

THE ZnT4 MUTATION IN *LETHAL MILK* MICE AFFECTS INTESTINAL ZINC HOMEOSTASIS THROUGH THE EXPRESSION OF OTHER Zn TRANSPORTERS

Chiara Murgia, Isabella Vespignani, Rita Rami and Giuditta Perozzi

INRAN, National Research Institute on Food and Nutrition, via Ardeatina 546, 00178 Roma, Italy

[Received February 28, 2006; Accepted March 30, 2006]

ABSTRACT: *The lethal milk mouse syndrome is caused by a point mutation in the zinc transporter gene ZnT4 resulting in defective zinc secretion in the milk of homozygous mutant dams. Pups of any genotype fed solely on lm milk die within the first two weeks of neonatal life, displaying zinc deficiency symptoms. Homozygous mutant pups survive when foster nursed by wild type dams and show signs of mild zinc deficiency in adulthood. To further investigate the role of ZnT4 in zinc secretion in the intestinal epithelium, we have studied the expression by real time quantitative PCR of mutant ZnT4 and of other zinc transporters of the Zip and ZnT families, in the jejunum of homozygous lm mice and of the isogenic wild type strain C57BL/6J. We report in this paper that expression of the mutant ZnT4 mRNA, carrying a premature translational termination codon (ZnT4/lm), is almost absent in tissues from lm mice, probably as a result of degradation by the Nonsense Mediated mRNA Decay (NMD) Pathway. In the jejunum of mutant mice, we also observed decreased expression of the uptake zinc transporter Zip4, paralleled by increased levels of both metallothionein genes MTI and MTII. Zinc supplementation of lm mice in the drinking water did not result in further decrease of Zip4 expression, but led to full induction of MT mRNAs. These results lead us to conclude that, although in the enterocytes of lm mice the absence of the zinc secretion activity mediated by ZnT4 results in increased intracellular zinc concentration, other zinc efflux activities are able to maintain the level of zinc ions below the threshold necessary for full induction of metallothioneins.*

KEY WORDS: Copper, Copper transporter, *lm* syndrome, Metallothionein, zinc deficiency, ZnT4, Zinc Transporter.

INTRODUCTION

The lethal milk (*lm*) mouse allele contains a recessive point mutation that occurred spontaneously in the inbred strain C57BL/6J (Green & Sweet, 1973). The phenotype of homozygous *lm/lm* mice is rather complex, its most prominent features occurring in lactating dams: irrespective of their genotype, pups raised on *lm* milk die before weaning, showing typical signs of systemic zinc deficiency. When suckled on wild type foster mothers, pups develop normally but, if homozygous for the *lm* mutation, later develop signs of mild zinc deficiency.

Zinc is an essential micronutrient that plays a pivotal role in several biochemical processes, either as catalytic components of enzymes or as structural elements in metalloproteins (Frausto da Silva & Williams, 1991). Adequate zinc intakes are essential for cellular metabolism; daily needs for this ion are especially high during embryonic and perinatal development. In the adult, zinc is essential for proper functioning of the immune system (Fraker et al., 2000) as well as of the nervous system (Frederickson et al., 2000) and of the airway epithelium (Murgia et al., 2006). The clinical picture of severe zinc deficiency involves skin lesions, growth retardation and cognitive defects (Van Wouwe, 1989). Excessive intracellular accumulation of zinc ions also leads to toxicity. It is essential therefore that zinc homeostasis be constantly maintained in cells and tissues. This is achieved at the cellular level through the coordinate regulation of zinc intake, intracellular compartmentalization and efflux. These steps are mediated by specific proteins belonging to two distinct gene families, SLC30 and SLC39, which differ in their tissue-specificity and intracellular localization. The constantly increasing number of characterized Zn transporters has been extensively reviewed elsewhere (Cousins & McMahon, 2000; Palmiter & Huang, 2004; Eide, 2004; Kambe et al., 2004; Murgia et al., 2006). Members of both families are transmembrane proteins containing an evolutionarily conserved zinc-binding motif, and distinct numbers of transmembrane segments (6 in SLC30 and 8 in SLC39).

Transporters of the SLC30 family (ZnT1-10) are mostly vesicular and responsible for zinc efflux and intracellular sequestration, while proteins belonging to the SLC39 family (ZIP1-14) are involved in zinc uptake and localize mainly to the plasma membrane.

The molecular basis of the *lm* mutation was mapped in the SLC30A4 gene on mouse chromosome 2, encoding the ZnT4 transporter (Huang & Gitschier, 1997), confirming earlier studies that pointed to defective transport of zinc into the milk of lactating *lm* dams (Ackland & Mercer, 1992; Lee et al., 1992). ZnT4 was isolated with two independent approaches, either as a gene upregulated during intestinal epithelial differentiation (Barilà et al., 1994) or as a gene mapping near the *pallid* locus (Huang & Gitschier, 1997). It displays the characteristic features of SLC30 transporters, with six transmembrane segments, a histidine rich cytosolic loop that binds metal ions and cytoplasmic N- and C-termini (Huang & Gitschier, 1997; Murgia et al., 1999). The point mutation in the *lm* allele is a C>T transition resulting in replacement of the arginine residue at position 297 with a translational stop codon (Huang & Gitschier, 1997). This mutation would be predicted to lead to the synthesis of a truncated protein lacking the sixth transmembrane domain and the entire hydrophilic C-terminus.

Full clarification of the specific role that each of the Zn transporters plays in zinc homeostasis has not yet been achieved, partly because functional assays are not easily available. This is especially true for the ZnT transporters (SLC30), most of which localize in intracellular vesicles (Palmiter et al., 1996a; Palmiter et al., 1996b; Murgia et al., 1999; Kambe et al., 2002; Huang et al., 2002; Michalczyk et al., 2003; Kirschke & Huang, 2003; Chimienti et al., 2004). Zinc absorption in the gut is mediated by the uptake activities of the ZIP4 and ZIP5 transporters at the apical and the basolateral side of enterocytes, respectively (Dufner-Beattie et al., 2004; Wang et al., 2004; Wang et al., 2002). In contrast, ZnT1 was proposed to mediate zinc efflux to the bloodstream in intestinal epithelial cells (McMahon & Cousins, 1998), although it is not yet clear whether other ZnT proteins might contribute to Zn secretion in gut cells. *Lethal milk* mice therefore appear a good model to investigate the molecular mechanisms involved in intestinal zinc homeostasis.

