



# HHS Public Access

Author manuscript

*J Trace Elem Med Biol.* Author manuscript; available in PMC 2018 September 01.

Published in final edited form as:

*J Trace Elem Med Biol.* 2018 September ; 49: 27–34. doi:10.1016/j.jtemb.2018.04.024.

## Differential expression of zinc transporters accompanies the differentiation of C2C12 myoblasts

Amanda L. Paskavitz<sup>a,b,1</sup>, Julia Quintana<sup>c,2</sup>, Daniella Cangussu<sup>a</sup>, Cristina Tavera-Montañez<sup>a,c</sup>, Yao Xiao<sup>b</sup>, Sonia Ortiz-Miranda<sup>d</sup>, Juan G. Navea<sup>b</sup>, Teresita Padilla-Benavides<sup>a,\*</sup>

<sup>a</sup>Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA, 01605, USA

<sup>b</sup>Department of Chemistry, Skidmore College, 815 North Broadway, Saratoga Springs, NY, 12866, USA

<sup>c</sup>Department of Chemistry and Biochemistry, Worcester Polytechnic Institute, 100 Institute Road, Worcester, MA, 01609, USA

<sup>d</sup>Department of Neurobiology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA, 01605, USA

### Abstract

Zinc transporters facilitate metal mobilization and compartmentalization, playing a key role in cellular development. Little is known about the mechanisms and pathways of Zn movement between Zn transporters and metalloproteins during myoblast differentiation. We analyzed the differential expression of ZIP and ZnT transporters during C2C12 myoblast differentiation. Zn transporters account for a transient decrease of intracellular Zn upon myogenesis induction followed by a gradual increase of Zn in myotubes. Considering the subcellular localization and function of each of the Zn transporters, our findings indicate that a fine regulation is necessary to maintain correct metal concentrations in the cytosol and subcellular compartments to avoid toxicity, maintain homeostasis, and for loading metalloproteins needed during myogenesis. This study advances our basic understanding of the complex Zn transport network during muscle differentiation.

### Keywords

Myogenesis; Zinc; Zn-transporters; Zn content; Gene Expression

---

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

\*Corresponding author: Teresita.Padilla@umassmed.edu (T. Padilla-Benavides).

<sup>1</sup>Current address: Center for Cancer Genome Discovery, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA, 02215, USA.

<sup>2</sup>Current address: Department of Molecular Genetics and Physiology of Plants, Ruhr University Bochum, Universitätsstrasse, 150, 44801, Bochum, Germany.

### Conflicts of interest

The authors declare no conflict of interest.

## 1. Introduction

Zinc (Zn) is an essential trace nutrient necessary for normal physiological functions in all forms of life [1,2]. Zn is a cofactor in up to 10% of mammalian proteins and it is crucial for proper folding and for the catalytic activity of numerous enzymes [1,3]. With a Zn-deficient diet, mammals develop anemia, growth retardation, hypogonadism, skin abnormalities, diarrhea, neurological and mental deficiencies, alopecia, taste disorders, chronic inflammation and compromised immune function [1–6]. Conversely, excess Zn is cytotoxic and causes deficient copper absorption [7–9]. Therefore, cellular Zn homeostasis must be tightly controlled. Two families of Zn transporters, ZnTs (solute-linked carrier 30, SLC30) and ZIPs (Zrt- and Irt-like proteins, SLC39) export and import Zn, respectively, between the cytosol, organelles, and extracellular milieu [1,2,10]. In mammals, ten ZnT (1–10) exporters and fourteen ZIP importers (1–14) have been identified. Nonetheless, the breadth of cellular functions for all these Zn transporters remains to be elucidated. ZnTs and ZIPs are transmembrane proteins predicted to have 6 or 8 transmembrane domains, respectively [1,2]. ZnT5 is the exception, with nine putative transmembrane domains [2]. Zn transport occurs in response to an ionic gradient. Most ZnTs dimerize and export Zn by a diffusion-mediated  $\text{Zn}^{2+}/\text{H}^{+}$  exchange, while ZIPs import the ion through a channel-like activity [2]. However, some ZIPs import Zn through a bicarbonate/ $\text{Zn}^{2+}$  symport mechanism [11]. Certain transporters like ZIP8 and ZIP14 also transport Fe, Mn, and Cd [12] while ZnT10 is the only ZnT known to primarily transport Mn over Zn [13,14].

Members of the ZnT and ZIP families have a prominent role in a wide range of developmental processes. For instance, in chondrocytes, increased levels of cellular Zn have been associated with the presence of ZIP8 in lysosomal vesicles, which may activate MTF1, a metal responsive transcription factor [15]. MTF1 in turn promotes the expression of metalloproteases (MMPs) that degrade different components of the extracellular matrix [16–19]. ZIP12 has been found to induce neuronal differentiation via activation of cAMP response element binding protein (CREB) [20]. ZIP14 transports Fe, Mn and Cd in addition to Zn [21–23]. Interestingly, severely affected phenotypes are observed in *Zip14* knock-out animals. For instance, these mice have impaired systemic growth, decreased body sizes, and impaired skeletal development due to impaired CREB activation [24]. Moreover, mice lacking *Zip14* present a proliferation defect in hepatocytes, are hypoglycemic, hyperinsulinemic, and have higher levels of glucose in the liver [21,25]. Importantly, CREB activation is also necessary for proper development and function of the skeletal muscle lineage. It is required for differentiation of embryonic skeletal muscle progenitors, for survival of adult skeletal muscle, for promoting myoblast proliferation in mice that were subject to muscle injury, and also for enhancing muscle regeneration in a dystrophic mouse model [26]. ZIP2 is induced by Zn deprivation in monocytes, and is necessary for keratinocyte differentiation [1,27,28]. Experimental evidence suggests a major role in development for ZIP2, as knock-out mice are sensitive to dietary Zn deficiency during pregnancy [29]. ZIP5 is required for systemic Zn excretion and to promote the development of the sclera and retina [30–32]. *Zip6* over-expression has been associated with the epithelial-mesenchymal transition of different cancer types [33–37]. ZIP6 also contributes to dendritic cell maturation [38]. ZIP1 is proposed to be involved in activation of

microglia, embryonic development, and prostate metabolism [2,39–42]. ZIP4 is required for early development, dietary Zn uptake in the intestine, and Zn levels regulate its expression [43,44]. ZIP10-dependent Zn signaling is required for early development of B-cells by suppressing caspase activity in the bone marrow [45]. The above mentioned ZIPs are located to the plasma membrane. Other ZIPs participate in Zn transport for protein maturation, a process that occurs in intracellular compartments. ZIP7, as an example, is located in the endoplasmic reticulum (ER) and the trans Golgi network (TGN), and it has an important role in glycemic control of skeletal muscle [46]. ZIP9 is located at the cell surface and TGN [47,48] and is considered to be an androgen receptor in addition to its role in Zn transport [48]. ZIP13 mobilizes Zn to the TGN and secretory vesicles, controls Zn homeostasis in the ER and is involved in BMP/TGF- $\beta$ /Smad signaling pathway [49–51].

ZnT1 is the only recognized Zn exporter located to the plasma membrane and is essential during embryonic development, as deletion of the *ZnT1* gene in mice is embryonic lethal [52,53]. On the other hand, the majority of the ZnT transporters are localized to secretory structures and the TGN [2]. Their involvement in growth and differentiation has been reported in several cellular systems. For instance, ZnT7 deletion causes growth retardation, decreased systemic Zn, reduced fat accumulation, and impaired glucose tolerance and insulin resistance in male mice [54–56]. ZnT10 is also located in endosomal vesicles, potentially contributing to Zn and Mn export [3]. ZnT5 acts in the early secretory pathway, and knock-out mice for this transporter present with growth delays, muscle weakness and cardiac deficiencies [57]. A ZnT5/6 dimer mobilizes Zn to activate enzymes such as alkaline phosphatases in the early secretory pathway [58]. Other transporters located to the early secretory pathway and TGN are ZnT2, ZnT3 and ZnT8. ZnT2 expression is induced by elevated Zn levels, and has been detected in mammary glands, prostate, pancreas, small intestine, kidney, and retina [31,59,60]. ZnT2 is necessary for secretion of Zn into breast milk in humans [61–64]. ZnT3 is located mainly in synaptic vesicles of glutamatergic neurons located in the hippocampus, but is proposed to also modulate insulin production in pancreatic  $\beta$  cells [65–67]. ZnT8 activity has important implications in diabetes mellitus [68]. ZnT8 knock-out mice fed a high-fat diet became glucose intolerant or diabetic, and islet cells became less responsive to glucose. This is due to a failure to transport Zn into insulin granules, which has been shown to co-crystallize with insulin in pancreatic beta cells [68,69].

