offspring were significantly underrepresented in the population. Homozygous implantation sites were represented in the expected (25%) frequency at day 10 of gestation, but these embryos had not progressed past the egg cylinder stage. Thus, in mice, *Zip4* is an essential gene for development

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of the early embryo. The early post-implantation death of homozygous *Zip4*-knockout mouse embryos was unexpected since AE patients generally present symptoms after birth and often benefit greatly from zinc therapy [23].Analyses of the patterns of expression of *Zip4* in the early mouse embryo suggest that the expression of this gene is restricted to the visceral endoderm cells of the visceral yolk sac. The visceral endoderm cells are the third cell type to differentiate from the inner cell mass, and, from day 7.5 of pregnancy to late in gestation, they surround the developing mouse conceptus. In humans, the visceral yolk sac is more of a vestigial organ, which may explain in part the difference in phenotypes between these species.

The embryonic lethality caused by the complete loss of ZIP4 function in mice could not be rescued by providing excess zinc to the mother, suggesting the lack of an adequate backup system for zinc uptake through the mother and into the embryonic environment at this critical stage of development. In addition to *Zip4*, the copper transporter *Ctr1*, the iron transporter ferroportin and the zinc efflux transporter *ZnT1* are also essential genes at this stage of development in mice (reviewed in [24]). The mouse embryo apparently requires the development of functional systems to maintain homoeostasis of essential metals at the egg cylinder stage. Interestingly, in zebrafish, a member of the ZIP superfamily (*zLIV-1*) is essential during early morphogenesis [25] and the *fear-of-intimacy* gene in *Drosophila* encodes a zinc transporter that is essential during early development of the fly [26]. *Zip4* is the first example of a mammalian *Zip* gene shown to have an essential function.

Heterozygous *Zip4*-knockout offspring were underrepresented by mid-gestation inmice fed on a diet with adequate zinc. Examination of embryos at day 10 of pregnancy revealed a wide range of abnormalities. Many embryos appeared to be normal, but, compared with their wild-type littermates, the $Zip4^{+/-}$ embryos often varied greatly in size and morphology. Many were smaller and some were severely growth retarded and exhibited abnormal craniofacial development. Heterozygous *Zip4*-knockout embryos with exencephalia were occasionally found at mid-gestation. These morphological abnormalities are reminiscent of previously reported teratology of maternal zinc deficiency [27,28], and were never seen in wild-type embryos within these litters.

After birth, a few heterozygous *Zip4*-knockout offspring died before weaning, and approx. 22% of those that survived were morphologically abnormal. Overall, nearly 40% of the heterozygous post-implantation embryos had developed abnormally. Examples of abnormal heterozygous *Zip4*-knockout mice are shown in Figure 2. High-field MRI (magnetic resonance imaging) revealed abnormal development of the eyes and brain. These heterozygous *Zip4*-knockout mice had no eyes or only one eye and were hydrocephalic. In addition, abnormal thickening of the ventricular septum and wall of the left ventricle and skeletal abnormalities were present in some of these affected $Zip4^{+/-}$ mice. These abnormalities were never seen among hundreds of wild-type offspring examined.

The effects of *Zip4* haploinsufficiency were largely ameliorated by feeding pregnant mice and their offspring excess zinc in the drinking water. At weaning, wild-type and heterozygous *Zip4*-knockout pups were present at the expected Mendelian frequency, and only one heterozygous pup (3%) displayed an overtly abnormal eye phenotype. In contrast, abnormal development of $Zip4^{+/-}$ embryos was exacerbated when $Zip4^{+/-}$ pregnant females were fed on a zinc-deficient diet (Figure 3). At mid-gestation, approx. 50% of the $Zip4^{+/-}$ embryos were lost, and approximately two-thirds of the remaining $Zip4^{+/-}$ embryos were morphologically abnormal. The majority (61%) of those were classified as severely abnormal. In contrast, approx. 71% of the wild-type embryos in these same litters appeared morphologically normal and only a small percentage (4.7%) were severely abnormal. These studies revealed that maternal zinc status modulates the effects of embryonic *Zip4* haploinsufficiency on

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development. Heterozygous *Zip4*-knockout mouse embryos are clearly hypersensitive to zinc deficiency relative to their wild-type littermates.