Several ZnT transporters were shown to be expressed in the gut (ZnT1, ZnT2, ZnT4, ZnT5, ZnT6, ZnT7) (Liuzzi et al., 2004). Since a mutation in ZnT4 is sufficient to cause a defect in Zn secretion in the mammary gland without apparently affecting intestinal zinc absorption, we studied the expression of the mutant protein and of the other intestinal zinc transporters in the ZnT4 mutant background represented by *lm* mice. We demonstrate that ZnT4 mRNA is almost absent in the intestine of *lm* mice and that altered expression of ZnT2 and of the metallothioneins is likely to contribute to the zinc deficiency syndrome displayed by surviving *lm* mice over the age of eight months.

METHODS

Animals

Two males and 2 females each of the homozygous *lm/lm* mice strain C57BL/6J-ZnT4 <lm> and of the isogenic wild type inbred strain C57BL/6J were purchased from The Jackson Laboratory, Bar Harbor, ME. 2 males and 2 females of the CD1 strain, a commonly used albino, outbred mouse strain originating from Switzerland, were purchased from Charles River Italia, Milan, Italy. All animals were housed in individual cages and fed ad libitum with a commercial pellet diet for rodents (4 RF 21, purchased from Mucedola, Settimo Milanese, Italy). Newborn pups from homozygous *lm* crosses were transferred to wild type dams for foster nursing. When required by the experimental setup, mice were supplemented for 6 months with 800 mg/l ZnCl₂ in the drinking water, acidified to pH 3.0 with HCl to prevent Zn precipitation (Erway & Grider, 1984).

Tissue preparation and RNA extraction

The portion of small intestine between the pylorus and the ileocecal valve was dissected from mice anesthetized by intraperitoneal injection of 20 mg/100 g body wt of Farmotal (Farmitalia-Carlo Erba, Milan, Italy) and further subdivided into three segments representing duodenum (4 cm from the pylorus), jejunum (5 cm in the middle of the small intestine) and ileum (4 cm from the ileocecal valve). The dissected tissue was quickly rinsed in cold 1X PBS and immediately frozen in liquid nitrogen. Frozen tissues were grinded under liquid nitrogen and RNA was extracted from a pool of tissues dissected from 10 animals with TRIzol reagent (Invitrogen) according to the protocol by the manufacturer.

Gene expression analysis

Complementary DNA was prepared from 1 µg of total RNA using M-MLV reverse transcriptase (Invitrogen, San Giuliano Milanese, Italy) and a combination of random hexamers and oligo d(T) as primers, according to the manufacturer's protocol. Sequences of zinc transporter genes were obtained from GenBank, and primers were designed using the web-based software Primer3 (Rozen & Skaletsky, 2000). Primer sequences are given in Table 1. 1/100 volume of the RT reaction was amplified using SYBR Green JumpStart Taq ReadyMix (Sigma Italia, Milan, Italy) on ABI PRISM 7900 HT (Applied Biosystems, Foster City, CA). A sample lacking Reverse Transcriptase was always included to test genomic DNA contamination. The cycle thresholds (Ct) were obtained for test genes and for the housekeeping reference gene Hypoxanthine guanine Phospho-Ribosyl Transferase (HPRT), their differences calculated (ΔCt). ΔCt values were transformed into absolute values according to the 2^{-ΔCt} calculation and expressed as relative to HPRT RNA levels as described by Applied Biosystems *User Bulletin #2ABI*. Primer pairs for each gene amplified with equal efficiency, as verified using serial dilutions of cDNAs.

Table 1. Oligonucleotide primers used for real-time Q-PCR analysis.

Name (GenBank Accession)	Forward primer	Reverse primer	PL	Gen. Fg.
mZnT1 (NM_009579)	TGGATGTACAAGTAAATGGGAATCT	GTCTTCAGTACAACCCTTCCAGTTA	184	184
mZnT2 (NM_011773)	CAGAAGGATTCTGGAAGTCACC	CGGGAAGACACCCAGAGG	211	1897
mZnT4 (NM_011774)	TAGGTGGATACATGGCAAATAGC	AGTTCATATGGATGGTTCTCTGC	243	5083
mZnT5 (NM_022885)	GCCTGTCAAGTTCTACTTCTGAGAC	TCTCGGTATGATTAATCCCTCAA	103	2646
mZnT6 (NM_144798)	TTAGAAGTCCTGGCTGTATTTGC	GAATAGAAAGCATCGTGAACAGG	172	
mZnT7 (NM_023214)	CGCTTTCTCTTATGGGTATGTTAGA	TCTCTCGACTCCTTCTGAGAAAATA	105	2213
mZIP1 (NM_013901)	CAGTGGAGACCTATTTGCTCTTTTA	CTACTTTTCTCTCACTCCAGACCAG	92	92
mZIP2 (XM_359330)	CATATGACTGCTGAAGCTCTGG	CGAGAAGAATTTCCCTTACTTCC	91	327
mZIP4 (NM_028064)	ACTTTGTGGACTTTGTGTTTCAGG	GAGTATGGAGCTCAGAGTCTTGG	193	326
mZIP5 (NM_028051) (NM_028092)	AGGACCTAGTGAGCAATCAGAGG	TTCTCCAAGATCCCTTTTGTTC	155	518
mHPRT* (NM_013556.1)	CTCATGGACTGATTATGGACAGG	TTAATGTAATCCAGCAGGTCAGC	135	3025

*HPRT = hypoxanthine phosphoribosyltransferase 1

cDNA constructs, transfection, cell culture and immunostaining

The ZnT4/*lm* cDNA construct used in this study was obtained by amplifying the ZnT4 cDNA fragment between the ATG codon and the premature stop codon present in lethal milk mice (codon 297), using the following primers: TTCCGGATCCATGGCCGGCCCCGGCGCGTGGAAAG (sense) TATTGCGGCCGCTCGTATGATGTATGCAGCTATAAT (antisense).

Restriction sites (underlined) were included in the primer sequences for subsequent cloning into the previously described vector pcDNA3-myc (Murgia et al., 1999). To obtain a ZnT4-GFP fusion protein the entire open reading frame of ZnT4 was excised from pcDNA3-ZnT4-myc and ligated in-frame with GFP into the vector pEGFP-N1 (Clontech, Mountain View, CA). The ZnT2 coding region was PCR amplified from rat intestinal cDNA with the following primers: CCAGAGGGAGAAGAGACTAATGAGC (sense) CTCGAATTCCTCGTCTGCTATGTCCC (antisense). The resulting fragment was cloned in frame with red fluorescent protein into the pDsRed1-N1 vector (Clontech, Mountain View, CA). MDCK cells were stably transfected with ZnT4 cDNA as described in (Ranaldi et al., 2002). For subcellular localization, cells were seeded on glass coverslips in 24-well tissue culture plates and transfected as previously described (Murgia et al., 1999). 24h after transfection, cells were fixed with 2% paraformaldehyde in 1X PBS for 30 min at RT and treated by conventional immunofluorescence techniques. Primary antibodies used include monoclonal anti-myc clone 9E10 (Sigma) and polyclonal anti-ZnT4 (Ranaldi et al., 2002). Secondary antibodies conjugated to FITC or tetramethylrhodamine isothiocyanate (TRITC) were affinity purified and species specific (Jackson ImmunoResearch Laboratories, West Grove, PA). Total protein extracts and western

blotting were performed as previously described (Ranaldi et al., 2002).

