Electronic Supplementary Material (ESI) for Metallomics.

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

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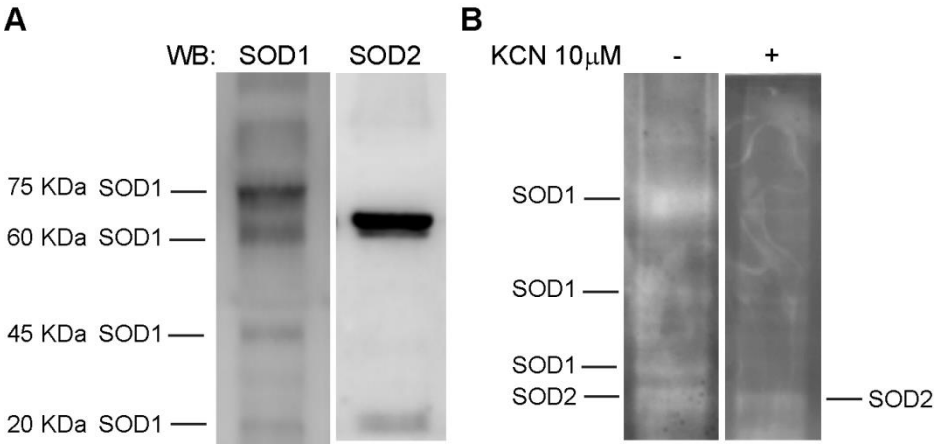
SUPPLEMENTARY INFORMATION