We also examined the contribution of maternal *Zip4* gene expression to the effects of embryonic *Zip4* heterozygosity on development. When wild-type females were mated with *Zip4*$^{+/-}$ males, heterozygous *Zip4*-knockout embryos were not underrepresented at mid-gestation (Figure 3) or at weaning, if dietary zinc was adequate. However, 18% (eight pups) of those heterozygous weaned pups display the same types of eye and brain abnormalities as shown in Figure 2. Maternal zinc deficiency also more profoundly affected the development of *Zip4*$^{+/-}$ embryos in wild-type females than it did wild-type embryos in the same litter. At mid-gestation, approximately two-thirds of the heterozygous *Zip4*- knockout embryos were morphologically abnormal, and 41% were severely abnormal in dams fed on a zinc-deficient diet. In contrast, almost all (94%) of the wild-type embryos were normal and none was severely abnormal.

Maternal *Zip4* haploinsufficiency clearly sensitized the developing embryo (wild-type and *Zip4*$^{+/-}$) to the effects of zinc deficiency. Furthermore, the combination of embryonic and maternal *Zip4* heterozygosity dramatically increased the chances of abnormal embryonic development and diminished the chances of survival of the embryo to parturition. These effects were exacerbated by dietary zinc deficiency during pregnancy and were largely ameliorated by providing excess zinc. Some of the abnormal phenotypes noted at weaning in heterozygous *Zip4*-knockout offspring reflect the diminished function of the embryonic *Zip4* gene, since they were noted in mice delivered from wild-type females.

Neurogenesis was particularly affected by embryonic *Zip4* haploinsufficiency. Exencephalia, hydrocephalus, anophthalmia and anopia each were noted even under normal dietary zinc conditions. These types of abnormalities have been associated with zinc deficiency in the rat [4,6,29,30]. Zinc is essential for brain development and function in monkeys as well [31].Diminished DNA and RNA synthesis in neurons during zinc deficiency may cause abnormal neurogenesis [32,33]. The retina is also rich in zinc, concentrated in photoreceptors and retinal pigment epithelial cells [34],where it may function as a neuromodulator [35,36]. Depletion of intracellular zinc induces caspase-dependent apoptosis in retinal cells [37].

There are no published studies indicating that *ZIP4* haploinsufficiency in humans is associated with effects on embryonic development, but our studies suggest that dietary zinc availability as well as gene dosage may modulate the severity and type of symptoms associated with AE mutations. Given the rare occurrence of this disease gene (~1 in 125 000 adults are heterozygous), the pleiotropic effects of zinc deficiency and the recent identification of this gene as the cause of AE, it is not surprising that the affects of *Zip4* heterozygosity in humans has not been examined. It should be noted, however, that gene dosage for *Atp7a*, the Menkes disease gene, controls the sensitivity of the developing zebrafish notochord to copper deficiency [38], and a transient neonatal zinc deficiency during breastfeeding has been associated with heterozygosity in the maternal *ZnT2* (*SLC30A2*) gene [39]. Our studies suggest that zinc nutritional status, interacting with mutations in human *ZIP4* may be associated with a wide range of developmental abnormalities.

Abbreviations used

AE, acrodermatitis enteropathica; MRI, magnetic resonance imaging; Slc39a, solute carrier 39A; ZIP, Zrt-Irt-like protein.

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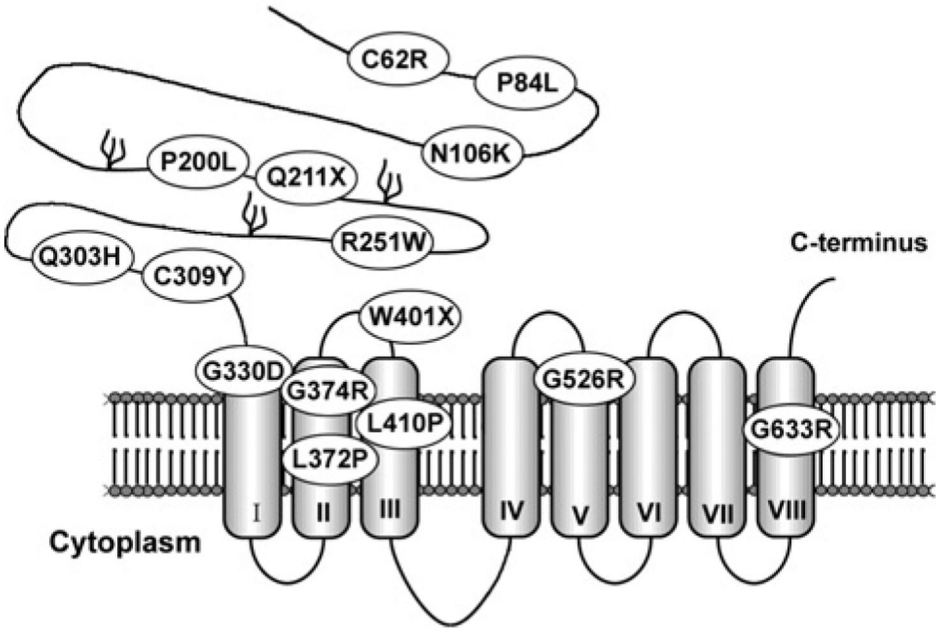