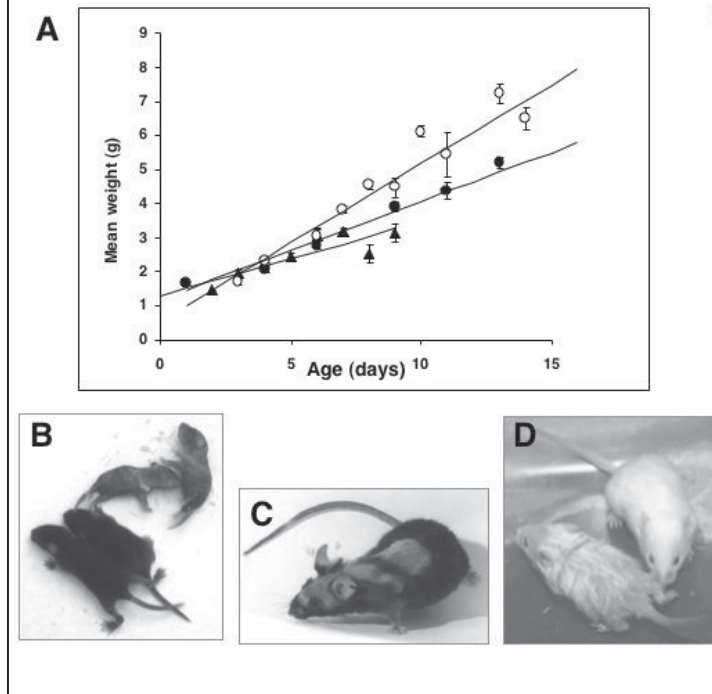
RESULTS

***lm* mice growth and phenotype**

Colonies of *lm* mice and of the wild type isogenic strain C57BL/6J were raised in our laboratory from two breeding pairs for each strain, purchased from The Jackson Laboratory (Bar Harbor, ME). Growth of the resulting offspring was monitored at regular intervals during lactation. As shown in Figure 1A, when the offspring of *lm/lm* x *lm/lm* crosses and that of isogenic wild type crosses were fed solely upon the milk of their natural mothers, growth was slower for the *lm* pups, which died by the tenth day of life displaying typical signs of zinc deficiency (Fig. 1B). However, it is interesting to note that the *lm* pups nursed by foster mothers of genetically unrelated background (the outbred strain CD1) grew much faster than pups of the isogenic wild type C57BL/6J strain nursed by their own mothers. The only difference between *lm* milk and that of the isogenic wild type was reported to reside in lower zinc content (Piletz & Ganschow, 1978), therefore such different growth rates are probably due to other nutrient components in CD1 milk. As expected, the surviving *lm* mice developed signs of mild zinc deficiency by the age of 8-10 months, including alopecia (Fig. 1C) and premature infertility. Since nursing on *lm* milk during the first 3 days of neonatal life seems to be crucial for the severity of the syndrome (Erway & Grider, 1984), newborn mice of the CD1 strain were fed for 4 days on their mother's milk, then half of the litter was given to a homozygous *lm* dam for two weeks. As shown in Figure 1D, although the CD1 pups fed on *lm* milk displayed hair loss as clear symptom of zinc deficiency, they survived until weaning. This experiment underlines the crucial role of zinc in the first few days of neonatal life.

Figure 1. Phenotypic characterization of the *lm* mice colony

(A) Growth curve of *lm* pups nursed by *lm/lm* dams (solid triangles), isogenic *wt/wt* C57BL/6J dams (open circles) or by dams of the outbred strain CD1 (solid circles). (B) 10 day old *lm/lm* littermates nursed from birth by either a C57BL/6J dam (bottom) or by an *lm/lm* dam (top). (C) male *lm* homozygous mouse at the age of 14 months showing widespread alopecia. (D) 17 day old CD1 littermates nursed either by their own mother (right) or for the first 4 days by their own mother and then switched to an *lm/lm* dam (left).



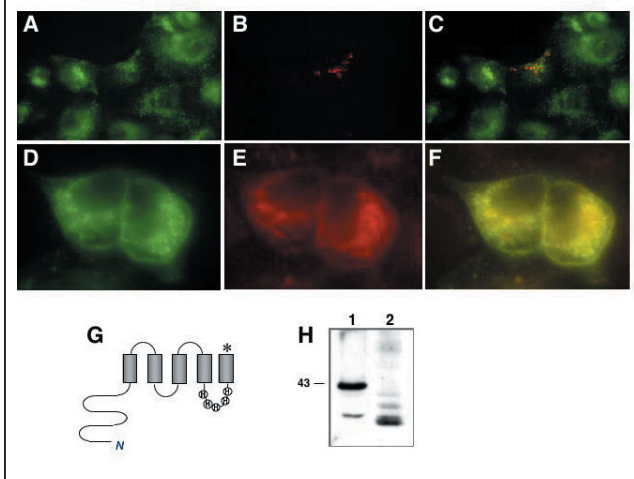
Intracellular localization of ZnT2 and ZnT4-*lm* constructs

ZnT4 and ZnT2 are very closely related among the SLC30 family members (Palmiter & Huang, 2004), although their expression profile in the different tissues overlaps only partially. Both proteins are present in intestinal epithelial cells and localize to a vesicular compartment (Palmiter et al., 1996a; Palmiter et al., 1996b; Murgia et al., 1999), therefore we wanted to investigate whether they might co-localize in the same vesicles and possibly exert overlapping functions. The ZnT2 ORF was cloned by RT-PCR from mouse intestinal RNA and fused in-frame at the C-terminus with the red fluorescent protein from the sea anemone *Discosoma*, as described in the Methods. Figure 2A shows the typical vesicular localization of ZnT4 in stably transfected epithelial MDCK cells, revealed by indirect immunofluorescence with an anti-myc primary antibody (Ranaldi et al., 2002). The ZnT2-DsRed fusion protein, transiently transfected in these cells, displayed a similar vesicular pattern revealed by red epifluorescence (Fig. 2B), but no co-localization of the two transporters was detected by merging the two images (Fig. 2C). We then focused on the intracellular localization of the mutant ZnT4-*lm* protein in epithelial cells by transfecting a construct containing the ZnT4 ORF truncated at codon 297 and followed by a myc-tag at the carboxyl-terminus (ZnT4/*lm*-myc). Figure 2H shows that the truncated ZnT4/*lm* protein is efficiently expressed from this

construct when compared to the complete ZnT protein, also cloned in the pcDNA3-myc vector. To compare the intracellular localization of the two proteins we co-transfected the ZnT4/*lm*-myc construct and a