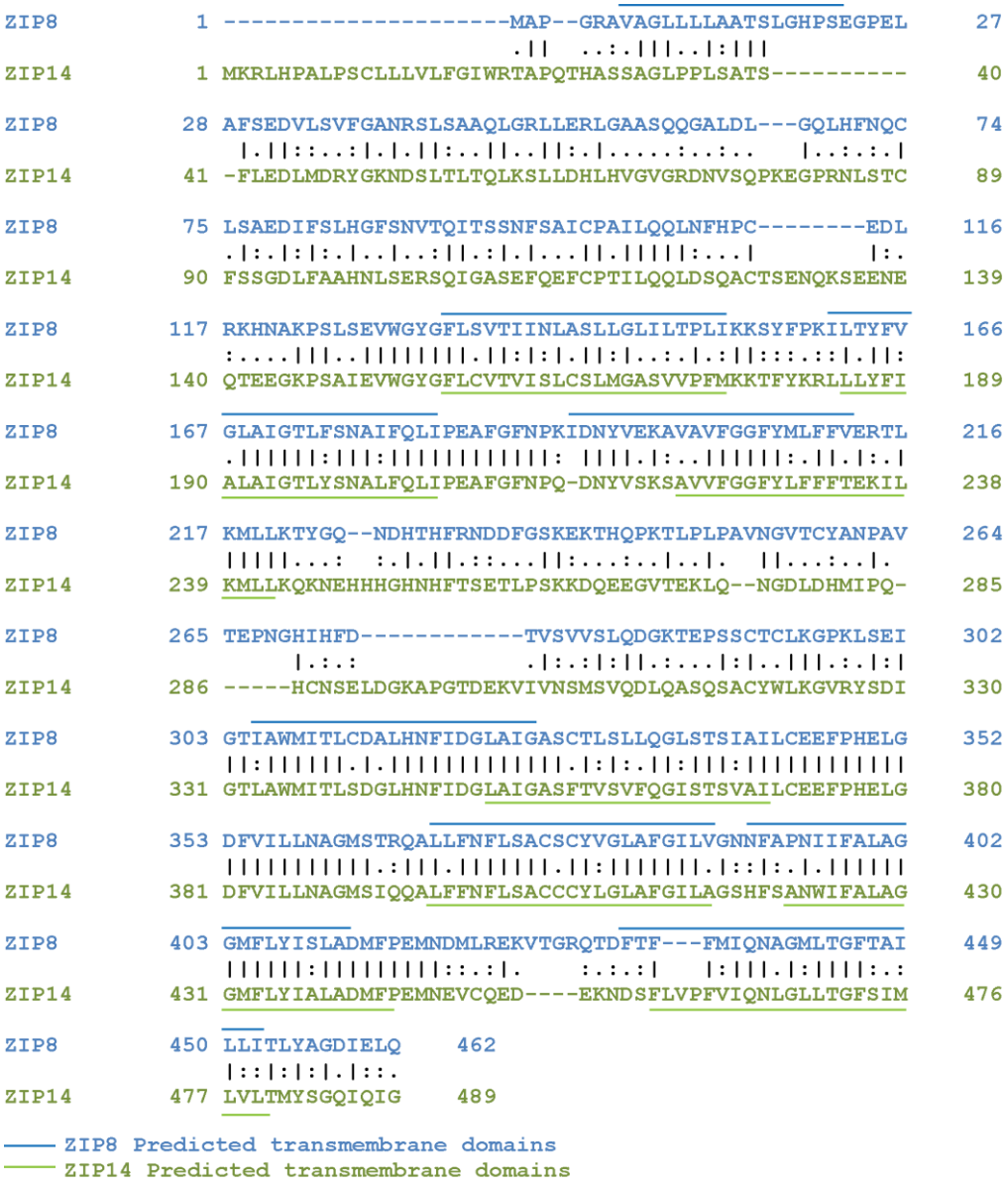
SUPPLEMENTARY FIGURES

Supplementary figure 1



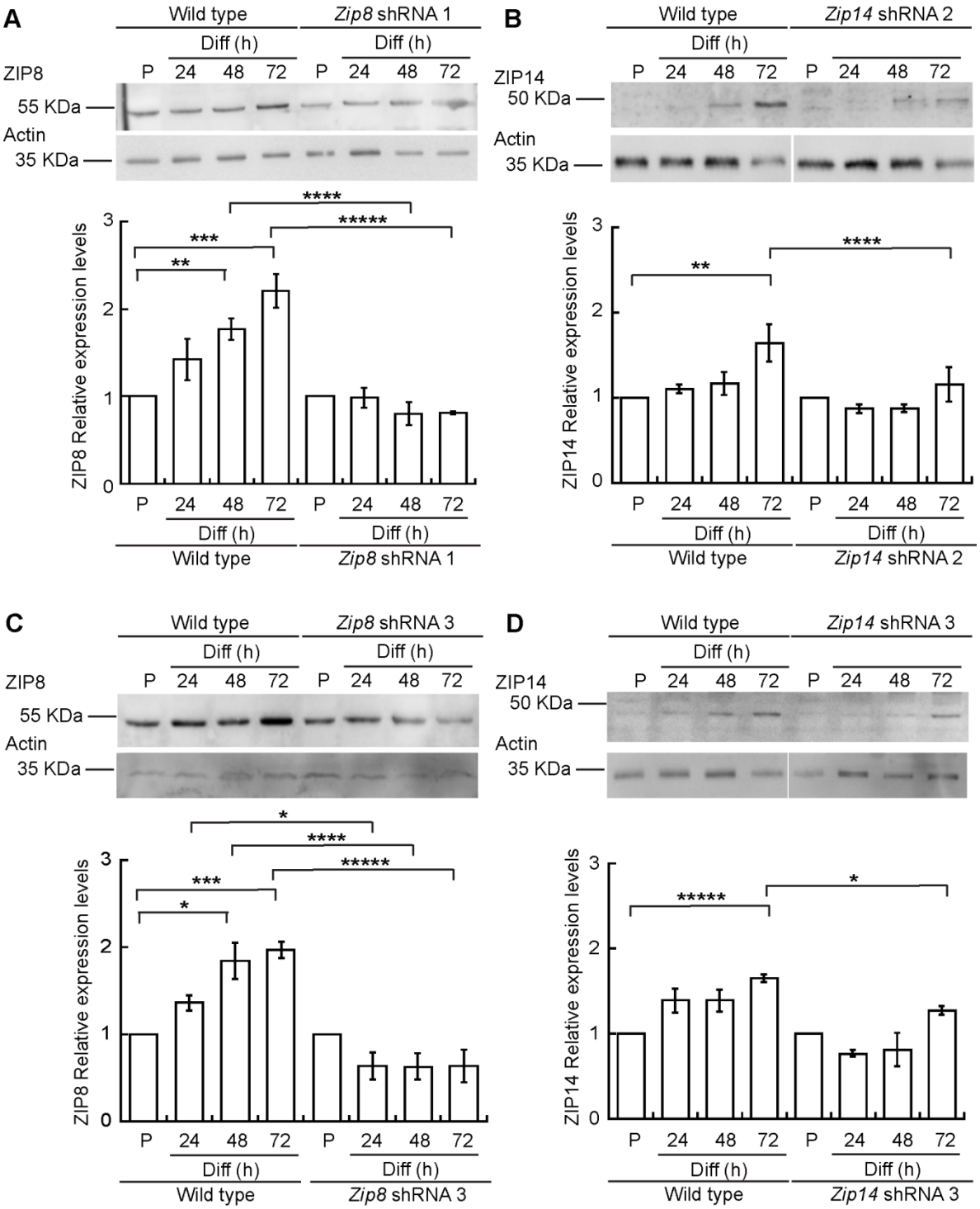
Supplementary Figure 1. In-gel assays for SOD1 and SOD2 activity. Representative figure showing how SOD1 and SOD2 activities were differentiated on native PAGE gels. **(A)** Immunoblot on a native gel loaded with cell lysates from proliferating wild type myoblasts and probed with antibodies against SOD1 and SOD2. **(B).** In-gel activity assay showing gel probed with nitro-blue tetrazolium on gels with and without cyanide treatment to strip the Cu cofactor from SOD1.