Figure 1. Mutations in *ZIP4 (SLC39A4)* cause acrodermatitis enteropathica

A predicted eight-transmembrane domain model of human ZIP4, indicating amino acid substitutions (one letter code) and nonsense mutations (X) identified in patients with AE. Three potential glycosylation sites are indicated in the extracellular N-terminal half of the protein.

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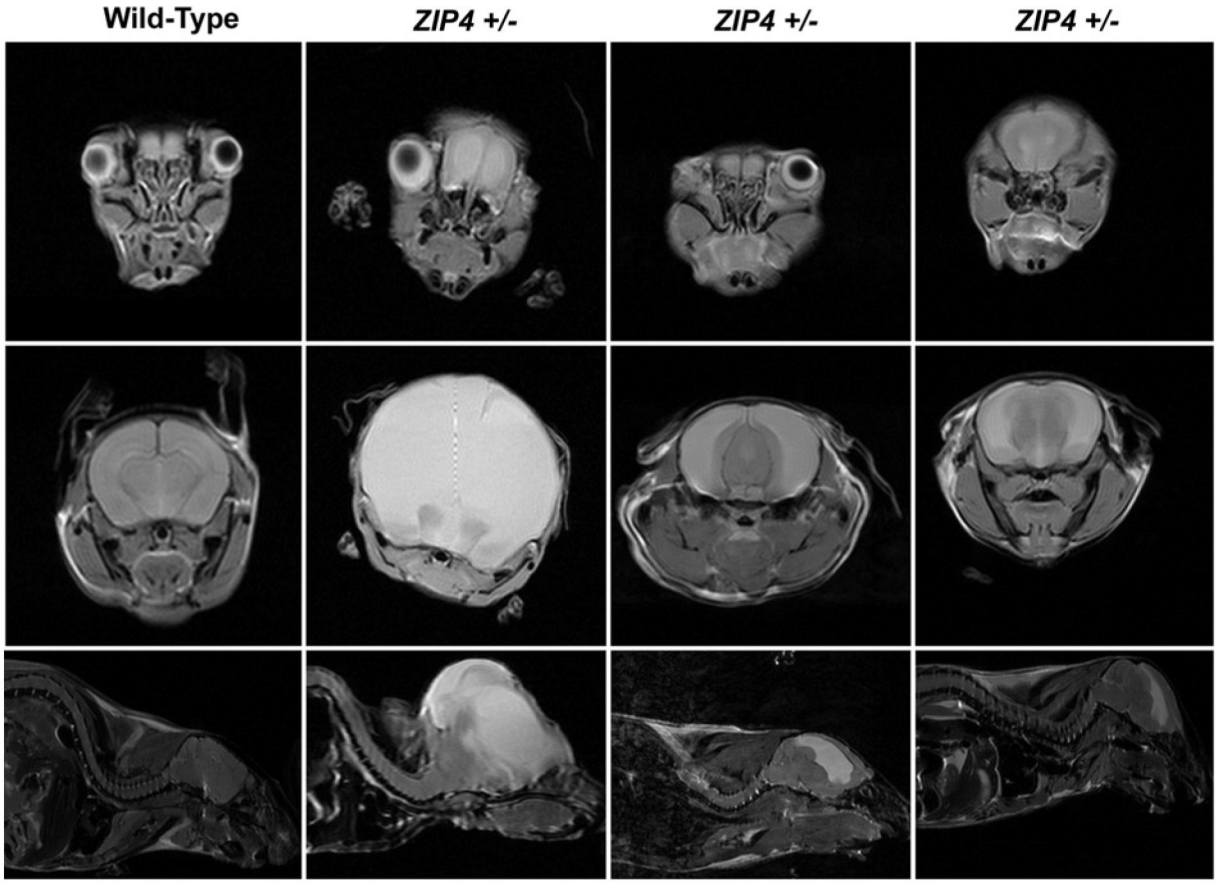