Figure 2. Expression of ZnT4 and ZnT2 by transfection in epithelial cells

(A) Indirect immunofluorescence detection of ZnT4 with anti-myc primary antibodies and FITC-labelled secondary antibodies in epithelial MDCK cells stably transfected with ZnT4-myc; (B) Epifluorescence of ZnT2-DsRed fusion protein transiently transfected in the MDCK cells shown in panel (A); (C) merged image. Parental MDCK cells co-transfected with a ZnT4-GFP construct (D) and with a ZnT4/*lm*-myc construct (E); merged image (F). Expression of the two proteins is detected by GFP epifluorescence (ZnT4) and by indirect immunofluorescence with anti-myc primary antibodies and TRITC-labelled secondary antibodies (ZnT4/*lm*). (G) Schematic representation of the truncated ZnT4/*lm* protein expressed by the transfected construct. The asterisk indicates the 10 amino acid myc tag at the carboxy-terminus. (H) Western blot analysis of ZnT4-myc (lane 1) and ZnT4/*lm*-myc (lane 2) in total protein extracts from transfected cells, stained with anti-myc primary antibodies.



ZnT4-GFP fusion construct in epithelial MDCK cells. The results in Figure 2 show complete overlap between the GFP epifluorescence of ZnT4 (panel D) and the indirect immunofluorescence with anti-myc antibodies of ZnT4/*lm* (panel E).

Comparative expression of ZnT2 and ZnT4 mRNAs in the small intestine of lethal milk mice

Expression of the ZnT4 and ZnT2 mRNAs was compared in the small intestine of adult (12-18 months old) *lm* mice and of their isogenic wild type controls by northern hybridization. Total RNA was extracted from the duodenum, jejunum and ileum of 10 mice for each genotype, blotted and hybridized to radioactively labelled cDNA probes. The results in figure 3A show that ZnT2 is expressed at similar levels along the three segments of small intestine in both mutant and wild type mice, while the ZnT4 transcript is absent in all the samples of *lm* mouse intestine. Lack of ZnT4 expression was observed also in other *lm* mouse tissues

(skin, kidney, brain; data not shown). As this result was quite unexpected, we sought confirmation by designing a real-time Q-PCR experiment, including also RNA samples extracted from the jejunum of mice supplemented with zinc in their drinking water. This treatment was previously reported to ameliorate the symptoms of zinc deficiency in aging *lm* mice (Grider & Erway, 1986) and regulates the expression of several zinc transporters, including ZnT2 and ZnT4 (Liuzzi et al., 2004). As further control we included RNA extracted from the jejunum of the genetically unrelated wild type CD1 mice of the same age. The results in figure 3B show very low levels of ZnT4 mRNA in the jejunum of *lm* mice when compared to both isogenic controls and CD1 mice (8-9 folds decrease). Such low levels are compatible with the results of the northern hybridization in panel A, as real-time Q-PCR is a much more sensitive technique. Zn supplementation did not affect ZnT4 mRNA levels in either strain of mice. In the case of ZnT2, on the contrary, Zn supplementation led to increased mRNA levels in both strains.

In vivo expression analysis of other zinc transporter and metallothionein mRNAs

To elucidate the molecular mechanisms by which enterocytes absorb and distribute Zn in the absence of ZnT4, the expression of several intestinal zinc transporters belonging to both the SLC30 and the SLC39 gene families was also investigated by real-time Q-PCR in the jejunum of unsupplemented and Zn-supplemented mice. As shown in Figure 4, the ZnT transcripts examined do not show any relevant quantitative difference in expression in mouse jejunum when comparing either the *lm* versus the wild type controls, or zinc supplemented versus unsupplemented animals (Fig 4A). As for the ZIP transcripts, they were expressed at very different levels in mice gut, ZIP1 and ZIP2 being barely detectable. However, we observed relevant differences in the expression of ZIP4, which is the main Zn uptake transporter in the intestinal epithelium. Mutations in the ZIP4 coding region were found to be responsible for the genetic zinc deficiency syndrome *Acrodermatitis enteropathica* (AE) (Kury et al., 2002; Wang et al., 2002) and ZIP4 expression was shown to

be up-regulated by zinc deficiency (Dufner-Beattie et al., 2003; Liuzzi et al., 2004). Figure 4B shows that ZIP4 mRNA levels are 6 folds lower in the jejunum of *lm* mutants than in control mice. Zn supplementation led to 2 fold decreased expression of ZIP4 in wild type mice, but did not cause any further decrease of the already low ZIP4 mRNA levels in *lm* mutant mice jejunum. The absence of Zn-dependent regulation of ZIP4 mRNA in *lm* mice could reflect higher intracellular concentrations of zinc ions, so we examined metallothionein expression in the different mice groups. Metallothionein (MT) is the main intracellular scavenger of zinc ions and transcription of the two major isoforms, MT1 and MT2, is strongly induced by zinc and other stimuli (Davis & Cousins, 2000; Coyle et al., 2002). The results of real time Q-PCR analysis of the expression of the MT I and MT II genes in the jejunum of *lm* mutant mice is shown in Figure 5. In the absence of Zn supplementation, both MT mRNAs were more abundantly expressed in *lm* than in wild type jejunum, 3 and 6 folds respectively (Fig 5A and table). Following Zn supplementation, however, induction of MT gene transcription led to comparable transcript levels in both strains (Fig 5B and

Figure 3. Expression of ZnT4 and ZnT2 in mouse intestine

(A) Northern blot hybridization of the indicated radioactively labelled cDNA probes to total RNA (20 µg/lane) extracted from the intestine of C57BL/6J mice (lanes 1,3,5) and homozygous *lm* mice (lanes 2,4,6) at the age of 12 months. Lanes 1 and 2: duodenum, lanes 3 and 4: jejunum, lanes 4 and 5: ileum. Equal loading and transfer of RNA was monitored by hybridization to a GAPDH cDNA probe. (B) Real time Q-PCR expression analysis of ZnT4 and ZnT2 in the jejunum of *lm* mice and of their isogenic C57BL/6J WT controls, with or without Zn supplementation. Unsupplemented CD1 mouse jejunum is included as further control. The RNAs are pools from 10 animals for each strain. Ct values are normalized to those of the housekeeping gene HPRT.