Supplementary figure 2



Supplementary Figure 2. ZIP8 and ZIP14 protein sequence alignment. Sequence homology analyses showed 46.6% identity and 62.0% similarity between these transporters. Since no crystal structure is available, the predicted transmembrane obtained from UniProtKB sites are indicated as lines in the top (ZIP8) and bottom (ZIP14) of the sequences.

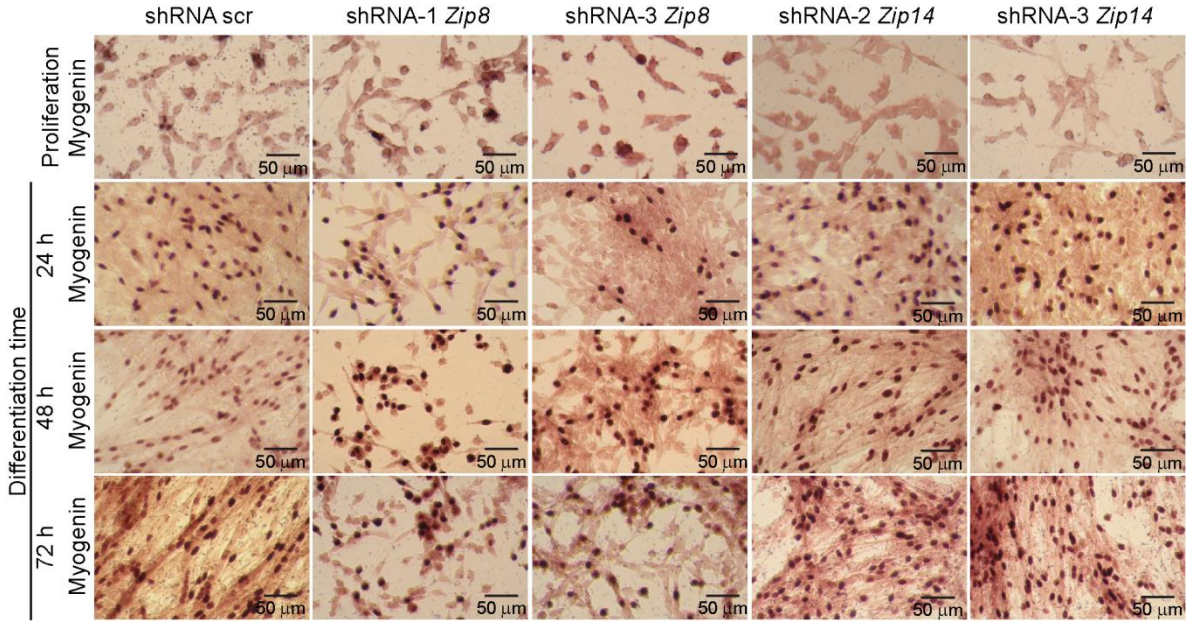
Supplementary figure 3



Supplementary Figure 3. ZIP8 and ZIP14 expression in proliferating and differentiating wild type and additional shRNA knockdown primary myoblasts derived from mouse satellite cells. ZIP8 and ZIP14 expression in cells transduced with alternate shRNAs utilized to knockdown *Zip8* and *Zip14*. **(A)** Representative immunoblot (top) and quantification (bottom) of ZIP8 levels in proliferating myoblasts and differentiated cells at 24, 48, and 72 h post-differentiation in wild type (left) and *Zip8* shRNA-1-transduced myoblasts (right). **(B)** Representative immunoblot of ZIP14 levels in proliferating and differentiating wild type (left) and *Zip14* shRNA-2-transduced myoblasts. **(C)** Representative immunoblot (top) and quantification (bottom) of ZIP8 levels in proliferating myoblasts and differentiated cells at 24, 48, and 72 h post-differentiation in

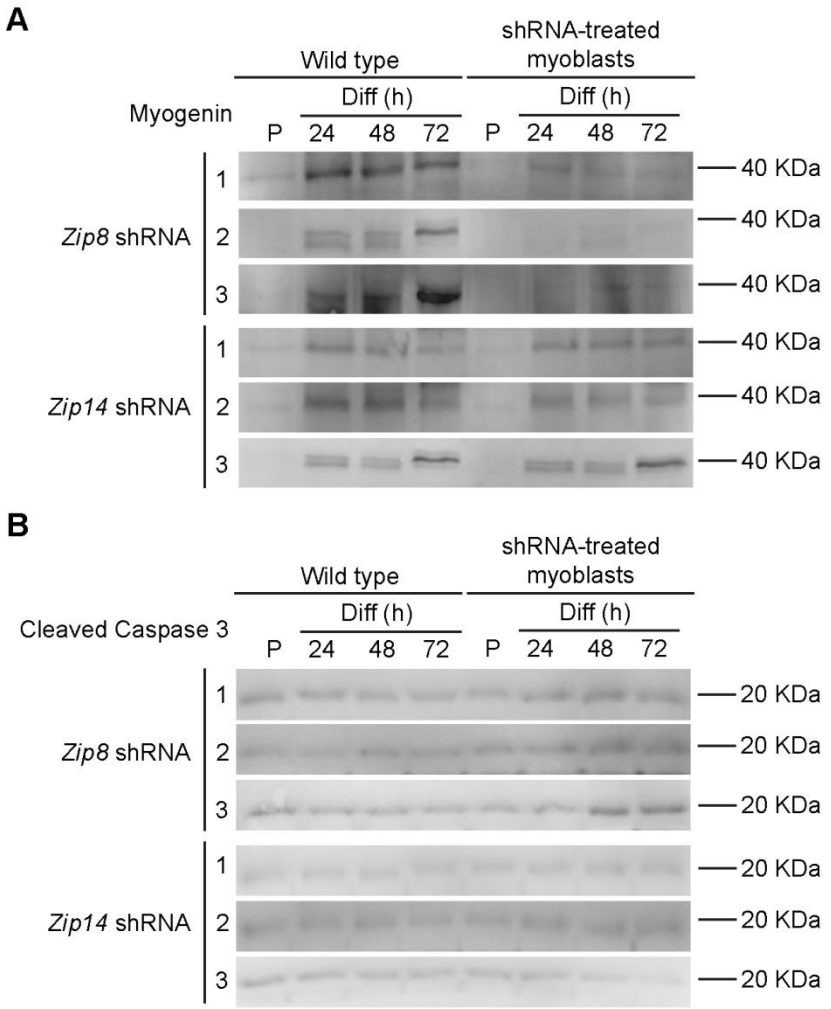
wild type (left) and *Zip8* shRNA-3-transduced cells (right). **(D)** Representative immunoblot of ZIP14 levels in proliferating and differentiating wild type (left) and *Zip14* shRNA-3-transduced myoblasts. For all samples, shown is mean ± SE of three independent biological replicates. Immunoblots against actin or Coomasie-stained membranes (Supp. Fig. 8) were used as loading controls. For wild type differentiating myoblasts, statistical analyses showed significant differences when compared to proliferating cells. Statistical analyses for *Zip8*-knockdown cells showed significant decrease in ZIP8 expression when compared to control cells at the corresponding time points. *Zip14*-knockdown cells showed significant decrease in ZIP14 expression when compared to control cells. *****P<0.0001 ****P<0.001, ***P < 0.005, **P < 0.01, *P ≤ 0.05