Figure 2. *Zip4* haploinsufficiency is associated with abnormal development in mice

Wild-type mice and *Zip4*$^{+/-}$-knockout mice (*Zip4*$^{+/-}$) that were obviously abnormal at weaning were examined using high-field MRI. Top row: axial sections through the face at the plane of the eyes. Second row: axial sections through the midbrain region. Third row: sagittal sections near the midline region. White areas in the brain indicate water accumulation. Modified from Dufner-Beattie, J., Weaver, B.P., Geiser, J., Bilgen, M., Larson, M., Xu, W. and Andrews, G.K. The mouse acrodermatitis gene *Slc39a4* (*ZIP4*) is essential for development and heterozygosity causes hypersensitivity to zinc deficiency. Hum. Mol. Genet., 2007, vol. **16** (12), pp. 1391–1399 by permission of Oxford University Press.

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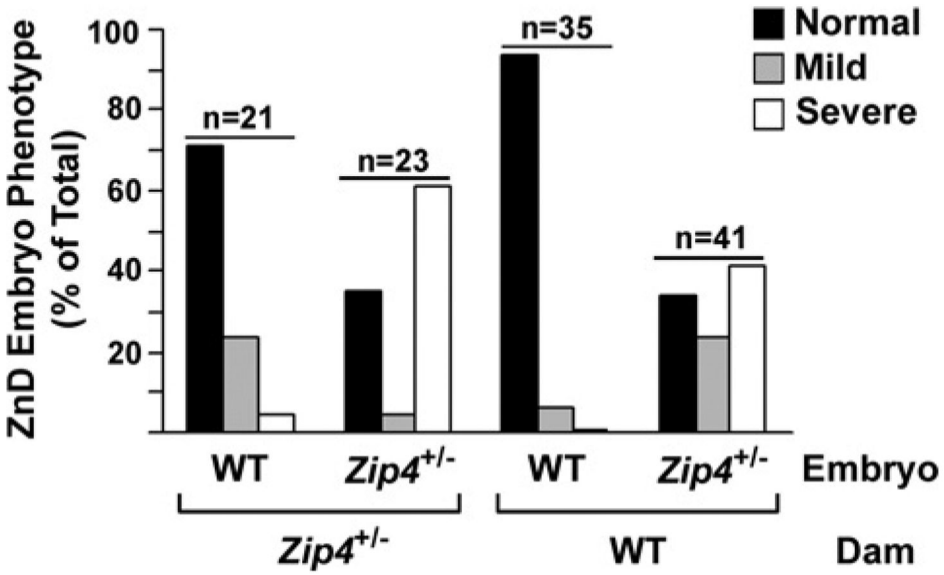

Figure 3. Heterozygous *Zip4*-knockout mice are hypersensitive to zinc deficiency

Zip4$^{+/-}$-knockout females (*Zip4*$^{+/-}$) or wild-type (WT) females were crossed with *Zip4*$^{+/-}$-knockout males. Pregnant females were fed on a zinc-deficient diet during pregnancy, and embryos were examined at mid-gestation. Severe: embryos were dramatically growth retarded and displayed multiple morphological abnormalities. Mild: embryos exhibited delayed limb development and retarded growth. Results are expressed as the percentage of embryos in each genotype of the total number examined (*n*). Reproduced from Dufner-Beattie, J., Weaver, B.P., Geiser, J., Bilgen, M., Larson, M., Xu, W. and Andrews, G.K. The mouse acrodermatitis gene *Slc39a4* (*ZIP4*) is essential for development and heterozygosity causes hypersensitivity to zinc deficiency. Hum. Mol. Genet., 2007, vol. **16**(12), pp. 1391–1399 by permission of Oxford University Press.