Transport of Zn to the nucleus is fundamental for cell growth and development. A large number of transcription factors require Zn for DNA binding and activation of gene expression [70–74]. Zn binding transcription factors can be metallated in the cytosol or subcellular compartments, although Zn acquisition may also occur in the nucleus [75]. Zn has also been found in the nucleolus and chromosomes, and is implicated in DNA replication and stabilization of DNA, RNA, and ribosomes [76]. ZIP11 is the only transporter proposed to be located at the nucleus, although no information on its cellular role is available [77]. Finally, ZnT9 homology to the ZnT family is under debate, as it lacks the distinguishing ZnT transmembrane Zn-binding domains and is mainly located in the cytoplasm [78,79]. ZnT9 acts as nuclear receptor coactivator and was renamed as GAC63 [78].

In spite of all this accumulated research, little is known about Zn requirements, regulation, and distribution in differentiating skeletal muscle cells. Because the majority of Zn in mammalian cells is bound to metalloenzymes and stored in organelles and cellular vesicles, free zinc levels remain quite low [2,80–84]. Within the cell, about 50% of Zn is found in the cytoplasm and in vesicles, 30–40% in the nucleus, and about 10% in the cell membrane [85,86]. Moreover, 60% of the total systemic Zn is sequestered and used in skeletal muscle, making this organ the greatest reservoir of Zn in the body [87]. Whether Zn is necessary for myoblast differentiation is a matter of controversy. Experimental evidence suggests that addition of Zn to differentiation medium inhibits myogenesis, but promotes myoblast proliferation and activation of quiescent myogenic satellite cells [88]. Yet other evidence demonstrated that Zn is required for differentiation, as myoblast differentiation in both C2C12 cells and chicken embryo myoblasts was inhibited in Zn-deficient medium [88,89]. Furthermore, mice fed with a Zn-deficient diet may have reduced muscle regeneration upon injury [4]. C2C12 myoblasts are an excellent model to study myogenesis due to their similarities to progenitor myogenic lines [90]. Upon serum depletion, the cells commit to differentiate and the myoblasts fuse together to become multinucleated myotubes. Myoblasts express specific regulators of the skeletal muscle lineage like MyoD, myogenic factor 5 (Myf5), and myogenin. Upon induction of differentiation, MyoD and Myf5 drive the expression of myogenin, a transcription factor fundamental for the coordination of skeletal muscle development and repair. Once the myoblasts commit to differentiate, myogenin expression continues to increase as myogenesis progresses leading to the expression of muscle-specific proteins such as myosin heavy chains, skeletal actin, muscle-specific creatine kinase and MMPs, among others. Additionally, myofibers express insulin receptors and require insulin binding for growth [90].

We hypothesized that the intracellular levels of Zn in differentiating myoblasts is tightly controlled by a timely and differential expression of ZnT and ZIP transporters that is specific to the skeletal muscle lineage. The C2C12 stable murine cell line was used as a model of myogenesis. We analyzed the kinetics of Zn accumulation at different stages of this process. Dynamic changes of Zn levels were observed over the course of myogenic differentiation. Gene and protein expression analyses suggest that cellular changes in Zn content in myogenesis may be dependent mainly on four ZIP transporters located at the plasma membrane (ZIP3, 8, 5 and 6), and four transporters located in subcellular compartments (ZnT7, 4, 8 and ZIP11). This study set the basis for understanding how Zn is distributed and potentially utilized during skeletal muscle differentiation.

## 2. Materials and methods

### 2.1. Cell culture

C2C12 cells are immortalized mouse myoblast used as a *in vitro* model for myogenesis studies. C2C12 cells proliferate rapidly under high serum conditions, and undergo myogenic differentiation under low serum conditions [91,92]. C2C12 myoblasts were seeded at  $1 \times 10^4$  cells/cm<sup>2</sup> in DMEM media containing 10% fetal bovine serum (Gibco) to support proliferation. After 48 h, cells reached confluence, and the media was changed to differentiation media (DMEM, 2% horse serum). At least three independent biological

replicates were collected for analysis at different time points of proliferation for 24 h (P) and throughout myogenic differentiation (0, 3, 6, 12, 24, 48, and 72 h).

## 2.2. Whole cell Zn content analysis

C2C12 cells from the indicated time points were rinsed three times and immediately re-suspended in 100  $\mu$ l of PBS. Cells were lysed by three sonication cycles (30 s on by 30 s off) for 5 min at medium intensity using a Bioruptor UCD-200 (Diagenode). Total protein content was determined by Bradford [93]. Then, samples were acid digested in concentrated HNO<sub>3</sub> (trace metal grade) for 1 h at 80 °C and overnight at 20 °C. Digestions were concluded by adding 30  $\mu$ l H<sub>2</sub>O<sub>2</sub> [94–97]. The resulting solution was measured by triplicate using atomic absorption spectroscopy (AAS) equipped with a graphite furnace (PerkinElmer, Analyst 800), which provides an ideal limit of detection (LOD) of Zn from currently available techniques [98]. The estimated LOD, calculated as 3 $\sigma$  of 0.01  $\mu$ mol L<sup>-1</sup>, allowed quantification of all samples. The quantification of Zn, at the ppb level, was completed using analytical grade standards diluted in 18 M $\Omega$  purified water. Concentration of Zn measured via AAS was normalized to the initial mass of protein in each sample.

## 2.3. Zinc imaging

Proliferating and differentiating C2C12 cells were loaded with 2  $\mu$ M Fluo-Zin3 AM, cell permeant (Invitrogen) in the corresponding culture media for 40 min at 37 °C. Fluo-Zin3 has a high affinity to bind Zn<sup>2+</sup>. Then, the cells were washed with fresh media and allowed to rest for another 20 min at 37 °C. For live imaging of Zn the media was exchanged for PBS right before microscopic analyses, to avoid fluorescent interference of the culture media. In addition, cells were fixed with 10% formalin-PBS and counterstained with DAPI. Fluorescence images were obtained with a Leica TCS SP5 II AOBS confocal laser-scanning microscope (Leica).

## 2.4. Gene expression analysis of Zn transporters

RNA was purified from at least three independent biological replicates of proliferating and differentiated primary myoblasts with TRIzol (Invitrogen). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (ThermoFisher Scientific). Quantitative RT-PCR was performed with Fast SYBR green master mix (ThermoFisher Scientific) on the ABI StepOne Plus Sequence Detection System (Applied Biosystems) using the primers listed in Supp. Table 1 and normalized to the levels of the housekeeping gene *Efl-a*.

## 2.5. Western blot analysis

C2C12 cells were washed with PBS and solubilized with RIPA buffer (10 mM piperazine-*N*, *N*-bis(2-ethanesulfonic acid), pH 7.4, 150 mM NaCl, 2 mM ethylenediamine-tetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, and 10% glycerol) containing complete protease inhibitor cocktail. Protein content was quantified by Bradford [93]. Samples (20  $\mu$ g) were resolved by SDS-PAGE and electro-transferred to PVDF membranes (Millipore). The proteins of interest were detected with the specific antibodies (Supp. Table 2), followed by species-appropriate HRP-conjugated secondary antibodies and

chemiluminescent detection (ECL PLUS; GE Healthcare). GAPDH was used as a loading control.

## 2.6. Immunocytochemistry

Proliferating and differentiating C2C12 cells were fixed overnight in 10% formalin-PBS at 4 °C. Samples were washed with PBS and incubated overnight with Hybridoma supernatants against myogenic markers (Supp. Table 2) in PBS buffer containing 5% horse serum and 0.2% Triton X-100. Secondary antibody binding and HRP staining were performed with the universal ABC kit (Vector Labs).