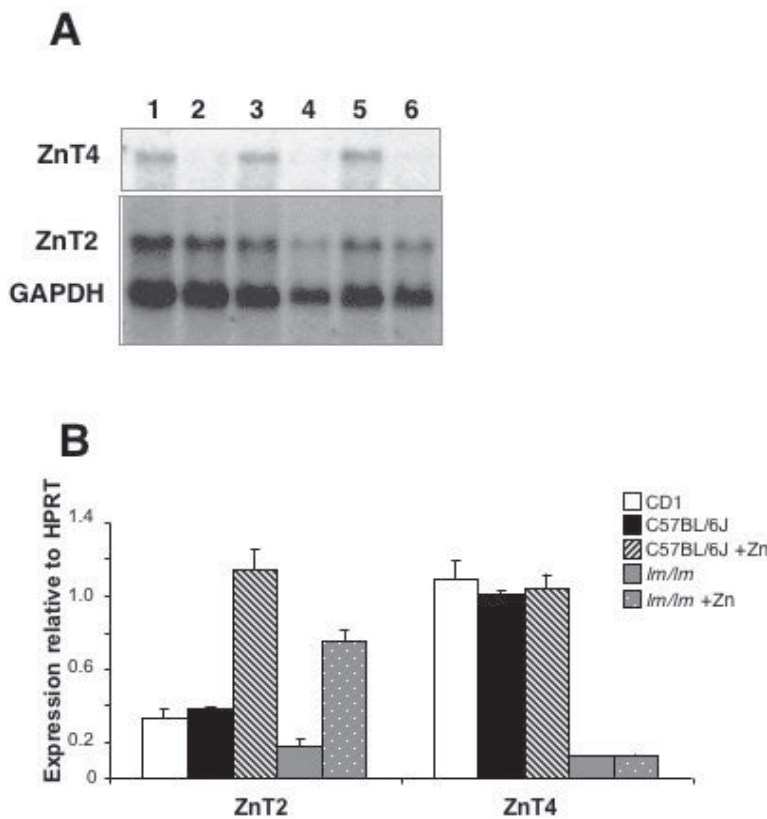


Figure 4. Expression of zinc transporter genes in mouse jejunum

(A) Real time Q-PCR expression analysis of (A) ZnT and (B) ZIP transporters in the jejunum of *lm* mice and of their isogenic C57Bl/6J WT controls, with or without Zn supplementation. Tables at the bottom contain the ratio of absolute values between target genes and HPRT, calculated from Ct values as described in the Methods section.

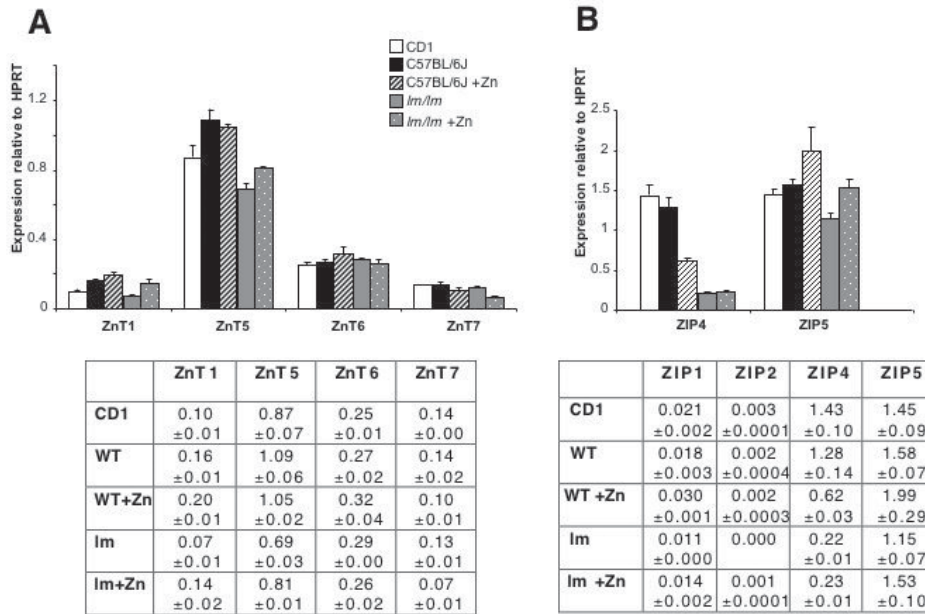


Figure 5. Expression of metallothionein genes in mouse intestine

Real time Q-PCR expression analysis of MT I and MT II genes in mouse jejunum (A) Expression in unsupplemented mice. (B) Expression in unsupplemented and zinc supplemented mice. MT I, white columns; MT II, grey columns. Ratios of the plotted values for both genes are listed in the table.

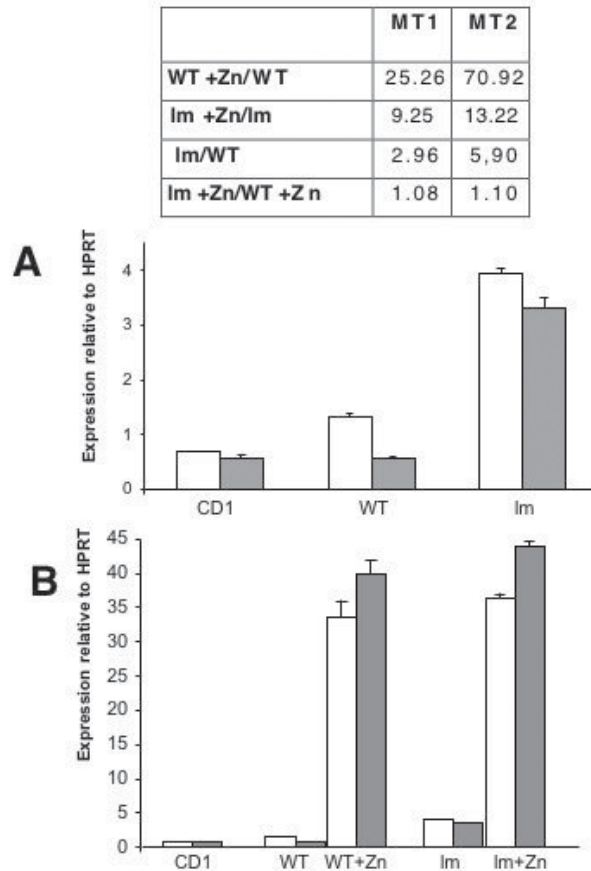


Figure 6. Model for zinc metabolism in intestinal cells of lethal milk mice.

The model was derived from the results of Northern hybridizations and Real Time Q-PCR experiments reported in this paper. Expression of the genes encoding the zinc transporters ZnT2 and ZnT4 and the metallothioneins MT1 and MT2 was different in intestinal cells of *lm* mice (C and D) as compared to wild type isogenic mice (A and B). Expression of the other zinc transporters examined did not display significant variations between the two strains, even following zinc supplementation (B and D).