Supplementary figure 4.



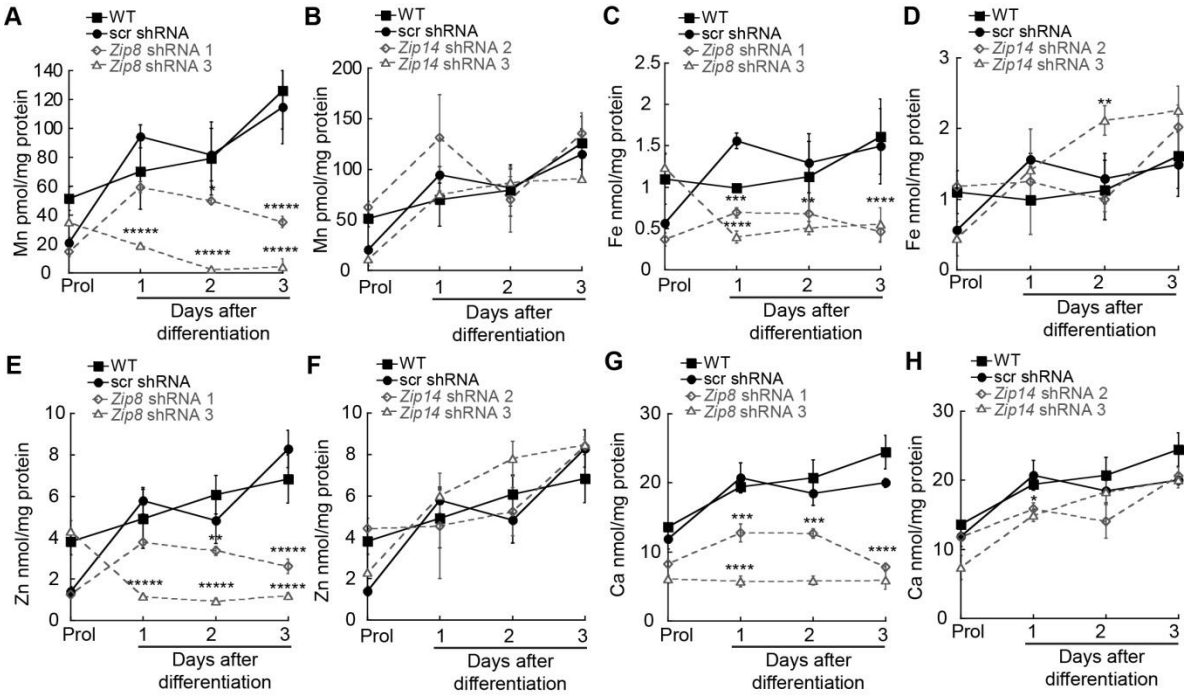
Supplementary Figure 4. Knockdown of *Zip8* with additional shRNAs impairs differentiation of primary myoblasts. Representative light micrographs of proliferating and differentiating myoblasts expressing alternate shRNAs against *Zip8* (shRNA-1 and -3 *Zip8*) and *Zip14* (shRNA-2 and -3 *Zip14*) knockdown myoblasts at 24, 48 and 72 h after inducing differentiation. Cells were immunostained with an anti-Myogenin antibody.

Supplementary figure 5.