## 3. Results

We first asked whether cellular concentration of Zn varies as a function of the myogenic program. To address this question, we analyzed Zn levels in proliferating and differentiating C2C12 cells (Fig. 1). Samples were collected and whole cell metal content was determined by AAS. Analyses detected  $0.93 \pm 0.09$   $\mu\text{mol Zn/mg}$  of protein in proliferating myoblasts (Fig. 1A). Confocal microscopy of live and fixed proliferating myoblasts using Fluo-Zin3 showed that free  $\text{Zn}^{2+}$  is located in cytosolic vesicles (Figs. 1B; Supp. S2). Whole cell Zn content significantly decreased when the differentiation program was initiated at 0 h ( $0.32 \pm 0.02$   $\mu\text{mol Zn/mg}$  of protein; Fig. 1A). Fluorescent labelling of  $\text{Zn}^{2+}$  is in agreement with the quantitative analysis by AAS, and showed a large decrease of unbound Zn-containing vesicles (Figs. 1B; Supp. S2). After 12 h of differentiation, a gradual increase in cytosolic Zn was detected, reaching a maximal level in mature myotubes at 72 h ( $2.3 \pm 0.3$   $\mu\text{mol Zn/mg}$  of protein; Fig. 1A). Fluo-Zin3 imaging also showed a gradual and sustained increase of cytosolic  $\text{Zn}^{2+}$  after 12 h, and until 72 h (Figs. 1B; Supp. S2), suggesting that the cellular Zn content is dynamic over the course of myogenesis. AAS analysis of Zn levels in both, proliferation and differentiation culture media showed no significant differences as indicated in Supp. Table 3, which support the idea that the Zn variations in the cells are independent of the metal levels in the media.

We then asked which transporters, among all the recognized ZIPs and ZnTs, might be responsible for those changes. Therefore, we investigated whether myogenesis prompts transcriptional changes in Zn transporters located in the cell membrane of differentiating C2C12 myoblasts. Most of the transporters in the cell membrane belong to the ZIP family [1,2,11]. We found that *Zip3* and *Zip8* were upregulated over the course of myogenesis (Fig. 2). *Zip3* showed the greatest increase in expression, starting at 12 h and going up to 20 fold-change by 72 h (Fig. 2A). ZIP3 is proposed to participate in Zn reuptake in other cellular systems [99], which is consistent with its expression at early stages of myogenesis (Fig. 2E). *Zip8* mRNA exhibited a 4-fold increase 12–48 h after initiation of differentiation (Fig. 2B), but protein accumulation peaked in mature myotubes (Fig. 2E). Considering the gradual increase in Zn levels after 12 h of differentiation (Fig. 1), ZIP3 and 8 might participate in Zn re-uptake after the initial stages of myogenesis. ZIP8 could also contribute to the establishment of the differentiated phenotype by promoting the expression of MTF1 and trans-activation of MMPs, which are necessary for myotube fusion [15]. *Zip5* and *Zip6* transcripts were upregulated during the early stages of C2C12 differentiation, showing a

peak at 12 h after induction of myogenesis (Fig. 2C, D); in both cases, the protein levels were constant during the first 12 h of differentiation, and decreased after 24 h. Additional genes encoding for Zn importers located at the cell membrane (*Zip1, 2, 4, 10, 12, 14*) had moderate changes in expression during myogenesis (Supp. Fig. 3A–F), and basal protein levels were sustained over time (Supp. Fig. S3H). Some of these transporters have been implicated in the development and differentiation of several tissues [2]. Nonetheless, it seems from our data that their role in skeletal muscle differentiation might be secondary. Altogether, our data indicate that changes in *Zip* expression, both transient and sustained over time, accompany changes in Zn content during myogenesis.

ZnT1 is the only proposed Zn exporter located to the plasma membrane [52]. Considering the significant decrease in the cellular levels of Zn during the initial hours of differentiation of C2C12 cells (Figs. 1, Supp. S2), we expected a major change in the expression of ZnT1. However, ZnT1 gene expression and protein abundance remained stable during differentiation of C2C12 cells (Supp. Fig. 3G, H); suggesting that the early decrease in Zn in differentiating myoblasts may occur via additional transporters localized to distinct secretory elements.

Metal acquisition by enzymes is a post-translational modification critical for folding and catalytic activity, which in some cases occurs in the ER and TGN [100–102]. Therefore, we analyzed the expression patterns of transporters located in these compartments. *ZnT7* mRNA expression peaked 12 h after induction of differentiation of C2C12 cells (Fig. 3A). ZnT7 participates in signaling pathways which involve the insulin receptor substrate 2 and AKT, which may have an important role for myogenesis [56]. Expression of other ER and TGN transporters, like ZIP7, remained constant during C2C12 differentiation, suggesting its constitutive expression (Supp. Fig. S4A), which is in agreement with a role for this transporter in glycemic control of skeletal muscles [46]. A modest increase in *Zip9* and *ZnT10* transcript levels was observed (Supp. Fig. S4B, C); however, Western blot analyses showed a differential increase in abundance of these two proteins at early (ZIP9) or late (ZnT10) stages of differentiation (Supp. Fig. S4J). Importantly, homozygous mutations of *ZnT10* produces muscle-related pathologies like Parkinsonism and dystonia with hypermanganesemia [103,104]. These muscular defects are consistent with abnormalities of mature tissue, supporting a role for ZnT10 in fully developed skeletal muscle cells.

Then, we analyzed the expression of Zn transporters located in compartments of the secretory route: lysosomes, endosomal vesicles, and secretory granules. These transporters potentially contribute to the metallation of secreted Zn-binding proteins. ZnT4 is an exporter associated with the secretory route. Interestingly, *ZnT4* mRNA expression was induced during myogenesis (Fig. 3B). However, a decrease in the protein abundance was observed at early time points (Fig. 3E). Considering our quantification of Zn by AAS and confocal microscopy observations of Zn distribution (Figs. 1; Supp. S2), it is plausible that ZnT4 is responsible for Zn export via cytoplasmic vesicles, which is partially supported by Western blot analyses showing a decrease in protein levels early in differentiation (Fig. 3E). However, the mechanisms by which ZnT4 may fulfill this role remain to be elucidated and are beyond the scope of this paper. ZIP13, ZnT6, and ZnT5 are localized to secretory vesicles [2,68]. *Zip13* and *ZnT6* showed a modest but significant increase in

differentiating C2C12 cells (Supp. Fig. S4D, E). A role for ZIP13 in myogenesis might be hypothesized, as lack of functional ZIP13 is related to Ehlers-Danlos syndrome, which is characterized by skeletal and connective tissue abnormalities [49,51,105]. Both ZnT5 gene and protein showed a non-significant but observable 2-fold increase in expression after 72 h of differentiation (Supp. Fig. S4F, J). Constitutive expression of ZnT5 in developing skeletal muscle cells is consistent with the observation that knock-out mice for this transporter have delayed growth, muscle weakness and cardiac deficiencies [57]. ZnT2 also located in secretory vesicles, exhibited a small but significant increase in expression at late stages of myogenesis, when the myotubes are formed (Supp. Fig. S4G, J). Expression of *ZnT2* has been associated with elevated Zn levels; considering that differentiated myotubes contain higher levels of Zn, ZnT2 expression was expected [59].

The exporter ZnT8 is localized to secretory granules, insulin granules, and endosomal membranes [68]. Of all transporters examined during C2C12 myoblast differentiation, *ZnT8* was the only down-regulated gene. *ZnT8* reached its lowest expression level at 24 h and was expressed once the cells were fully differentiated (Fig. 3C, E). Insulin is critical for muscle growth and development as more than 80% of systemic glucose usage occurs in skeletal muscle and because failure in insulin signaling leads to muscle atrophy [90,106]. Considering the high demand for glucose transport for carbohydrate metabolism in mature myotubes, we hypothesize that ZnT8-dependent Zn transport and/or compartmentalization has a role in insulin metabolism at late stages of myogenesis, which may explain the late expression of ZnT8. In line with this rationale, ZnT3 is also implicated in insulin metabolism [67]. A transient increase on ZnT3 mRNA and protein expression was found during early stages of differentiation of C2C12 cells, (Supp. Fig. S4H, J), suggesting that ZnT3 might partially participate in the insulin signaling pathways during early stages of myogenesis, when ZnT8 is downregulated.