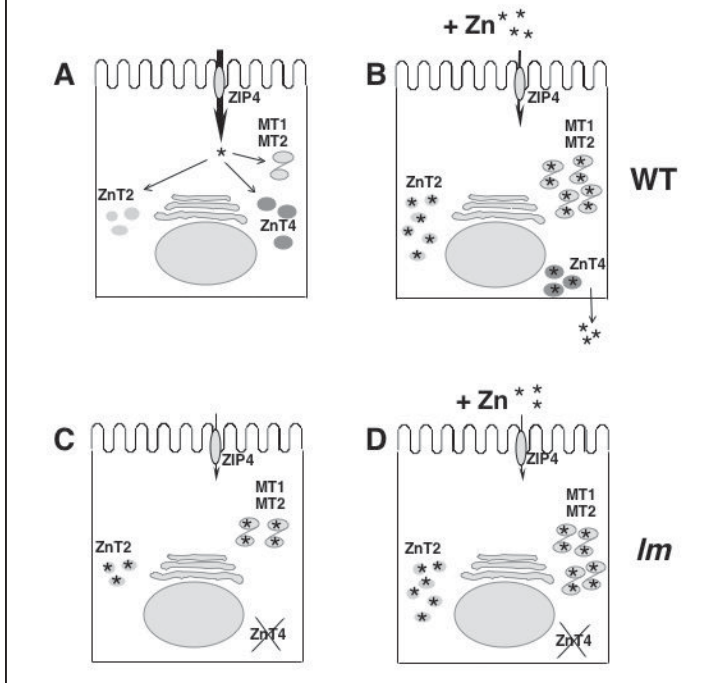


table). These data further indicate that the absence of zinc secretory activity of ZnT4 in the intestinal enterocytes of *lm* mice leads to higher intracellular zinc content.

DISCUSSION

Zinc deficiency in humans can be caused by severe malnutrition or by gastrointestinal and infectious diseases decreasing intestinal absorption. Two genetic diseases characterized by severe zinc deficiency in newborns have been described in mammals: *Acrodermatitis enteropathica* (AE) and *lethal milk* (*lm*). AE is an inherited form of zinc deficiency, is a human rare, autosomal recessive syndrome characterized by systemic zinc deficiency that leads to skin lesions, alopecia, diarrhea, neuropsychological disturbance and reduced immune function. In the absence of Zn supplementation AE leads to death of the patients (reviewed in: Ackland and Michalczyk, 2006). The gene responsible for this syndrome was mapped to chromosome 8 (Wang et al., 2001) and identified as ZIP4, a transporter responsible for intestinal Zn uptake (Kury et al., 2002; Wang et al., 2002). The newly identified gene was shown to harbor a range of mutations in patients with the disorder (Kury et al., 2003) and was induced by Zn deficiency (Dufner-Beattie et al., 2003; Liuzzi et al., 2004). Unlike in AE, the most prominent phenotype of the *lethal milk* mouse syndrome is a decrease in the milk Zn content that dramatically affects survival of the pups,

which die within the first two weeks of neonatal life unless they are foster nursed by wild type dams. The fact that normal milk Zn content is sufficient to ensure survival indicates that the defect in *lm* mutant mice resides in Zn secretion by mammary epithelial cells into the milk, rather than in intestinal absorption. Identification of a premature stop codon in the ZnT4 gene as the molecular basis of the *lm* mutation (Huang & Gitschier, 1997) suggested therefore that this protein might be responsible for Zn secretion in epithelial cells of the mammary gland. ZnT4 is ubiquitously expressed in mammalian tissues and displays vesicular localization in tissue culture cells of various origins (Murgia et al., 1999; Michalczyk et al., 2002). In support for the involvement of ZnT4 in secretion, ZnT4 vesicles appear to move from the trans-Golgi region to the cell periphery upon Zn addition (Huang et al., 2002 and our unpublished observations), suggesting a mechanism similar to that mediated by the Menkes copper transporter (Petris et al., 1996; Pascale et al., 2003). Further evidence that a fraction of ZnT4 traffics to the plasma membrane comes from surface biotinylation studies in cultured fibroblasts (Henshall et al., 2003). In our experiments, we observed plasma membrane localization of ZnT4 only when this transporter was overexpressed in fully differentiated, polarized epithelial cells by transfection. Although we cannot distinguish between integral membrane insertion and sub-membrane localization of ZnT4 in these cells, the circular staining points at basolateral rather than apical localization. It was therefore possible that expression of the truncated form of ZnT4 predicted by the *lm* mutation, lacking the last transmembrane segment and the hydrophilic carboxyl-terminus, might lead to decreased zinc secretion to bloodstream through the inability to bind proteins responsible for proper vesicular trafficking. However, we show in this paper by Northern hybridization and real time quantitative PCR that the mutant ZnT4 mRNA carrying a premature termination codon is barely detectable in the intestine of *lm* mice, even following Zn supplementation that was shown to induce ZnT4 expression (Liuzzi et al., 2004). Since the *lm* mutation lies within the open reading frame, a possible explanation is that the ZnT4/*lm* mRNA is efficiently degraded by the NMD (Nonsense-Mediated mRNA Decay) pathway, the quality control mechanism that prevents cells from synthesizing truncated and potentially harmful proteins by acting on mRNAs that contain open reading frames after translational stop codons (reviewed in: Conti & Izaurralde, 2005). In support of this hypothesis, when we forced expression of a mutant ZnT4/*lm* protein from a construct in which the open reading frame downstream of the stop codon at position 297 is deleted, the mutant protein fully co-localizes with full length ZnT4.

Although, as previously mentioned, the lactating phenotype is the most prominent feature of the *lm* syndrome, surviving *lm* mice develop signs of mild Zn deficiency in adulthood, thus implying a key role for ZnT4-mediated zinc secretion also in other tissues. To further investigate this, we chose to compare by real time-PCR the expression of other zinc transporters, as well as of the metallothioneins, in the small intestine of *lm* and wild type

