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Manganese influx and expression of ZIP8 is essential in primary myoblasts and contributes to activation of SOD2

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Abstract

Trace elements such as copper (Cu), zinc (Zn), iron (Fe), and manganese (Mn) function as enzyme cofactors and second messengers in cell signaling. Trace elements are emerging as key regulators of differentiation and development of mammalian tissues including blood, brain, and skeletal muscle. We previously reported an influx of Cu and dynamic expression of metal transporters during differentiation of skeletal muscle cells. Here, we demonstrate that during differentiation of skeletal myoblasts an increase of Mn, Fe and Zn also occurs. Interestingly the Mn increase is concomitant with increased Mn-dependent SOD2 levels. To better understand the Mn import pathway in skeletal muscle cells, we probed the functional relevance of the closely related proteins ZIP8 and ZIP14, which are implicated in Zn, Mn, and Fe transport. Partial depletion of ZIP8 severely impaired growth of myoblasts and led to cell death under differentiation conditions, indicating that ZIP8-mediated metal transport is essential in skeletal muscle cells. Moreover, knockdown of *Zip8* impaired activity of the Mn-dependent SOD2. Growth defects were partially rescued only by Mn supplementation to the medium, suggesting additional functions for ZIP8 in the skeletal muscle lineage. Restoring wild type *Zip8* into the knockdown cells rescued the proliferation and differentiation phenotypes. On the other hand, knockdown of *Zip14*, had only a mild effect on myotube size, consistent with a role for ZIP14 in muscle hypertrophy. Simultaneous knockdown of both *Zip8* and *Zip14* further impaired differentiation and led cell death. This is the first report on the functional relevance of two members of the ZIP family of metal transporters in the skeletal muscle lineage, and further supports the paradigm that trace metal transporters are important modulators of mammalian tissue development.

Introduction

Transition metals such as copper (Cu), zinc (Zn), iron (Fe), and manganese (Mn), are essential for normal cellular function due to their roles as catalytic or structural cofactors

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Conflicts of interest

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and as signalling molecules. Transition metals also function as key regulators of mammalian development^{1–8}. For example, Fe is a well-characterized regulator of erythropoiesis due to the high demand for heme biosynthesis in erythroid cells (reviewed by Ganz, *et al.*, 2012, ⁹). Recent studies have implicated other transition metals such as Cu in the differentiation of muscle, neuronal, and blood cells^{1, 2, 10}. Like Cu and Fe, Mn acts as a catalytic or structural cofactor in enzymes involved in removal of oxygen radicals, gluconeogenesis, and protein glycosylation^{11–13}. Excess Mn is toxic, and Mn accumulation leads to a neurodegenerative condition with symptoms similar to Parkinson's disease and dystonia^{14–16}. While few cases of dietary Mn deficiency have been reported, mutations in the Mn transporter, ZIP8, have been associated with skeletal and neuromuscular symptoms¹⁷. However, few studies have shown how Mn homeostasis and other transition metals impact skeletal muscle development.

Skeletal muscle largely consists of elongated, post-mitotic myofibers and a closely associated pool of stem cells, termed satellite cells^{18–25}. Satellite cells are critical for embryonic and early postnatal muscle development, muscle regeneration, and may be important for maintenance of basal muscle function^{26–28}. In response to injury or muscle damage, quiescent satellite cells are activated, proliferate as myoblasts, and differentiate into myocytes to regenerate skeletal muscle myofibers^{19–22, 24, 25, 29–38}. During this process, multiple metabolic changes occur to meet the fluctuating energetic demands. Quiescent satellite cells typically rely on fatty acid oxidation while proliferating myoblasts primarily utilize glycolysis for energy generation^{37, 39}. During myoblast differentiation to myocytes, there is increased mitochondrial biogenesis and utilization of oxidative metabolism³⁹. Given the importance of the mitochondrial Mn-superoxide dismutase (SOD2), Mn-influx and dynamic expression of Mn-transporting proteins are likely important factors in differentiation of skeletal muscle cells.

Several membrane metal transporters have been proposed to mobilize Mn in mammals^{40–45}. Among these, two members of the family of Zrt- and Irt-like proteins (ZIP), ZIP8 (SLC39A8) and ZIP14 (SLC39A14), are considered the major Mn transporters in mammals^{41, 46–48}. ZIP transporters have been mostly characterized for their function in importing Zn from the extracellular milieu to the cytosol^{48–50}. They contain eight transmembrane helices (TM), form dimers, and have nanomolar to micromolar affinity for Zn^{40, 46, 48, 51–53}. ZIP8 and ZIP14 have also been implicated in transport of Zn, Mn, Fe, Cu, and cadmium (Cd)⁵⁴. These two proteins are the most closely related members of the LIV-1 subfamily of ZIPs, which contains the Zn-binding motif HEXXH in transmembrane domain 5 (TM5) (reviewed by Kambe *et al.*, 2015, and Jenkitkasemwong, *et al.*, 2015, ^{48, 55}). Both ZIP8 and ZIP14 contain a modified EEEXH motif in TM5; this single amino acid substitution may allow these proteins to transport other metals (reviewed by Jenkitkasemwong, *et al.*, 2015, ⁵⁵). Thus, the specific metal binding residues in the TM domains of ZIP proteins may confer metal specificity.