ZIP11 is the only transporter proposed to be located in the nucleus and TGN [2]. *Zip11* mRNA and was upregulated in differentiating C2C12 myoblasts (Fig. 3D). However, the protein expression pattern was unusual, as ZIP11 could not be detected at 24 and 48 h and increased in mature myoblasts, at 72 h (Fig. 3E). This effect is probably due to protein de-stabilization related to signaling induced by the differentiation stimulus, which is followed by protein induction at later times, due to the increased mRNA production. The contributions of ZIP11 to myogenesis remain to be elucidated and are beyond the scope of this study. Finally, we analyzed the expression of ZnT9 which is thought to be a nuclear receptor coactivator [78]. Both ZnT9 gene expression and protein levels increased after 72 h of differentiation (Supp. Fig. S4I, J). The later recovery of ZIP11 and ZnT9 reflects a preferential role for these proteins and for nuclear Zn in mature myotubes, which is worth exploring in the future.

#### 4. Conclusions

The expression, localization, and roles of Zn transporters in different tissues and stages of development are largely unknown. In this study we investigated the differential expression of Zn transporters and how intracellular levels of Zn vary during differentiation of C2C12 myoblasts. We found that Zn levels decrease during early stages of myogenesis, only to

be restored by 12 h after the initiation of the myogenic program. Zn levels continue to increase until the end of the differentiation period. We also found that the majority of the transporters were transiently expressed as a consequence of the myogenic program and were often detected during the first 12 h upon induction of differentiation. Only ZnT8 was down-regulated in differentiating C2C12 cells. Our data suggest that myogenesis requires specific and optimal Zn levels in different subcellular compartments. Where most of this Zn is needed remains unclear, but our data indicate that a variety of Zn-binding proteins secreted from cellular organelles and granules are most likely necessary during myogenesis. Our findings also suggest that a dynamic network of transport and distribution of Zn occurs as a consequence of initiation of myoblast differentiation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

### Funding information

This work was supported by the Faculty Diversity Scholars Award from the University of Massachusetts Medical School to T.P.-B. A.L.P. was partially supported by UMMS funding for the Summer Undergraduate Research Experience program. S.O.-M. is supported by the grant NIH NS093384.

The authors thank Drs. Anthony N. Imbalzano and Dominic T. Haokip for critical reviews of this manuscript.

## References

1. Dufner-Beattie J, Langmade SJ, Wang F, Eide D, Andrews GK. Structure, function, and regulation of a subfamily of mouse zinc transporter genes. *J Biol Chem.* 278 (50) 2003; :50142–50150. [PubMed: 14525987]
2. Kambe T, Tsuji T, Hashimoto A, Itsumura N. The physiological, biochemical, and molecular roles of zinc transporters in zinc homeostasis and metabolism. *Physiol Rev.* 95 (3) 2015; :749–784. [PubMed: 26084690]
3. Sekler I, Sensi SL, Hershinkel M, Silverman WF. Mechanism and regulation of cellular zinc transport. *Mol Med.* 13 (7–8) 2007; :337–343. [PubMed: 17622322]
4. Jinno N, Nagata M, Takahashi T. Marginal zinc deficiency negatively affects recovery from muscle injury in mice. *Biol Trace Elem Res.* 158 (1) 2014; :65–72. [PubMed: 24488209]
5. Prasad AS, Halsted JA, Nadimi M. Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism and geophagia. *Am J Med.* 31 1961; :532–546. [PubMed: 14488490]
6. Devirgiliis C, Zalewski PD, Perozzi G, Murgia C. Zinc fluxes and zinc transporter genes in chronic diseases. *Mutat Res.* 622 (1–2) 2007; :84–93. [PubMed: 17374385]
7. Broun ER, Greist A, Tricot G, Hoffman R. Excessive zinc ingestion. A reversible cause of sideroblastic anemia and bone marrow depression. *Jama.* 264 (11) 1990; :1441–1443. [PubMed: 2094240]
8. Fischer PW, Giroux A, L'Abbe MR. The effect of dietary zinc on intestinal copper absorption. *Am J Clin Nutr.* 34 (9) 1981; :1670–1675. [PubMed: 7282591]
9. Ogiso T, Ogawa N, Miura T. Inhibitory effect of high dietary zinc on copper absorption in rats. II. Binding of copper and zinc to cytosol proteins in the intestinal mucosa. *Chem Pharm Bull.* 27 (2) 1979; :515–521.
10. Eide DJ. Zinc transporters and the cellular trafficking of zinc. *Bba-Bioenergetics.* 1763 (7) 2006; :711–722. [PubMed: 16675045]

11. Jeong J, Eide DJ. The SLC39 family of zinc transporters. *Mol Aspects Med.* 34 (2–3) 2013; :612–619. [PubMed: 23506894]
12. Jenkitkasemwong S, Wang CY, Mackenzie B, Knutson MD. Physiologic implications of metal-ion transport by ZIP14 and ZIP8. *Biometals.* 25 (4) 2012; :643–655. [PubMed: 22318508]
13. Leyva-Illades D, Chen P, Zogzas CE, Hutchens S, Mercado JM, Swaim CD, Morrisett RA, Bowman AB, Aschner M, Mukhopadhyay S. SLC30A10 is a cell surface-localized manganese efflux transporter, and parkinsonism-causing mutations block its intracellular trafficking and efflux activity. *J Neurosci.* 34 (42) 2014; :14079–14095. [PubMed: 25319704]
14. Nishito Y, Tsuji N, Fujishiro H, Takeda TA, Yamazaki T, Teranishi F, Okazaki F, Matsunaga A, Tuschl K, Rao R, Kono S, Miyajima H, Narita H, Himeno S, Kambe T. Direct comparison of manganese Detoxification/Efflux proteins and molecular characterization of ZnT10 protein as a manganese transporter. *J Biol Chem.* 291 (28) 2016; :14773–14787. [PubMed: 27226609]
15. Kim JH, Jeon J, Shin M, Won Y, Lee M, Kwak JS, Lee G, Rhee J, Ryu JH, Chun CH, Chun JS. Regulation of the catabolic cascade in osteoarthritis by the zinc-ZIP8-MTF1 axis. *Cell.* 156 (4) 2014; :730–743. [PubMed: 24529376]
16. Wang W, Pan H, Murray K, Jefferson BS, Li Y. Matrix metalloproteinase-1 promotes muscle cell migration and differentiation. *Am J Pathol.* 174 (2) 2009; :541–549. [PubMed: 19147819]
17. Caron NJ, Asselin I, Morel G, Tremblay JP. Increased myogenic potential and fusion of matrilysin-expressing myoblasts transplanted in mice. *Cell Transplant.* 8 (5) 1999; :465–476. [PubMed: 10580341]
18. Ohtake Y, Tojo H, Seiki M. Multifunctional roles of MT1-MMP in myofiber formation and morphostatic maintenance of skeletal muscle. *J Cell Sci.* 119 (Pt 18) 2006; :3822–3832. [PubMed: 16926191]
19. Chen X, Li Y. Role of matrix metalloproteinases in skeletal muscle: migration, differentiation, regeneration and fibrosis. *Cell Adh Migr.* 3 (4) 2009; :337–341. [PubMed: 19667757]
20. Chowanadisai W, Graham DM, Keen CL, Rucker RB, Messerli MA. Neurulation and neurite extension require the zinc transporter ZIP12 (slc39a12). *Proc Natl Acad Sci U S A.* 110 (24) 2013; :9903–9908. [PubMed: 23716681]
21. Aydemir TB, Chang SM, Guthrie GJ, Maki AB, Ryu MS, Karabiyik A, Cousins RJ. Zinc transporter ZIP14 functions in hepatic zinc, iron and glucose homeostasis during the innate immune response (endotoxemia). *PloS One.* 7 (10) 2012; :e48679. [PubMed: 23110240]
22. Fujishiro H, Yano Y, Takada Y, Tanihara M, Himeno S. Roles of ZIP8, ZIP14, and DMT1 in transport of cadmium and manganese in mouse kidney proximal tubule cells. *Metallomics.* 4 (7) 2012; :700–708. [PubMed: 22534978]
23. Pinilla-Tenas JJ, Sparkman BK, Shawki A, Illing AC, Mitchell CJ, Zhao N, Liuzzi JP, Cousins RJ, Knutson MD, Mackenzie B. Zip14 is a complex broad-scope metal-ion transporter whose functional properties support roles in the cellular uptake of zinc and nontransferrin-bound iron. *Am J Physiol Cell Physiol.* 301 (4) 2011; :C862–71. [PubMed: 21653899]
24. Hojyo S, Miyai T, Fujishiro H, Kawamura M, Yasuda T, Hijikata A, Bin BH, Irie T, Tanaka J, Atsumi T, Murakami M, Nakayama M, Ohara O, Himeno S, Yoshida H, Koseki H, Ikawa T, Mishima K, Fukada T. Zinc transporter SLC39A10/ZIP10 controls humoral immunity by modulating B-cell receptor signal strength. *Proc Natl Acad Sci U S A.* 111 (32) 2014; :11786–11791. [PubMed: 25074919]
25. Aydemir TB, Sitren HS, Cousins RJ. The zinc transporter Zip14 influences c-Met phosphorylation and hepatocyte proliferation during liver regeneration in mice. *Gastroenterology.* 142 (7) 2012; :1536–1546. e5. [PubMed: 22374166]
26. Stewart R, Flechner L, Montminy M, Berdeaux R. CREB is activated by muscle injury and promotes muscle regeneration. *PloS One.* 6 (9) 2011; :e24714. [PubMed: 21931825]
27. Cousins RJ, Blanchard RK, Popp MP, Liu L, Cao J, Moore JB, Green CL. A global view of the selectivity of zinc deprivation and excess on genes expressed in human THP-1 mononuclear cells. *Proc Natl Acad Sci U S A.* 100 (12) 2003; :6952–6957. [PubMed: 12756304]
28. Inoue Y, Hasegawa S, Ban S, Yamada T, Date Y, Mizutani H, Nakata S, Tanaka M, Hirashima N. ZIP2 protein, a zinc transporter, is associated with keratinocyte differentiation. *J Biol Chem.* 289 (31) 2014; :21451–21462. [PubMed: 24936057]