isogenic mice in the presence or in the absence of Zn supplementation. As a further control, we included RNA extracted from the jejunum of wild type CD1 mice, a commonly used outbred strain of mice representative of a different genetic background. Our results indicate that in the intestinal cells of *lm* mice expression of the ZnT2 and ZIP4 genes is not regulated by zinc, while expression of both metallothioneins 1 and 2 is higher than in the wild type in the absence of zinc supplementation. ZnT2 is a transporter implicated in vesicular sequestration of excess intracellular zinc (Palmiter et al., 1996a). According to its proposed role, ZnT2 expression is downregulated by zinc deficiency in mouse small intestine (Liuzzi et al., 2004). The Zip4 gene, which is defective in AE, is responsible for intestinal Zn uptake and is normally upregulated in Zn deficiency (Dufner-Beattie et al., 2003; Liuzzi et al., 2004) was almost threefold lower in the jejunum of unsupplemented *lm* mice and was not further downregulated by Zn supplementation. Overall, our results on zinc transporter expression in the intestinal cells of adult *lm* mice point to a Zn secretion defect leading to higher intracellular Zn content, which in turn triggers increased expression of the metallothionein genes. Such higher levels of metallothionein transcripts in unsupplemented *lm* mice confirm previous reports on increased levels of the corresponding proteins (Grider & Erway, 1986). However, induction of MT1 and MT2 expression by zinc supplementation is not impaired in *lm* mice, as both genes reach the same levels of expression as those observed in the jejunum of isogenic wild type. Figure 6 presents the model that we envisage for Zn metabolism in *lm* mice, showing the relevant transporters, which are significant in our experiments. Zinc uptake from the intestinal lumen, under normal conditions in wild type enterocytes, is mediated by Zip4 (Fig. 6A). Intracellular zinc is then distributed to ZnT2 and ZnT4 vesicles. Following Zn supplementation the intracellular zinc content is higher, resulting in induction of the metallothioneins, ZnT2 levels are higher and Zip4 activity is downregulated (Fig. 6B). Intracellular zinc homeostasis is maintained by vesicular sequestration (ZnT2) and by ZnT4 mediated Zn secretion to bloodstream, possibly through vesicular fusion with the plasma membrane. In the enterocytes of mutant *lm* mice, in contrast, ZnT4 secretion activity is abolished, leading to increased intracellular zinc (Fig. 6C). As a consequence, Zip4 expression is decreased and the metallothionein mRNA levels are increased. However, other zinc efflux activities, among which ZnT1, must be operating in *lm* enterocytes to keep the intracellular zinc concentration below a threshold level that leads to full induction of MT1, MT2 and ZnT2. Higher intracellular zinc concentration have been shown to induce ZnT1 transcription by the Zn-dependent transcription factor MTF1 (Lichtlen & Schaffner, 2001; Langmade et al., 2000) which also regulates transcription of the metallothionein genes. Zinc concentration in the milk of *lm* mutant dams is decreased by 34% with respect to wild type (Piletz & Ganschow, 1978; Ackland & Mercer, 1992; Lee et al., 1992), therefore zinc secretion is mediated by other transporters, other than ZnT4, also in mammary epithelial cells.

Finally, in Zn supplemented *lm* mice (Fig. 6D), the expression profiles of ZnT2, Zip4 and of the metallothionein genes is very similar to that observed in the intestine of supplemented wild type mice (Fig. 6B).

ACKNOWLEDGEMENTS

The Authors wish to thank Piera Rami for invaluable help with animal care. This work was supported in part by the FISIR grant "Improvement of lipid and mineral contents of milk to enhance its nutraceutical and safety properties" from the Italian Ministry of Research (MIUR).

REFERENCES

- Ackland, M.L. and Mercer, J.F.B. (1992) The murine mutation, lethal milk, results in production of zinc-deficient milk. *Journal of Nutrition* **122**, 1214-1218.
- Ackland, M.L. and Michalczyk, A. (2006) Zinc Deficiency and its Inherited Disorders - a Review. *Genes & Nutrition* **1**, this issue.
- Barilà, D., Murgia, C., Nobili, F., Gaetani, S. and Perozzi, G. (1994) Subtractive hybridization cloning of novel genes differentially expressed during rat intestinal development. *European Journal of Biochemistry* **223**, 701-709.
- Chimienti, F., Devergnas, S., Favier, A. and Seve, M. (2004) Identification and cloning of a β -cell-specific zinc transporter, ZnT8, localized into insulin secretory granules. *Diabetes* **53**, 2330-2337.
- Conti, E. and Izaurralde, E. (2005) Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species. *Current Opinion in Cell Biology* **17**, 316-25.
- Cousins, R.J. and McMahon, R.J. (2000) Integrative aspects of zinc transporters. *Journal of Nutrition* **130**, 1384S-1387S.
- Coyle, P., Philcox, J.C., Carey, L.C. and Rofe, A.M. (2002) Metallothionein: the multipurpose protein. *Cellular & Molecular Life Sciences* **59**, 627-47.
- Davis, S.R. and Cousins, R.J. (2000) Metallothionein expression in animals: a physiological perspective on function. *Journal of Nutrition* **130**, 1085-8.
- Dufner-Beattie, J., Kuo, Y.M., Gitschier, J. and Andrews, G.K. (2004) The adaptive response to dietary zinc in mice involves the differential cellular localization and zinc regulation of the zinc transporters ZIP4 and ZIP5. *Journal of Biological Chemistry* **279**, 49082-90.
- Dufner-Beattie, J., Wang, F., Kuo, Y.M., Gitschier, J., Eide, D. and Andrews, G.K. (2003) The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. *Journal of Biological Chemistry* **278**, 33474-81.