Both ZIP8 and ZIP14 are associated with human disease. Individuals with mutations in ZIP8 experience severe neurodevelopmental defects, profound hypotonia, and skeletal abnormalities^{17, 56}. Tissue-wide and liver-specific *Zip8* knockout in mice revealed that ZIP8-mediated Mn uptake from the bile in hepatocytes is required for systemic Mn availability⁵⁷. Individuals with mutations in *ZIP14* experience parkinsonism-dystonia due to

accumulation of toxic Mn levels⁵⁸. Subsequent animal studies revealed that ZIP14 modulates systemic Mn levels by importing circulating Mn into hepatocytes^{45, 59–62}. However, mice with liver-specific ZIP14 knockout did not accumulate Mn in the brain, indicating an important function for ZIP14 in other tissues⁶⁰. Although both ZIP8 and ZIP14 are involved in liver-mediated regulation of systemic Mn, tissue-specific functions cannot be ruled out. A similar situation exists for ATP7B, which is important for copper excretion to bile in the liver, but also has important tissue-specific functions in intestinal and neuronal tissue^{63, 64}. The fact that individuals with mutations in ZIP8 and ZIP14 experience hypotonia suggests that these proteins may be important in the development or function of skeletal muscle. However, muscle phenotypes, such as regeneration after induced injury or early postnatal myogenesis have not been tested in murine ZIP8 and ZIP14 knockouts^{45, 57, 59–62}.

Despite the potential role for these ZIP transporters to mobilize a variety of ions, limited information is available regarding the participation of these metals and transporters in mammalian skeletal muscle development. Importantly, our group demonstrated dynamic changes in the steady-state levels of mRNAs and proteins encoding the putative Mn transporters ZIP8 and ZIP14 during differentiation of immortalized muscle stem cell-derived C2C12 myoblasts⁶⁵. Therefore, we hypothesized that a ZIP8- and/or ZIP14-mediated influx of intracellular Mn is necessary for myoblast differentiation.

We investigated the role of Mn in skeletal muscle differentiation using murine satellite cell-derived primary myoblasts. We detected increased cellular Mn in primary myoblasts starting at 24 h after inducing differentiation along with increased levels and activity of the manganese-dependent SOD2. We also detected increased expression of ZIP8 and ZIP14 during myoblast differentiation, suggesting that these two transporters are important mediators of metal mobilization in differentiating skeletal muscle cells. Partial loss of ZIP8 by shRNA-mediated knockdown led to cell death when myoblasts were induced to differentiate. ZIP8 knockdown also impaired proliferation of primary myoblasts, which supports an essential role for this transporter in the skeletal muscle lineage. Knockdown of *Zip14* partially impaired myotube formation but did not cause cell death indicating that ZIP14 contributes to, but is not essential for myogenesis. This study demonstrates that Mn, partially targeted to SOD2, is indispensable for the differentiation of the skeletal muscle lineage and that ZIP8 contributes to this process in primary cells. These results further support a critical role for transition metals in skeletal muscle differentiation, and this work lays the groundwork for future mechanistic studies to understand the tissue-specific transport functions of ZIP family proteins.

Results

Manganese and Mn-dependent SOD2 accumulate in differentiating myoblasts

Previous reports from our group showed that the concentrations of intracellular Zn and Cu increase during myogenesis^{2, 65}. To better understand how other metals fluctuate during myogenesis, we utilized ICP-OES to measure total metal levels in proliferating and differentiating primary myoblasts. We detected that cellular Mn levels trended higher starting 24 h after inducing differentiation and were significantly increased at 72 h after

differentiation (Fig. 1A). We also detected a small but not significant increase in cellular Fe after 72 h of differentiation (Fig. 1B). Consistent with our study of Zn in immortalized C2C12 myoblasts^{65, 66}, we detected a slight increase in intracellular Zn 48 h after inducing differentiation in primary myoblasts (Fig. 1C). In all cases, intracellular Ca levels, which increase starting 24 h after inducing differentiation, were used as a positive control for differentiated muscle cells (Fig. 1D).

During differentiation, myoblasts switch from primarily glycolytic to a more oxidative metabolism^{39, 67–69}. This is associated with increased mitochondrial biogenesis and reactive oxygen species generation^{39, 60–62}. Therefore, the mitochondrial Mn-dependent SOD2 is a likely target of the increased influx of intracellular manganese during myogenic differentiation. Immunoblot analysis and in-gel activity staining revealed an increased level of SOD2 protein and activity during differentiation of primary myoblasts when compared to proliferating cells (Fig. 2). Importantly, SOD2 was distinguished from SOD1, the Cu/Zn superoxide dismutase by potassium cyanide (KCN) inhibition⁷⁰ (Supp. Fig. 1). These data suggest that SOD2 is an important target for Mn during myogenic differentiation.

Expression of ZIP8 and ZIP14 increases during differentiation of primary myoblasts derived from mouse satellite cells.

To better understand how Mn is transported into primary myoblasts during differentiation, we looked to the ZIP family of metal transporters. ZIP8 and ZIP14 are two homologous Zn, Mn and Fe transporters that share 46.6% identity and 62.0% similarity (Supp. Fig. 2). Previous investigations from our laboratory showed that in immortalized C2C12 myoblasts, expression of ZIP8 but not ZIP14 was induced during myogenesis⁶⁵. Here, we studied expression of ZIP8 and ZIP14 in primary myoblasts derived from mouse hind limb muscle stem cells. To determine whether the increase in ZIP8 was recapitulated in primary myoblasts, we performed immunoblots using antibodies to ZIP8 and ZIP14 in proliferating cells and cells differentiated for 24, 48, and 72 h. As shown in Fig. 3A, levels of ZIP8 increased by approximately 50% starting 24 h after inducing differentiation and more than doubled after 72 h. This is similar to the increase detected in differentiating C2C12 myoblasts⁶⁵. Levels of ZIP14 increased at 48 and 72 h after inducing differentiation (Fig. 3B). These results indicate that, unlike ZIP14 in C2C12 myoblast differentiation, levels of ZIP14 increase during later stages of primary myoblast differentiation.