29. Peters JL, Dufner-Beattie J, Xu W, Geiser J, Lahner B, Salt DE, Andrews GK. Targeting of the mouse *Slc39a2* (*Zip2*) gene reveals highly cell-specific patterns of expression, and unique functions in zinc, iron, and calcium homeostasis. *Genesis*. 45 (6) 2007; :339–352. [PubMed: 17506078]
30. Geiser J, De Lisle RC, Andrews GK. The zinc transporter *Zip5* (*Slc39a5*) regulates intestinal zinc excretion and protects the pancreas against zinc toxicity. *PLoS One*. 8 (11) 2013; :e82149. [PubMed: 24303081]
31. Leung KW, Liu M, Xu X, Seiler MJ, Barnstable CJ, Tombran-Tink J. Expression of ZnT and ZIP zinc transporters in the human RPE and their regulation by neurotrophic factors. *Invest Ophthalmol Vis Sci*. 49 (3) 2008; :1221–1231. [PubMed: 18326752]
32. Guo H, Jin X, Zhu T, Wang T, Tong P, Tian L, Peng Y, Sun L, Wan A, Chen J, Liu Y, Li Y, Tian Q, Xia L, Zhang L, Pan Y, Lu L, Liu Q, Shen L, Xiong W, Li J, Tang B, Feng Y, Zhang X, Zhang Z, Pan Q, Hu Z, Xia K. *SLC39A5* mutations interfering with the BMP/TGF- $\beta$  pathway in non-syndromic high myopia. *J Med Genet*. 51 (8) 2014; :518–525. [PubMed: 24891338]
33. Hogstrand C, Kille P, Ackland ML, Hiscox S, Taylor KM. A mechanism for epithelial-mesenchymal transition and anoikis resistance in breast cancer triggered by zinc channel ZIP6 and STAT3 (signal transducer and activator of transcription 3). *Biochem J*. 455 (2) 2013; :229–237. [PubMed: 23919497]
34. Lue HW, Yang X, Wang R, Qian W, Xu RZ, Lyles R, Osunkoya AO, Zhou BP, Vessella RL, Zayzafoon M, Liu ZR, Zhou HE, Chung LW. LIV-1 promotes prostate cancer epithelial-to-mesenchymal transition and metastasis through HB-EGF shedding and EGFR-mediated ERK signaling. *PLoS One*. 6 (11) 2011; :e27720. [PubMed: 22110740]
35. Sansone P, Bromberg J. Targeting the interleukin-6/Jak/stat pathway in human malignancies. *J Clin Oncol*. 30 (9) 2012; :1005–1014. [PubMed: 22355058]
36. Unno J, Satoh K, Hirota M, Kanno A, Hamada S, Ito H, Masamune A, Tsukamoto N, Motoi F, Egawa S, Unno M, Horii A, Shimosegawa T. LIV-1 enhances the aggressive phenotype through the induction of epithelial to mesenchymal transition in human pancreatic carcinoma cells. *Int J Oncol*. 35 (4) 2009; :813–821. [PubMed: 19724917]
37. Takatani-Nakase T, Matsui C, Maeda S, Kawahara S, Takahashi K. High glucose level promotes migration behavior of breast cancer cells through zinc and its transporters. *PLoS One*. 9 (2) 2014; :e90136. [PubMed: 24587242]
38. Kitamura H, Morikawa H, Kamon H, Iguchi M, Hojyo S, Fukada T, Yamashita S, Kaisho T, Akira S, Murakami M, Hirano T. Toll-like receptor-mediated regulation of zinc homeostasis influences dendritic cell function. *Nat Immunol*. 7 (9) 2006; :971–977. [PubMed: 16892068]
39. Dufner-Beattie J, Huang ZL, Geiser J, Xu W, Andrews GK. Mouse ZIP1 and ZIP3 genes together are essential for adaptation to dietary zinc deficiency during pregnancy. *Genesis*. 44 (5) 2006; :239–251. [PubMed: 16652366]
40. Zou J, Milon BC, Desouki MM, Costello LC, Franklin RB. hZIP1 zinc transporter down-regulation in prostate cancer involves the overexpression of ras responsive element binding protein-1 (RREB-1). *Prostate*. 71 (14) 2011; :1518–1524. [PubMed: 21360563]
41. Costello LC, Franklin RB, Zou J, Feng P, Bok R, Swanson MG, Kurhanewicz J. Human prostate cancer ZIP1/zinc/citrate genetic/metabolic relationship in the TRAMP prostate cancer animal model. *Cancer Biol Ther*. 12 (12) 2011; :1078–1084. [PubMed: 22156800]
42. Higashi Y, Segawa S, Matsuo T, Nakamura S, Kikkawa Y, Nishida K, Nagasawa K. Microglial zinc uptake via zinc transporters induces ATP release and the activation of microglia. *Glia*. 59 (12) 2011; :1933–1945. [PubMed: 22253048]
43. Dufner-Beattie J, Weaver BP, Geiser J, Bilgen M, Larson M, Xu W, Andrews GK. The mouse acrodermatitis enteropathica gene *Slc39a4* (*Zip4*) is essential for early development and heterozygosity causes hypersensitivity to zinc deficiency. *Hum Mol Genet*. 16 (12) 2007; :1391–1399. [PubMed: 17483098]
44. Dufner-Beattie J, Wang F, Kuo YM, Gitschier J, Eide D, Andrews GK. The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. *J Biol Chem*. 278 (35) 2003; :33474–33481. [PubMed: 12801924]