- Eide, D.J. (2004) The SLC39 family of metal ion transporters. *Pflügers Archives European Journal of Physiology* **447**, 796-800.
- Erway, L.C. and Grider, A. (1984) Zinc metabolism in lethal-milk mice. Otolith, lactation, and aging effects. *Journal of Heredity* **75**, 480-484.
- Fraker, P.J., King, L.E., Laakko, T. and Vollmer, T. (2000) The dynamic link between the integrity of the immune system and zinc status. *Journal of Nutrition* **130**, 1399S-1406S.
- Frausto da Silva, J.J.R. and Williams, R.J.P. (1991) *The Biological Chemistry of the Elements*, Clarendon Press, Oxford, U.K.
- Frederickson, C.J., Suh, S.W., Silva, D., Frederickson, C.J. and Thompson, R.B. (2000) Importance of zinc in the central nervous system: the zinc-containing neuron. *Journal of Nutrition* **130**, 1471S-1483S.
- Green, M. and Sweet, H. (1973) Locus order *lm - a - Ra*. *Mouse News Letters* **49**, 32.
- Grider, A., Jr. and Erway, L.C. (1986) Intestinal metallothionein in lethal-milk mice with systemic zinc deficiency. *Biochemical Genetics* **24**, 635-42.
- Henshall, S.M., Afar, D.E., Rasiah, K.K., Horvath, L.G., Gish, K., Caras, I., Ramakrishnan, V., Wong, M., Jeffry, U., Kench, J.G., Quinn, D.I., Turner, J.J., Delprado, W., Lee, C.S., Golovsky, D., Brenner, P.C., O'Neill, G.F., Kooner, R., Stricker, P.D., Grygiel, J.J., Mack, D.H. and Sutherland, R.L. (2003) Expression of the zinc transporter ZnT4 is decreased in the progression from early prostate disease to invasive prostate cancer. *Oncogene* **22**, 6005-12.
- Huang, L. and Gitschier, J. (1997) A novel gene involved in zinc transport is deficient in the lethal milk mouse. *Nature Genetics* **17**, 292-297.
- Huang, L., Kirschke, C.P. and Gitschier, J. (2002) Functional characterization of a novel mammalian zinc transporter, ZnT6. *Journal of Biological Chemistry* **277**, 26389-95.
- Kambe, T., Narita, H., Yamaguchi-Iwai, Y., Hirose, J., Amano, T., Sugiura, N., Sasaki, R., Mori, K., Iwanaga, T. and Nagao, M. (2002) Cloning and characterization of a novel mammalian zinc transporter, zinc transporter 5, abundantly expressed in pancreatic beta cells. *Journal of Biological Chemistry* **277**, 19049-55.
- Kambe, T., Yamaguchi-Iwai, Y., Sasaki, R. and Nagao, M. (2004) Overview of mammalian zinc transporters. *Cellular & Molecular Life Sciences* **61**, 49-68.
- Kirschke, C.P. and Huang, L. (2003) ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. *Journal of Biological Chemistry* **278**, 4096-102.
- Kury, S., Dreno, B., Bezieau, S., Giraudet, S., Kharfi, M., Kamoun, R. and Moisan, J. P. (2002) Identification of SLC39A4, a gene involved in acrodermatitis enteropathica. *Nature Genetics* **31**, 239-40.
- Kury, S., Kharfi, M., Kamoun, R., Taieb, A., Mallet, E., Baudon, J.J., Glastre, C., Michel, B., Sebag, F., Brooks, D., Schuster, V., Scoul, C., Dreno, B., Bezieau, S. and Moisan, J.P. (2003) Mutation spectrum of human SLC39A4 in a panel of patients with acrodermatitis enteropathica. *Human Mutation* **22**, 337-8.
- Langmade, S.J., Ravindra, R., Daniels, P.J. and Andrews, G.K. (2000) The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. *Journal of Biological Chemistry* **275**, 34803-9.
- Lee, D.Y., Shay, N.F. and Cousins, R.J. (1992) Altered zinc metabolism occurs in murine lethal milk syndrome. *Journal of Nutrition* **122**, 2233-8.
- Lichtlen, P. and Schaffner, W. (2001) Putting its fingers on stressful situations: the heavy metal-regulatory transcription factor MTF-1. *Bioessays* **23**, 1010-7.
- Liuzzi, J.P., Bobo, J.A., Lichten, L.A., Samuelson, D.A. and Cousins, R.J. (2004) Responsive transporter genes within the murine intestinal-pancreatic axis form a basis of zinc homeostasis. *Proceedings of the National Academy of Sciences U S A* **101**, 14355-60.
- McMahon, R.J. and Cousins, R.J. (1998) Mammalian zinc transporters. *Journal of Nutrition* **128**, 667-70.
- Michalczyk, A., Allen, J., Blomeley, R.C. and Ackland, M.L. (2002) Constitutive expression of hZnT4 zinc transporter in human breast epithelial cells. *Biochemical Journal* **364**, 105-13.
- Michalczyk, A., Varigos, G., Catto-Smith, A., Blomeley, R.C. and Ackland, M.L. (2003) Analysis of zinc transporter, hZnT4 (Slc30A4), gene expression in a mammary gland disorder leading to reduced zinc secretion into milk. *Human Genetics* **113**, 202-10.
- Murgia, C., Lang, C., Grosser, D., Ruffin, R., Perozzi, G., Truong-Tran, A.-Q., Ho, L. and Zalewski, P. (2006) The role of zinc and its specific transporters in airway physiology and disease. *Current Drug Targets* **7**, in press.
- Murgia, C., Vespignani, I., Cerase, J., Nobili, F. and Perozzi, G. (1999) Cloning, expression, and vesicular localization of zinc transporter Dri 27/ZnT4 in intestinal tissue and cells. *American Journal of Physiology* **277**, G1231-G1239.
- Palmiter, R.D., Cole, T.B. and Findley, S.D. (1996a) ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. *EMBO Journal* **15**, 1784-1791.

Palmiter, R.D., Cole, T.B., Quaife, C.J. and Findley, S.D. (1996b) ZnT-3, a putative transporter of zinc into synaptic vesicles. *Proceedings of the National Academy of Sciences USA* **93**, 14934-14939.

Palmiter, R.D. and Huang, L. (2004) Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. *Pflugers Archives European Journal of Physiology* **447**, 744-51.

Pascale, M. C., Franceschelli, S., Moltedo, O., Belleudi, F., Torrisci, M. R., Bucci, C., La Fontaine, S., Mercer, J.F. and Leone, A. (2003) Endosomal trafficking of the Menkes copper ATPase ATP7A is mediated by vesicles containing the Rab7 and Rab5 GTPase proteins. *Experimental Cell Research* **291**, 377-85.

Petris, M.J., Mercer, J.F., Culvenor, J.G., Lockhart, P., Gleeson, P.A. and Camakaris, J. (1996) Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. *EMBO Journal* **15**, 6084-95.

Piletz, J. E. and Ganschow, R. E. (1978) Zinc deficiency in murine milk underlies expression of the lethal milk (*lm*) mutation. *Science* **199**, 181-183.

Ranaldi, G., Perozzi, G., Truong-Tran, A., Zalewski, P. and Murgia, C. (2002) Intracellular distribution of labile Zn(II) and zinc transporter expression in kidney and MDCK cells. *American Journal of Physiology* **283**, F1365-F1375.

Rozen, S. and Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology* **132**, 365-86.

Van Wouwe, J. (1989) Clinical and laboratory diagnosis of Acrodermatite enteropathica. *European Journal of Pediatrics* **149**, 2-8.

Wang, F., Kim, B.E., Petris, M.J. and Eide, D.J. (2004) The mammalian Zip5 protein is a zinc transporter that localizes to the basolateral surface of polarized cells. *Journal of Biological Chemistry* **279**, 51433-41.

Wang, K., Pugh, E.W., Griffen, S., Doheny, K.F., Mostafa, W.Z., al-Aboosi, M.M., el-Shanti, H. and Gitschier, J. (2001) Homozygosity mapping places the acrodermatitis enteropathica gene on chromosomal region 8q24.3. *American Journal of Human Genetics* **68**, 1055-60.

Wang, K., Zhou, B., Kuo, Y.M., Zemansky, J. and Gitschier, J. (2002) A novel member of a zinc transporter family is defective in acrodermatitis enteropathica. *American Journal of Human Genetics* **71**, 66-73.