Zip8 and Zip14 knockdowns have differential effects on the differentiation of primary myoblasts

Given the increased expression of ZIP8 and ZIP14 observed during primary myoblast differentiation (Fig. 3) and the roles of these proteins in Mn transport^{41, 71, 72}, we hypothesized that ZIP8- and/or ZIP14-dependent Mn transport may contribute to the maturation of the skeletal muscle lineage. To determine the role of ZIP8 and ZIP14 in myoblast differentiation, we used three lentiviral constructs encoding small hairpin RNAs (shRNA) to either *Zip8* or *Zip14* knock down these two transporters in proliferating and differentiating myoblasts (Supp. Table 1). The virus-infected cells were selected with puromycin and the levels of ZIP8 and ZIP14 were examined by immunoblot (Fig. 3, Supp. Fig. 3). Cells containing *Zip8* shRNA failed to increase expression of ZIP8 protein upon

inducing differentiation (Fig. 3A, Supp. Fig. 3A). Cells containing *Zip14* shRNA showed increased levels of ZIP14 during differentiation but to a much lesser degree and at a later time point than wild type cells (Fig. 3B, Supp. Fig. 3B).

To determine whether the partial loss of ZIP8 or ZIP14 impairs myogenesis, *Zip8* and *Zip14* shRNA primary myoblasts were grown to confluence and induced to differentiate by serum deprivation⁷³. Immunocytochemical staining and immunoblot analyses for the lineage specific differentiation marker myogenin were used as indicators of myogenesis in samples taken during the proliferation stage and at 24, 48 and 72 h post-differentiation (Fig. 4, Supp. Fig. 4 and 5). *Zip8* knockdown cells did form elongated myocytes 24 h after inducing differentiation, but also detached from the plates and failed to form myotubes at later time points (Fig. 4A, Supp. Fig. 4A). The *Zip8* knockdown cells had decreased myogenin positive nuclei and showed a significantly lower fusion index than wild type cells at similar time points (Fig. 4A, B, Supp. Fig. 4, 5). Cells containing *Zip14* shRNA formed myotubes but also showed a lower fusion index than control cells (Fig. 4A, B, Supp. Fig. 4, 5). These results suggest that ZIP14 contributes to robust myotube formation, but ZIP8 is essential for differentiation.

The detachment detected in *Zip8* differentiating knockdown cells suggests that partial loss of ZIP8 leads to cell death. A major cell death pathway is caspase 3 dependent apoptosis⁷⁴. Myoblast differentiation requires the disassembly and reorganization of actin fibres and the activation of myosin light chain kinase, events that also occur during apoptosis^{75–77}. Previous studies have shown that myogenic differentiation and apoptosis undergo similar mechanisms of cleaving and activation of caspase 3^{78–81}. We employed immunoblot assay to detect cleaved Caspase 3 in proliferating and differentiating primary myoblasts. We found the expected low levels of cleaved caspase 3 in wild type proliferating and differentiating myoblasts (Supp. Fig. 5B). Furthermore, we observed an increase in the cleaved caspase 3 at 48 h after inducing myogenesis in cells transduced with the three different *Zip8* shRNA (Supp. Fig. 5B). No changes in the levels of cleaved caspase 3 were detected in the *Zip14*-treated shRNA myoblasts, which is consistent with the fact that these cells did not detach upon inducing differentiation (Supp. Fig. 5B).

***Zip8* knockdown cells fail to expand intracellular Mn after being induced to differentiate**

Both ZIP8 and ZIP14 have been implicated in transport of Mn, Zn and Fe (reviewed by Jenikikasemwong, *et al.*, 2015, ⁵⁵), which are critical for cell growth and survival⁴¹. To understand how partial loss of ZIP8 and ZIP14 affect accumulation of Mn, Fe, and Zn, we used ICP-OES to measure total metal levels in differentiating *Zip8* and *Zip14* knockdown cells (Fig. 5). Similar to wild type cells (Fig. 1A), myoblasts transfected with scrambled (scr) shRNA showed increased intracellular Mn starting at 24 h after inducing myogenesis (Fig. 6A). Cells transduced with *Zip8* shRNA failed to accumulate Mn while those transduced with *Zip14* shRNA accumulated the same or higher levels of Mn compared to control cells (Fig. 5A; Supp. Fig. 6). Measurement of total levels of Fe (Fig. 5B; Supp. Fig. 6), Zn (Fig. 5C; Supp. Fig. 6) revealed similar results, where wild type and *Zip14* knockdown cells showed accumulation at 24 h after inducing differentiation. *Zip8* knockdown cells accumulated Mn to a much lesser degree at 24h after inducing

differentiation and showed no accumulation at later time points (Figure 5A). Total Ca was measured as a positive control for myogenesis (Fig. 5D; Supp. Fig. 6). Total Ca levels increased in wild type cells and in cells transfected with scrambled shRNA or *Zip14* shRNA at 24 h after inducing differentiation (Fig. 5C; Supp. Fig. 6). However, *Zip8* knockdown cells failed to accumulate Ca after the differentiation stimulus (Fig. 5C; Supp. Fig. 6). These results are consistent with the severe differentiation defect observed in *Zip8* knockdown cells (Fig. 4; Supp. Fig. 5).