45. Miyai T, Hojyo S, Ikawa T, Kawamura M, Irie T, Ogura H, Hijikata A, Bin BH, Yasuda T, Kitamura H, Nakayama M, Ohara O, Yoshida H, Koseki H, Mishima K, Fukada T. Zinc transporter SLC39A10/ZIP10 facilitates antiapoptotic signaling during early B-cell development. *Proc Natl Acad Sci U S A.* 111 (32) 2014; :11780–11785. [PubMed: 25074913]
46. Myers SA, Nield A, Chew GS, Myers MA. The zinc transporter, Slc39a7 (Zip7) is implicated in glycaemic control in skeletal muscle cells. *PLoS One.* 8 (11) 2013; :e79316. [PubMed: 24265765]
47. Matsuura W, Yamazaki T, Yamaguchi-Iwai Y, Masuda S, Nagao M, Andrews GK, Kambe T. SLC39A9 (ZIP9) regulates zinc homeostasis in the secretory pathway: characterization of the ZIP subfamily I protein in vertebrate cells. *Biosci Biotechnol Biochem.* 73 (5) 2009; :1142–1148. [PubMed: 19420709]
48. Thomas P, Pang Y, Dong J, Berg AH. Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: II. Role of human ZIP9 in testosterone-induced prostate and breast cancer cell apoptosis. *Endocr Metab Immune Disord: Drug Targets.* 155 (11) 2014; :4250–4265.
49. Bin BH, Fukada T, Hosaka T, Yamasaki S, Ohashi W, Hojyo S, Miyai T, Nishida K, Yokoyama S, Hirano T. Biochemical characterization of human ZIP13 protein: a homo-dimerized zinc transporter involved in the spondylocheiro dysplastic Ehlers-Danlos syndrome. *J Biol Chem.* 286 (46) 2011; :40255–40265. [PubMed: 21917916]
50. Fukada T, Civic N, Furuichi T, Shimoda S, Mishima K, Higashiyama H, Idaira Y, Asada Y, Kitamura H, Yamasaki S, Hojyo S, Nakayama M, Ohara O, Koseki H, Dos Santos HG, Bonafe L, Ha-Vinh R, Zankl A, Unger S, Kraenzlin ME, Beckmann JS, Saito I, Rivolta C, Ikegawa S, Superti-Furga A, Hirano T. The zinc transporter SLC39A/ZIP13 is required for connective tissue development; Its involvement in BMP/TGF-beta signaling pathways. *PLoS One.* 3 (11) 2008; :e3642. [PubMed: 18985159]
51. Jeong J, Walker JM, Wang F, Park JG, Palmer AE, Giunta C, Rohrbach M, Steinmann B, Eide DJ. Promotion of vesicular zinc efflux by ZIP13 and its implications for spondylocheiro dysplastic Ehlers-Danlos syndrome. *Proc Natl Acad Sci U S A.* 109 (51) 2012; :E3530–E3538. [PubMed: 23213233]
52. Palmiter RD, Findley SD. Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. *EMBO J.* 14 (4) 1995; :639–649. [PubMed: 7882967]
53. Andrews GK, Wang H, Dey SK, Palmiter RD. Mouse zinc transporter 1 gene provides an essential function during early embryonic development. *Genesis.* 40 (2) 2004; :74–81. [PubMed: 15452870]
54. Kirschke CP, Huang L. ZnT7, a novel mammalian zinc transporter, accumulates zinc in the golgi apparatus. *J Biol Chem.* 278 (6) 2003; :4096–4102. [PubMed: 12446736]
55. Huang L, Kirschke CP, Lay YA, Levy LB, Lamirande DE, Zhang PH. ZnT7-null mice are more susceptible to diet-induced glucose intolerance and insulin resistance. *J Biol Chem.* 287 (40) 2012; :33883–33896. [PubMed: 22854958]
56. Huang L, Yu YY, Kirschke CP, Gertz ER, Lloyd KK. ZnT7 (Slc30a7)-deficient mice display reduced body zinc status and body fat accumulation. *J Biol Chem.* 282 (51) 2007; :37053–37063. [PubMed: 17954933]
57. Inoue K, Matsuda K, Itoh M, Kawaguchi H, Tomoike H, Aoyagi T, Nagai R, Hori M, Nakamura Y, Tanaka T. Osteopenia and male-specific sudden cardiac death in mice lacking a zinc transporter gene, ZnT5. *Hum Mol Genet.* 11 (15) 2002; :1775–1784. [PubMed: 12095919]
58. Fukunaka A, Suzuki T, Kurokawa Y, Yamazaki T, Fujiwara N, Ishihara K, Migaki H, Okumura K, Masuda S, Yamaguchi-Iwai Y, Nagao M, Kambe T. Demonstration and characterization of the heterodimerization of ZnT5 and ZnT6 in the early secretory pathway. *J Biol Chem.* 284 (45) 2009; :30798–30806. [PubMed: 19759014]
59. Liuzzi JP, Bobo JA, Cui L, McMahon RJ, Cousins RJ. Zinc transporters 1, 2 and 4 are differentially expressed and localized in rats during pregnancy and lactation. *J Nutr.* 133 (2) 2003; :342–351. [PubMed: 12566465]
60. Guo L, Lichten LA, Ryu MS, Liuzzi JP, Wang F, Cousins RJ. STAT5-gluco-corticoid receptor interaction and MTF-1 regulate the expression of ZnT2 (Slc30a2) in pancreatic acinar cells. *Proc Natl Acad Sci U S A.* 107 (7) 2010; :2818–2823. [PubMed: 20133611]