ZIP8 has been previously shown to transport Mn⁴⁺, which is a cofactor for the mitochondrial SOD⁸². SOD2 has a well-characterized role in maintaining redox balance and mitochondrial function in skeletal muscle, as well as in promoting proliferation and differentiation of myoblasts^{83–85}. To determine whether differentiation defects in *Zip8* knockdown myoblasts are associated with a loss of SOD2 activity, we performed immunoblot and *in-gel* activity assays of SOD2. As shown in Fig. 2, increased steady-state levels and activity of SOD2 were detected in differentiating wild type myoblasts. Therefore we asked whether the partial knockdown of ZIP8 or ZIP14 would impair the expression or activity of SOD2. In differentiating *Zip8* knockdown myoblasts, activity of SOD2 failed to increase and SOD 2 levels increased to a much lesser degree compared to wild type cells (Fig. 6A; Supp. Fig. 7A, C). SOD2 protein levels and activity in proliferating and differentiating *Zip14* knockdown myoblasts were similar to wild type and scramble control (Fig. 6B, Supp. Fig. 7B, D). Protein levels and activity of SOD2 was normalized to total protein (Supp. Fig. 8). These results could support a role for ZIP8, but not ZIP14, in providing Mn for SOD2 in differentiating myoblasts. However, impaired SOD2 activity could also be an indirect result of impaired differentiation in *Zip8* knockdown cells.

Knockdown of *Zip8* impairs proliferation of primary myoblasts

Considering that knockdown of *Zip8* in primary myoblasts led to cell death after inducing differentiation and that Mn, Fe, and Zn are important for cellular growth and survival, we sought to understand how myoblasts proliferation is affected. We used cell counting to as a proxy measurement for proliferation in *Zip8* knockdown cells. While numbers of wild type myoblasts and myoblasts transduced with scrambled shRNA increased 50 fold over three days (Fig. 7, Supp. Fig. 9), cells containing *Zip8* shRNA increased in number only 10 fold during the same period (Fig. 7; Supp. Fig. 9). Expression of PAX7 was normal in proliferating *Zip8* knockdown cells, indicating that growth defects are not related to loss of this myoblast-specific transcription factor (Fig. 7E, F).

Expression of exogenous ZIP8 rescues the proliferation and differentiation phenotypes of *Zip8* knockdown

To assess whether the proliferation and differentiation defects observed in *Zip8* partially depleted myoblasts was in fact due to the loss of this transporter, we performed rescue experiments using exogenous HA-tagged murine ZIP8. Myoblasts were transduced with plasmids encoding wild type *Zip8* and expression was confirmed by immunoblot for ZIP8 and for the HA-tag (Fig. 8A). Differentiation defects detected in *Zip8* knockdown cells were rescued upon expression of wild type ZIP8 as shown by the expression of the myogenic marker Myogenin (Fig. 8A,B). Consistently, the levels of cleaved Caspase 3 were decreased

relative to those detected in *Zip8* knockdown cells (Fig. 8A). Interestingly, protein levels of SOD2 were higher proliferating cells expressing exogenous ZIP8 than levels of SOD2 in wild type proliferating cells (Fig. 8A). Importantly, the proliferation defect observed in *Zip8* knockdown myoblasts was rescued upon expression of the exogenous wild type ZIP8 (Fig. 8C). Consistent with restored ability to differentiate, *Zip8* knockdown cells expressing exogenous wild type ZIP8 showed increased levels of Mn, Fe, and Zn (Fig. 8D–F).

Simultaneous knockdown of *Zip8* and *Zip14* exacerbates defects caused by *Zip8* knockdown

To further demonstrate the importance of ZIP8 and ZIP14 in myogenesis, we transduced cells with shRNAs targeting *Zip8* and *Zip14* and investigated the effect of the double knockdown on differentiation and proliferation of primary myoblasts. Simultaneous knockdown of both transporters prevented differentiation as shown by the lack of myotube formation and Myogenin expression (Fig. 9A, B). We also detected increased levels of cleaved caspase 3, indicating that double knockdown of *Zip8* and *Zip14* causes cell death (Fig. 9A). In fact these cells largely detached within 24 h after applying the differentiation stimulus, thus experiments could not be performed at later time points. SOD2 protein was undetectable by immunoblot in double knockdown cells extracted 24 h after being induced to differentiate (Fig. 9A). As expected, these cells showed nearly zero proliferation (Fig. 9C). Importantly, the levels of Mn, Fe and Zn were dramatically decreased in double knockdown cells (Fig. 9D–F). Taken together, these data show an additive effect of knocking down both *Zip8* and *Zip14*. This result suggests these two transporters act in different pathways and confirm their biological relevance in the skeletal muscle lineage.

Manganese supplementation partially rescues the proliferation defect of *Zip8* knockdown myoblasts

To determine whether impaired metal import is responsible for the growth defect detected in *Zip8* knockdown cells, we supplemented the growth medium with increasing concentrations of Mn (Fig. 10A, Supp. Fig. 10), Fe (Fig. 10B, Supp. Fig. 10), and Zn (Fig. 10C, Supp. Fig. 10). We found that only addition of 300 μ M Mn to the medium partially rescued the growth defects detected in *Zip8* knockdown cells while this same concentration impaired growth in wild type cells (Fig. 10A, Supp. Fig. 10). Cell counting assay revealed that control and Mn-treated cells expressing *Zip14* shRNA proliferated similarly to wild type and shRNA scrambled cells (Fig. 10A, Supp. Fig. 10). Interestingly, Both *Zip8* and *Zip14* knockdown cells were more sensitive to Fe and Zn compared to control cells (Fig. 10B, C, Supp. Fig. 10). These results indicate that partial loss of ZIP8 impacts myoblast proliferation and can be partially rescued by supplementation with exogenous Mn.

To determine whether impaired Mn import is responsible for differentiation defects detected in *Zip8* and *Zip14* knockdown cells, we supplemented differentiation medium with variable concentrations of Mn, Fe, or Zn. None of the supplemented metals restored the differentiation defect of *Zip8* knockdown cells. Fig. 10D (and Supp. Fig. 10D) show representative light microscopy images of the cells cultured at the highest non-toxic concentration of the metals to wild type cells. Higher levels of these metals were also toxic

to the mutant cells. Interestingly, both *Zip8* and *Zip14* knockdown cells were sensitive to Fe and Zn concentrations that are not toxic to wild type cells (Fig. 10D, Supp. Fig. 10D).

Considering the minimal rescue effect of metal supplementation to the phenotypes observed in *Zip8* and *Zip14* knockdown cells, we used ICP-OES to assay Mn, Fe, or Zn accumulation after these metals were added to the medium. Proliferating and differentiating *Zip8* and *Zip14* knockdown cells were able to accumulate Mn when it was added to the medium (Table 1). However, differentiated *Zip8* and *Zip14* knockdown cells accumulated 83% and 76% less Mn than Mn-treated wild type cells, respectively (Table 1). A 64% decrease Fe accumulation was detected in *Zip8* knockdown cells, but *Zip14* knockdown cells showed a nearly ten-fold increase in Fe accumulation relative to wild type cells (Table 1). Similar results were obtained after Zn treatment in *Zip8* knockdown cells but to a much lesser degree. Variations in metal accumulations were also detected based on the shRNA construct transduced into the myoblasts (Supp. Table 2).

Discussion

Myogenesis encompasses metabolic and morphological changes that depend on the bioavailability of transition metals to enable energy production and redox homeostasis^{86, 87}. Here, we show that the intracellular concentration of Mn increases within 24 h of inducing differentiation in primary myoblasts. Mn influx is associated with increased expression and activity of SOD2, which is consistent with mitochondrial biogenesis in differentiating myoblasts^{39, 67, 68}. We sought to understand the functional relevance of metal transporters in myogenesis and we focused on ZIP8 and ZIP14 due to their function in Mn, Fe, and Zn transport^{40, 41, 44, 71, 72}. We detected increased expression of ZIP8 24 h after inducing differentiation of primary myoblasts, which was consistent with our previous findings from C2C12 cells⁶⁵. We also detected a small increase in the expression of ZIP14 after 48 to 72 h of differentiation. The increases in ZIP8 and ZIP14 expression suggest they are important for myoblast differentiation.

To determine the functional significance of ZIP8 expression, we knocked down *Zip8* in primary myoblasts and induced them to differentiate. *Zip8* knockdown cells failed to form myotubes and did not increase expression of the differentiation marker myogenin. We observed no increase in Mn levels and a much smaller expansion of SOD2 levels and activity in *Zip8* knockdown cells, which could be an indirect result of impaired differentiation or cell death. Indeed, we detected an increase in the apoptotic marker cleaved caspase-3 and a severe growth defect in *Zip8* knockdown cells. Taken together, these data indicate that ZIP8 is essential for growth, survival, and differentiation of primary myoblasts. All defects were rescued by expression of exogenous wild type ZIP8, indicating that ZIP8 is essential in muscle cells. Previous studies have demonstrated that ZIP8 expression in skeletal muscle is low relative to other tissues, and that depletion of ZIP8 does not impair embryonic muscle development or cause obvious muscle degeneration^{88, 89}. However, no previous studies have probed the functional importance of ZIP8 in muscle regeneration, post-natal satellite cells, or satellite cell-derived primary myoblasts. Given that several reports have demonstrated a connection between ZIP8 and cancer cell proliferation and survival, ZIP8 expression may be a general requirement for growth and survival in

mammalian cells^{90, 91}. Further studies are needed to determine the link between ZIP8 and cell survival in the context of muscle and other cell types.

Zip8 knockdown caused impaired expansion of SOD2 levels and activity. A previous study of *Sod2*^{+/-} myoblasts showed that partial loss of SOD2 impaired proliferation and differentiation, but not to the degree we observed⁸⁵. The discrepancy between *Sod2*^{+/-} and *Zip8* knockdown myoblasts suggests ZIP8 performs other functions in addition to import of Mn for SOD2. For example, ZIP8 expression is correlated with activation of the metal-sensing transcription factor (MTF1)⁹², which contributes to the expression of skeletal muscle genes in myoblasts⁹³. This model is supported by the fact that Mn supplementation only partially reversed the growth defect in *Zip8* knockdown myoblasts. The exact substrates of ZIP8 in myoblasts remain to be elucidated but are likely Mn, Fe, and Zn and these may vary in different contexts. Although we detected decreased levels of Mn, Zn, and Fe in *Zip8* knockdown cells, we cannot rule out the possibility that this decrease is an indirect effect of the severe growth and differentiation defect caused by partial loss of ZIP8.

To identify additional metal transporters important for myoblast differentiation, we also studied the functional relevance of the closely related transporter, ZIP14. *Zip14* knockdown cells grow normally and are able to form myotubes. However, myotubes were fewer and smaller in *Zip14* knockdown cells. This result suggests that ZIP14 contributes to later stages of myogenesis or myotube hypertrophy rather than myoblast differentiation. We did not detect major changes in Mn, Fe, or Zn accumulation in *Zip14* knockdown cells. However, we observed that Mn accumulation was decreased and Fe accumulation was increased upon metal supplementation compared to wild type cells. Additional studies are needed to further elucidate the specific function of ZIP14 in myoblasts.