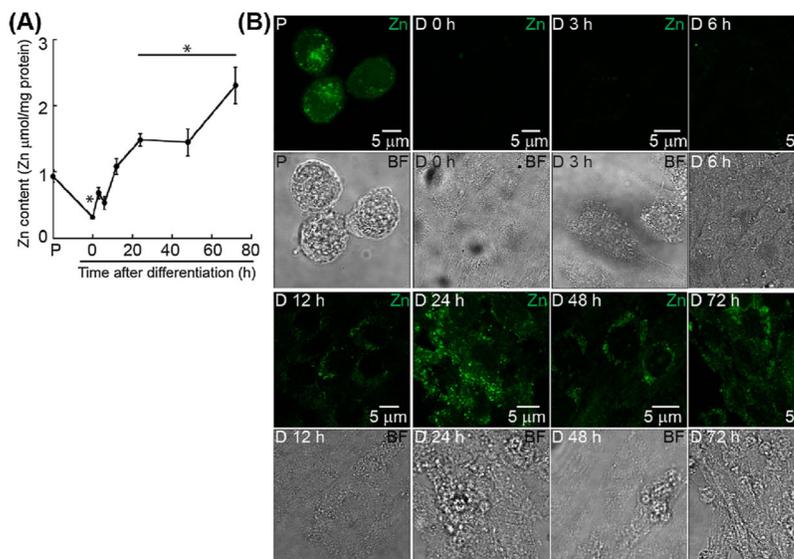
61. Chowanadisai W, Lonnerdal B, Kelleher SL. Identification of a mutation in SLC30A2 (ZnT-2) in women with low milk zinc concentration that results in transient neonatal zinc deficiency. *J Biol Chem.* 281 (51) 2006; :39699–39707. [PubMed: 17065149]
62. Isumura N, Inamo Y, Okazaki F, Teranishi F, Narita H, Kambe T, Kodama H. Compound heterozygous mutations in SLC30A2/ZnT2 results in low milk zinc concentrations: a novel mechanism for zinc deficiency in a breast-fed infant. *PLoS One.* 8 (5) 2013; :e64045. [PubMed: 23741301]
63. Lasry I, Seo YA, Ityel H, Shalva N, Pode-Shakked B, Glaser F, Berman B, Berezovsky I, Goncarenco A, Klar A, Levy J, Anikster Y, Kelleher SL, Assaraf YG. A dominant negative heterozygous G87R mutation in the zinc transporter, ZnT-2 (SLC30A2), results in transient neonatal zinc deficiency. *J Biol Chem.* 287 (35) 2012; :29348–29361. [PubMed: 22733820]
64. Miletta MC, Bieri A, Kernland K, Schoni MH, Petkovic V, Fluck CE, Eble A, Mullis PE. Transient neonatal zinc deficiency caused by a heterozygous G87R mutation in the zinc transporter znT-2 (SLC30A2) Gene in the mother highlighting the importance of zn (2+) for Normal growth and development. *Int J Endocrinol.* 2013 2013; :259189. [PubMed: 24194756]
65. Palmiter RD, Cole TB, Quaife CJ, Findley SD. ZnT-3, a putative transporter of zinc into synaptic vesicles. *Proc Natl Acad Sci U S A.* 93 (25) 1996; :14934–14939. [PubMed: 8962159]
66. Cole TB, Wenzel HJ, Kafer KE, Schwartzkroin PA, Palmiter RD. Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. *Proc Natl Acad Sci U S A.* 96 (4) 1999; :1716–1721. [PubMed: 9990090]
67. Smidt K, Jessen N, Petersen AB, Larsen A, Magnusson N, Jeppesen JB, Stoltenberg M, Culvenor JG, Tsatsanis A, Brock B, Schmitz O, Wogensen L, Bush AI, Rungby J. SLC30A3 responds to glucose- and zinc variations in beta-cells and is critical for insulin production and in vivo glucose-metabolism during beta-cell stress. *PLoS One.* 4 (5) 2009; :e5684. [PubMed: 19492079]
68. Myers SA, Nield A, Myers M. Zinc transporters, mechanisms of action and therapeutic utility: implications for type 2 diabetes mellitus. *J Nutr Metab.* 2012 2012; :173712. [PubMed: 23304467]
69. Lemaire K, Ravier MA, Schraenen A, Creemers JW, Van de Plas R, Granvik M, Van Lommel L, Waelkens E, Chimienti F, Rutter GA, Gilon P, in't Veld PA, Schuit FC. Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. *Proc Natl Acad Sci U S A.* 106 (35) 2009; :14872–14877. [PubMed: 19706465]
70. Polozov RV, Sivozhelzov VS, Chirgadzhe YN, Ivanov VV. Recognition rules for binding of Zn-Cys2His2 transcription factors to operator DNA. *J Biomol Struct Dyn.* 33 (2) 2015; :253–266. [PubMed: 24460547]
71. Elrod-Erickson M, Benson TE, Pabo CO. High-resolution structures of variant Zif268-DNA complexes: implications for understanding zinc finger-DNA recognition. *Structure.* 6 (4) 1998; :451–464. [PubMed: 9562555]
72. Miller JC, Pabo CO. Rearrangement of side-chains in a Zif268 mutant highlights the complexities of zinc finger-DNA recognition. *J Mol Biol.* 313 (2) 2001; :309–315. [PubMed: 11800559]
73. Nardelli J, Gibson T, Charnay P. Zinc finger-DNA recognition: analysis of base specificity by site-directed mutagenesis. *Nucleic Acids Res.* 20 (16) 1992; :4137–4144. [PubMed: 1508708]
74. Pavletich NP, Pabo CO. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. *Science.* 252 (5007) 1991; :809–817. [PubMed: 2028256]
75. Rutherford JC, Bird AJ. Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. *Eukaryot Cell.* 3 (1) 2004; :1–13. [PubMed: 14871932]
76. Wu FY, Wu CW. Zinc in DNA replication and transcription. *Annu Rev Nutr.* 7 1987; :251–272. [PubMed: 2440465]
77. Kelleher SL, Velasquez V, Croxford TP, McCormick NH, Lopez V, MacDavid J. Mapping the zinc-transporting system in mammary cells: molecular analysis reveals a phenotype-dependent zinc-transporting network during lactation. *J Cell Physiol.* 227 (4) 2012; :1761–1770. [PubMed: 21702047]
78. Chen YH, Kim JH, Stallcup MR. GAC63, a GRIP1-dependent nuclear receptor coactivator. *Mol Cell Biol.* 25 (14) 2005; :5965–5972. [PubMed: 15988012]

79. Sim DL, Chow VT. The novel human HUEL (C4orf1) gene maps to chromosome 4p12-p13 and encodes a nuclear protein containing the nuclear receptor interaction motif. *Genome Res.* 59 (2) 1999; :224–233.
80. Fraústo da Silva, JJR, Williams, RJP. *The Biological Chemistry of the Elements: the Inorganic Chemistry of Life. 2.* Oxford University Press; Oxford: 2001.
81. Outten CE, O'Halloran TV. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science.* 292 (5526) 2001; :2488–2492. [PubMed: 11397910]
82. Qin Y, Dittmer PJ, Park JG, Jansen KB, Palmer AE. Measuring steady-state and dynamic endoplasmic reticulum and golgi Zn<sup>2+</sup> with genetically encoded sensors. *Proc Natl Acad Sci U S A.* 108 (18) 2011; :7351–7356. [PubMed: 21502528]
83. Sensi SL, Canzoniero LM, Yu SP, Ying HS, Koh JY, Kerchner GA, Choi DW. Measurement of intracellular free zinc in living cortical neurons: routes of entry. *J Neurosci.* 17 (24) 1997; :9554–9564. [PubMed: 9391010]
84. Vinkenborg JL, Nicolson TJ, Bellomo EA, Koay MS, Rutter GA, Merckx M. Genetically encoded FRET sensors to monitor intracellular Zn<sup>2+</sup> homeostasis. *Nat Methods.* 6 (10) 2009; :737–740. [PubMed: 19718032]
85. Haase H, Rink L. Zinc signals and immune function. *Biofactors.* 40 (1) 2014; :27–40. [PubMed: 23804522]
86. Thiers RE, Vallee BL. Distribution of metals in subcellular fractions of rat liver. *J Biol Chem.* 226 (2) 1957; :911–920. [PubMed: 13438880]
87. Jackson, MJ. *Physiology of Zinc: General Aspects.* Springer; New York: 1989.
88. Ohashi K, Nagata Y, Wada E, Zammit PS, Shiozuka M, Matsuda R. Zinc promotes proliferation and activation of myogenic cells via the PI3K/Akt and ERK signaling cascade. *Exp Cell Res.* 333 (2) 2015; :228–237. [PubMed: 25773777]
89. Petrie L, Chesters JK, Franklin M. Inhibition of myoblast differentiation by lack of zinc. *Biochem J.* 276 (Pt 1) 1991; :109–111. [PubMed: 2039464]
90. Burattini S, Ferri P, Battistelli M, Curci R, Luchetti F, Falcieri E. C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *Eur J Histochem: EJH.* 48 (3) 2004; :223–233. [PubMed: 15596414]
91. Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature.* 270 (5639) 1977; :725–727. [PubMed: 563524]
92. Bajaj P, Reddy B Jr, Millet L, Wei C, Zorlutuna P, Bao G, Bashir R. Patterning the differentiation of C2C12 skeletal myoblasts. *Integr Biol: Quant Biosci Nano Macro.* 3 (9) 2011; :897–909.
93. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72 1976; :248–254. [PubMed: 942051]
94. Raimunda D, Padilla-Benavides T, Vogt S, Boutigny S, Tomkinson KN, Finney LA, Argüello JM. Periplasmic response upon disruption of transmembrane Cu<sup>++</sup> transport in *Pseudomonas aeruginosa*. *Metallomics.* 5 (2) 2013; :144–151. [PubMed: 23354150]
95. Raimunda D, Long JE, Padilla-Benavides T, Sasseti CM, Arguello JM. Differential roles for the Co(2+)/Ni(2+) transporting ATPases, CtpD and CtpJ, in *Mycobacterium tuberculosis* virulence. *Mol Microbiol.* 91 (1) 2014; :185–197. [PubMed: 24255990]
96. Padilla-Benavides T, Long JE, Raimunda D, Sasseti CM, Argüello JM. A novel PIB-type Mn<sup>2+</sup>-transporting ATPase is required for secreted protein metallation in mycobacteria. *J Biol Chem.* 288 (16) 2013; :11334–11347. [PubMed: 23482562]
97. Cheng L, Wang F, Shou H, Huang F, Zheng L, He F, Li J, Zhao FJ, Ueno D, Ma JF, Wu P. Mutation in nicotianamine aminotransferase stimulated the Fe(II) acquisition system and led to iron accumulation in rice. *Plant Physiol.* 145 (4) 2007; :1647–1657. [PubMed: 17951455]
98. Stevens BJ, Hare DJ, Volitakis I, Cherny RA, Roberts BR. Direct determination of zinc in plasma by graphite furnace atomic absorption spectrometry using palladium/magnesium and EDTA matrix modification with high temperature pyrolysis. *J Anal At Spectrom.* 32 (4) 2017; :843–847.
99. Kelleher SL, Lopez V, Lonnerdal B, Dufner-Beattie J, Andrews GK. Zip3(Slc39a3) functions in zinc reuptake from the alveolar lumen in lactating mammary gland. *Am J Physiol Regul Integr Comp Physiol.* 297 (1) 2009; :R194–201. [PubMed: 19458277]