Mutations in human *ZIP14* have been linked to symptoms of the early onset of Parkinsonism and dystonia¹⁶. This phenotype has been proposed to be related to Mn accumulation in the nervous system, rather than direct effects in skeletal muscle¹⁶. However, muscle phenotypes were not directly studied in patient tissues or animal models. Therefore, a muscle-specific function of ZIP14 cannot be ruled out. An RNA-Seq study comparing quiescent and activated satellite cells showed that steady-state levels of *Zip14* mRNA increase two-fold in newly activated satellite cells relative to quiescent satellite cells suggesting that ZIP14 has additional functions in satellite cells beyond differentiation⁹⁴. Interestingly, levels of *Zip8* mRNA are also upregulated during early myogenic activation⁹⁴. These two transporters may work cooperatively to mediate metal transport in activated muscle stem cells. Indeed, the subtle phenotype detected in *Zip14* knockdown cells would suggest that metal transport via ZIP8 can partially compensate for the loss of ZIP14. This model is supported by the additive defects we detected in *Zip8* and *Zip14* double knockdown cells. However, future studies are needed to confirm the mechanistic details of ZIP8 and ZIP14 functional overlap in myoblasts.

Conclusions

This study demonstrates the role for manganese influx and SOD2 metallation in the context of skeletal muscle differentiation. We found that ZIP8 is essential in primary myoblasts

derived from mouse satellite cells, and that ZIP14 plays a non-essential role in myotube hypertrophy. Future studies are needed to determine the mechanistic details of the interaction between ZIP8 and ZIP14 and the specific metallo-substrates of each transporter in myoblasts.

Experimental procedures

Isolation, growth and differentiation of primary myoblasts—Mice were housed in the University of Massachusetts Medical School animal care facility as specified by Institutional Animal Care and Use Committee guidelines. Primary myoblasts derived from mouse satellite cells were isolated from hind limb muscles of wild type C57Bl/6 mice as previously described³⁰. The myoblasts were grown in culture plates coated in 0.02% collagen (Advanced BioMatrix), in a 1:1 mix of DMEM and F-12 media (Gibco) supplemented with 20% fetal bovine serum, and 25 ng/ml recombinant basic FGF (Millipore). Myoblasts were induced to differentiate in DMEM containing 2% horse serum and 1% Insulin-Transferrin-Selenium-Sodium Pyruvate (Thermo Fisher Scientific). All cells were maintained in a humidified 5% CO₂ incubator at 37 °C.

Plasmids and lentivirus production—Mission plasmids (Sigma) encoding for three different shRNA against *Zip8* and *Zip14* (Table S1) were isolated by using the Pure yield plasmid midiprep system (Promega) following the manufacturer's instructions. shRNA (15 µg) and the packing vectors pLP1 (15 µg), pLP2 (6 µg), pSVG (3 µg) were transfected using lipofectamine 2000 (Thermo Fisher) into HEK293T cells for lentiviral production. After 24 and 48 h the supernatants containing viral particles were collected and filtered using a 0.22 µm syringe filter (Millipore). Primary myoblasts were transduced with lentivirus in the presence of 8 mg/ml polybrene and selected with 1.5 µg/ml puromycin (Invitrogen). The codon-optimized cDNA of *Zip8* including a C-terminal HA-tag sequence was purchased from GENEWIZ, and subsequently cloned into the pBABE retroviral vector containing a blasticidin resistance gene. The retroviral vector expressing ZIP8 was transfected into BOSC23 cells using Lipofectamine 2000 according to the manufacturer's instructions, and the retroviral particles were collected and transduced into the primary myoblasts as described above. Transduced cells were selected with media containing 4 µg/ml blasticidin (Invitrogen). After selection, the cells were maintained with 1 µg/ml of puromycin and 2 µg/ml of blasticidin. **Whole cell metal content analysis**

Three independent biological replicates of proliferating and differentiating (24, 48, and 72 h) primary myoblasts were rinsed three times with PBS without Ca⁺² and Mg⁺² (Gibco). Cells were resuspended in 100 µl PBS and lysed by sonication using a Diagenode Bioruptor UCD-200. Total protein content was quantified using Bradford assay⁹⁵. The samples were resuspended in concentrated HNO₃ (trace metal grade) and analyzed by inductively coupled plasma-optical emissions spectroscopy (ICP-OES) as previously described⁹⁶. Concentrations of Mn, Zn, Fe, and Ca measured via ICP-OES were normalized to the initial mass of protein in each sample.

Western blot analyses—Protein samples from proliferating and differentiating primary myoblasts (control and *Zip8*- and *Zip14*-knockdown) were 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) supplemented with Complete protease inhibitor cocktail (Roche). Protein content was quantified by Bradford⁹⁵. Samples (20 µg) were separated by SDS-PAGE and electrotransferred to PVDF membranes (Millipore). The proteins of interest were detected using the primary antibodies rabbit anti-ZIP8 (A10395), anti-ZIP14 (A1043), anti-SOD1 (A0274), anti-SOD2 (A1340), anti-Caspase 3 (A2156), anti-HA (AE008) from Abclonal. Mouse anti-actin (sc-8432) from Santa Cruz Biotechnologies was used as a loading control. Secondary antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-rabbit (Thermo Fisher). Chemiluminescent detection was performed using ECL PLUS (GE Healthcare).

SOD Activity Gels—Protein samples from proliferating and differentiating primary myoblasts (control and *Zip8*- and *Zip14*-knockdown) were solubilized with RIPA buffer plus protease inhibitor cocktail and quantified by Bradford assay⁹⁵. Fifty micrograms of each sample were separated using native PAGE gels. Gels were incubated for 30 min in 2.5 mM nitro blue tetrazolium (Sigma), followed by a 20-min incubation with 30 mM potassium phosphate, 30 mM TEMED, 30 mM riboflavin, pH 7.8⁹⁷. Superoxide dismutase activity was visualized by illuminating gels with white light for 10 min on a transilluminator. Areas of activity were visible as white bands against a dark background. SOD2 activity was detected as a cyanide-insensitive 26 KDa band, while SOD1 activity was detected as a 17 KDa band and a series of higher molecular weight oligomers (Supp. Fig. 4).

Immunocytochemistry—Proliferating and differentiating primary myoblasts (control and *Zip8*- and *Zip14*-knockdown) were fixed overnight in 10% formalin-PBS at 4 °C. Samples were washed with PBS and permeabilized for 10 min in PBS containing 0.2% Triton X-100. Immunocytochemistry was performed using universal ABC kit (Vector Labs) following manufacturer's instructions. Hybridoma supernatants from the Developmental Studies Hybridoma Bank (University of Iowa) were used against myogenin (F5D, deposited by W.E. Wright), and Pax7 (deposited by A. Kawakami).

Statistical analysis—Statistical analyses were performed using Kaleidagraph (Version 4.1). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison tests. Experiments where $P < 0.05$ were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance to metallomics

Trace elements are emerging as critical components for mammalian tissue development but are potentially toxic, so their homeostasis must be tightly regulated. Recent studies have reported influxes of copper and zinc during skeletal muscle cell differentiation. This study demonstrates that muscle differentiation is tightly coupled with increased manganese (Mn) and Mn-dependent superoxide dismutase 2 (SOD2). Here, we probe the functional requirements for the Mn, Zn, and Fe transporters ZIP8 and ZIP14, in skeletal muscle cells, and demonstrate that ZIP8 is essential in this cell type while ZIP14 is dispensable. This study is the first to report on the functional relevance of two members of the ZIP family of transporters in the skeletal muscle lineage.

Figure 1. Metal intake in differentiating primary myoblasts.

Whole cell metal content analyses of proliferating and differentiating wild type myoblasts. (A) Manganese. (B) Iron. (C) Zinc. (D) Calcium. All data were obtained using ICP-OES and normalized to total protein. Statistical analyses showed significant differences in metal accumulation in differentiating myoblasts compared to proliferating cells. Data is the mean $\pm$ SE for three independent biological replicates. ****P < 0.001, ***P < 0.005, **P < 0.01

Figure 2. SOD2 expression and activity increases as a consequence of myogenic differentiation. Representative immunoblots and activity gels analyses for SOD2 in proliferating and differentiating myoblasts expressing, Actin and Coomassie-stained membranes were used as loading controls (Supp. Fig. 8).

Figure 3. ZIP8 and ZIP14 expression in proliferating and differentiating wild type and shRNA knockdown primary myoblasts derived from mouse satellite cells.

(A) Representative immunoblot (top) and quantification (bottom) of ZIP8 levels in proliferating myoblasts and differentiated cells for 24, 48, and 72 h in wild type (left) and *Zip8* shRNA expressing cells (right). (B) Representative immunoblot of ZIP14 levels in proliferating and differentiating wild type (left) and *Zip14* shRNA myoblasts (right). For all samples, shown is the mean $\pm$ SE of three independent biological replicates. Immunoblots against actin or Coomassie-stained membranes (Supp. Fig. 8) were used as loading controls. Samples were compared to the corresponding wild type or mutant proliferation time point, and the; mutants were compared also to the equivalent time point in wild type cells.

****P<0.0001 ****P<0.001, ***P < 0.005, **P < 0.01

Figure 4. Knockdown of *Zip8* impairs differentiation of primary myoblasts.

(A) Representative light micrographs of wild type, *Zip8* and *Zip14* knockdown myoblasts grown in proliferating conditions or at 24, 48 and 72 h after inducing differentiation. Cells were immunostained with an anti-Myogenin antibody. Calculated fusion index for *Zip8*- (B) and *Zip14*-shRNA treated myoblasts. (C) Data represent mean $\pm$ SE for three independent experiments. ****P<0.0001

Figure 5. Knockdown of *Zip8*, but not *Zip14* impairs metal intake in differentiating primary myoblasts.

Whole cell metal content analyses of proliferating and differentiating wild type or myoblasts transduced with scrambled (scr), *Zip8* or *Zip14* shRNA. Statistical analyses showed significant differences in metal accumulation in differentiating myoblast comparing to the corresponding time point of wild type cells. (A) Manganese. (B) Iron. (C) Zinc. (D) Calcium. All data were obtained using ICP-OES and normalized to total protein. Shown is mean $\pm$ SE for three independent biological replicates. **** $P < 0.001$, *** $P < 0.005$.

Figure 6. SOD2 expression and activity is decreased in *Zip8*, but not *Zip14* knockdown primary myoblasts.