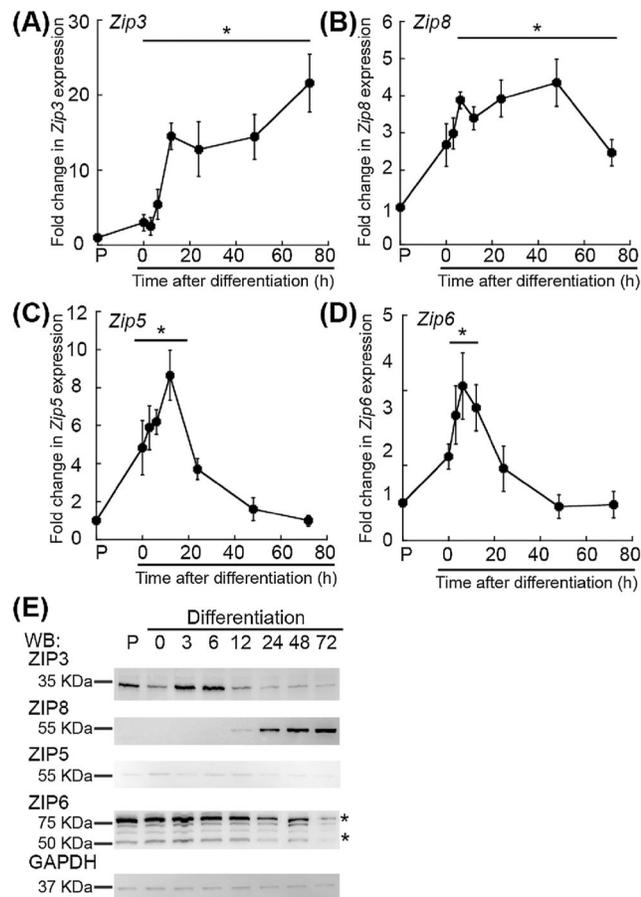
100. McCormick NH, Kelleher SL. ZnT4 provides zinc to zinc-dependent proteins in the trans-Golgi network critical for cell function and Zn export in mammary epithelial cells. *Am J Physiol Cell Physiol.* 303 (3) 2012; :C291–C297. [PubMed: 22621784]
101. Culotta VC, Yang M, O’Halloran TV. Activation of superoxide dismutases: putting the metal to the pedal. *Bba-Bioenergetics.* 1763 (7) 2006; :747–758. [PubMed: 16828895]
102. Qiao W, Ellis CD, Wu CY, Eide DJ. Zinc metallation of yeast alkaline phosphatase and its protein level response to zinc. *Faseb J.* 22 2008;
103. Tuschl K, Clayton PT, Gospe SM Jr, Gulab S, Ibrahim S, Singhi P, Aulakh R, Ribeiro RT, Barsottini OG, Zaki MS, Del Rosario ML, Dyack S, Price V, Rideout A, Gordon K, Wevers RA, Chong WK, Mills PB. Syndrome of hepatic cirrhosis, dystonia, polycythemia, and hypermanganesemia caused by mutations in SLC30A10, a manganese transporter in man. *Am J Hum Genet.* 90 (3) 2012; :457–466. [PubMed: 22341972]
104. Quadri M, Federico A, Zhao T, Breedveld GJ, Battisti C, Delnooz C, Severijnen LA, Di Toro Mammarella L, Mignarri A, Monti L, Sanna A, Lu P, Punzo F, Cossu G, Willemsen R, Rasi F, Oostra BA, van de Warrenburg BP, Bonifati V. Mutations in SLC30A10 cause parkinsonism and dystonia with hypermanganesemia, polycythemia, and chronic liver disease. *Am J Hum Genet.* 90 (3) 2012; :467–477. [PubMed: 22341971]
105. Giunta C, Elcioglu NH, Albrecht B, Eich G, Chambaz C, Janecke AR, Yeowell H, Weis M, Eyre DR, Kraenzlin M, Steinmann B. Spondylocheiro dysplastic form of the Ehlers-Danlos syndrome—an autosomal-recessive entity caused by mutations in the zinc transporter gene SLC39A13. *Am J Hum Genet.* 82 (6) 2008; :1290–1305. [PubMed: 18513683]
106. Miranda ER, Dey CS. Effect of chromium and zinc on insulin signaling in skeletal muscle cells. *Biol Trace Element Res.* 101 (1) 2004; :19–36.

## Appendix A. Supplementary data

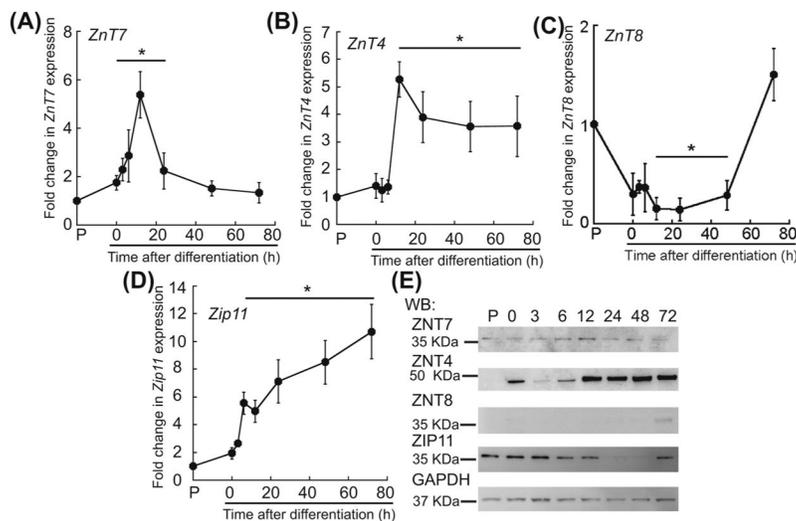
Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jtemb.2018.04.024>.



**Fig. 1.** Changes in Zn content in proliferating and differentiating C2C12 murine myoblasts. (A) Whole cell Zn content was determined by furnace AAS and normalized to protein content in the sample. Data represent the average of three independent experiments  $\pm$  S.E; Student *T*-test comparing the differentiation time points to proliferating cells; \* $P < 0.005$ . (B) Representative confocal images of three independent biological experiments of live proliferating and differentiating myoblasts labeled with Fluo-Zin3 (green) and the corresponding bright field image (BF) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



**Fig. 2.** Expression profile of Zn importers located at the cell membrane during C2C12 differentiation. Gene expression profiles of *Zip3* (A), *Zip8* (B), *Zip5* (C), *Zip6* (D). Data represent the mean of at least three independent experiments  $\pm$  S.E; Student *T*-test comparing the differentiation time points to proliferating cells; \**P* < 0.01. (E) Representative Western blots of ZIP3, ZIP8, ZIP5 and ZIP6. GAPDH as loading control.



**Fig. 3.** Expression profiles of Zn transporters found in subcellular compartments during C2C12 differentiation. Gene expression profiles of the transporters located in the ER and TGN: *ZnT7* (A), *ZnT4* (B), *ZnT8* (C) and *Zip11* (D). Data represent the mean of at least three independent experiments  $\pm$  S.E; two-tailed unpaired *t*-test; \**P* < 0.01. Representative Western blots of ZnT7, ZnT4, ZnT8 and ZIP11. GAPDH as loading control.