Representative Western blots, activity gels (top panels) and quantification of SOD2 levels and activity (bottom panels) in wild type and *Zip8* shRNA cell (A), and *Zip14* shRNA cells. For all samples, blots against actin and Coomassie-stained membranes were used as loading controls (Supp. Fig. 8). Shown is mean $\pm$ SE for three biological replicates. For wild type differentiating myoblasts, statistical analyses showed significant differences when compared to proliferating cells. Bonferroni statistical analyses for *Zip8*-knock down cells showed significant differences when compared to control cells at the corresponding time points, and

also when compared to the mutant's proliferation point. Similar to control cells, *Zip14* knockdown cells showed significant differences when compared to proliferating *Zip14* mutant myoblasts; *****P<0.0001 *****P<0.001, ***P < 0.005, **P < 0.01, *P 0.05

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Figure 7. Partial depletion of *Zip8*, but not *Zip14*, impairs growth of primary myoblasts. Cell counting assay of proliferating wild type myoblasts, myoblasts transduced with scrambled shRNA (shRNA scr, B,C), *Zip8* (A) or *Zip14* shRNAs (B). Data in A-C are mean $\pm$ SE for three independent experiments. ***** $P < 0.0001$ relative to 0 h of proliferation. (C) Representative light micrographs of proliferating wild type myoblasts, *Zip8* or *Zip14* knockdown myoblasts immunostained for Pax7.

Figure 8. exogenous ZIP8 rescued the proliferation and differentiation phenotype of Zip8 knockdown myoblasts.

(A) Representative Western blot of myoblasts transduced with the murine wild type *Zip8*. Detection of ZIP8, an anti-HA antibody was used to detect the tag associated to the exogenous protein. Myogenin, Caspase 3 and SOD2 antibodies were used. Actin is the loading control. SOD2 activity was also evaluated in gel. (B) Representative light micrographs of differentiating wild type myoblasts and those transduced with an shRNA against *Zip8* (shRNA-2) complemented with the murine wild type *Zip8* at 48 h post differentiation. Cells were immunostained with an anti-Myogenin antibody. (C) Cell counting assay of proliferating wild type myoblasts, cells transduced with scrambled shRNA (scr shRNA), and cells complemented with *Zip8*. Data is the mean $\pm$ SE for three

independent experiments. ICP analysis of **(D)** Mn, **(E)** Fe, **(F)** Zn in cells Zip8 shRNA cells complemented with wild type Zip8.

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Figure 9. *Zip8* and *Zip14* double knockdown myoblasts present severe proliferation and differentiation defects.

(A) Representative Western blot of myoblasts transduced with the murine wild type *Zip8* and *Zip14*. The antibodies used were ZIP8, ZIP14, Myogenin, Caspase 3 and SOD2 antibodies were used. Actin is the loading control. SOD2 activity was also evaluated in gel.

(B) Representative light micrographs of differentiating wild type myoblasts and those transduced with scramble and with both shRNA against *Zip8* and *Zip14* at 24 h post differentiation. Cells were immunostained with an anti-Myogenin antibody. (C) Cell counting assay of proliferating wild type myoblasts, scrambled shRNA and double mutant. Data is the mean $\pm$ SE for three independent experiments. Whole cell metal content analyses of proliferating and differentiating wild type or myoblasts transduced with scrambled (scr),

Zip8 and *Zip14* shRNA. Statistical analyses showed significant differences in metal accumulation in differentiating myoblasts (**D**) Manganese. (**E**) Iron. (**F**) Zinc. Data was obtained using ICP-OES and normalized to total protein. Shown is mean $\pm$ SE for three independent biological replicates. ****P<0.001, ***P < 0.005

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Figure 10. Manganese supplementation partially rescues the proliferation defect, but not the differentiation phenotype of *Zip8* knockdown myoblasts.

Cell counting assays performed in myoblasts expressing *Zip8* and *Zip14* shRNAs grown in medium supplemented with exogenous Mn (A), Fe (B) or Zn (C). Statistical analyses showed significant differences when comparing differentiating myoblasts to proliferating cells. For all experiments, data represent mean $\pm$ standard error for three biological replicates. ****P<0.0001. (D) Representative light micrographs of differentiating wild type myoblasts and those transduced with scramble and shRNA against *Zip8* or *Zip14* at 48 h post differentiation. Cells were immunostained with an anti-Myogenin antibody.

Table 1. Mn, Fe and Zn levels in proliferating and differentiating cells supplemented with either metal.

Treatment	Cells	Mn (pmol/mg protein)		Treatment	Cells	Fe (nmol/mg protein)		Treatment	Cells	Zn (nmol/mg protein)	
		Proliferation	Differentiation			Proliferation	Differentiation			Proliferation	Differentiation
NT	Wild Type	60.96 ± 3.13	209.92 ± 26.93	NT	Wild Type	0.40 ± 0.32	1.24 ± 0.28	NT	Wild Type	3.70 ± 0.80	7.11 ± 1.84
	Zip8 shRNA-2	41.01 ± 3.03	79.44 ± 7.42		Zip8 shRNA-2	0.18 ± 0.11	0.42 ± 0.15		Zip8 shRNA-2	1.51 ± 0.15	2.72 ± 0.63
	Zip14 shRNA-1	29.75 ± 2.09	81.41 ± 5.85		Zip14 shRNA-1	0.22 ± 0.33	0.57 ± 0.26		Zip14 shRNA-1	2.32 ± 0.07	5.26 ± 1.23
Mn	Wild Type	498.54 ± 10.79	37782.14 ± 679.81	Fe	Wild Type	5.49 ± 1.03	33.63 ± 7.14	Zn	Wild Type	1.18 ± 0.21	7.39 ± 0.45
	Zip8 shRNA-2	441.74 ± 32.89	6223.61 ± 120.63		Zip8 shRNA-2	5.52 ± 0.23	12.23 ± 0.36		Zip8 shRNA-2	1.16 ± 0.05	3.26 ± 0.27
	Zip14 shRNA-1	638.29 ± 49.71	8718.94 ± 293.89		Zip14 shRNA-1	5.14 ± 0.63	215.94 ± 12.72		Zip14 shRNA-1	2.05 ± 0.03	8.04 ± 0.41