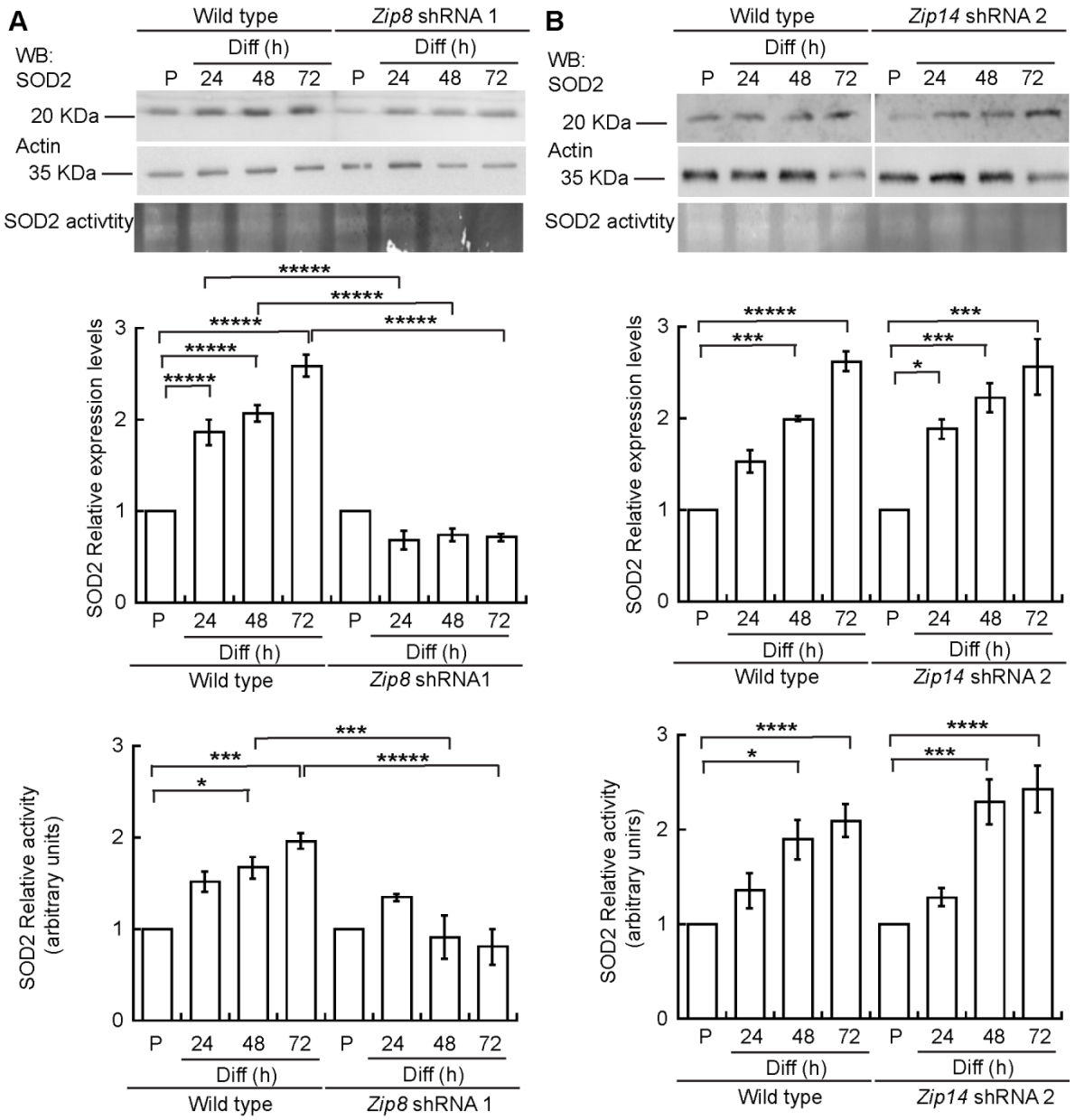
Supplementary Figure 5. Decreased expression of myogenin and increased activation of caspase 3 is observed in myoblasts exhibiting partial *Zip8* knockdown. Representative Western blots of the three independent biological replicates of wild type and each of the three clones of primary myoblasts treated with either with *Zip8* or *Zip14* shRNA developed an anti-myogenin (**A**) and anti-Caspase 3 (**B**) antibodies.

Supplementary figure 6.

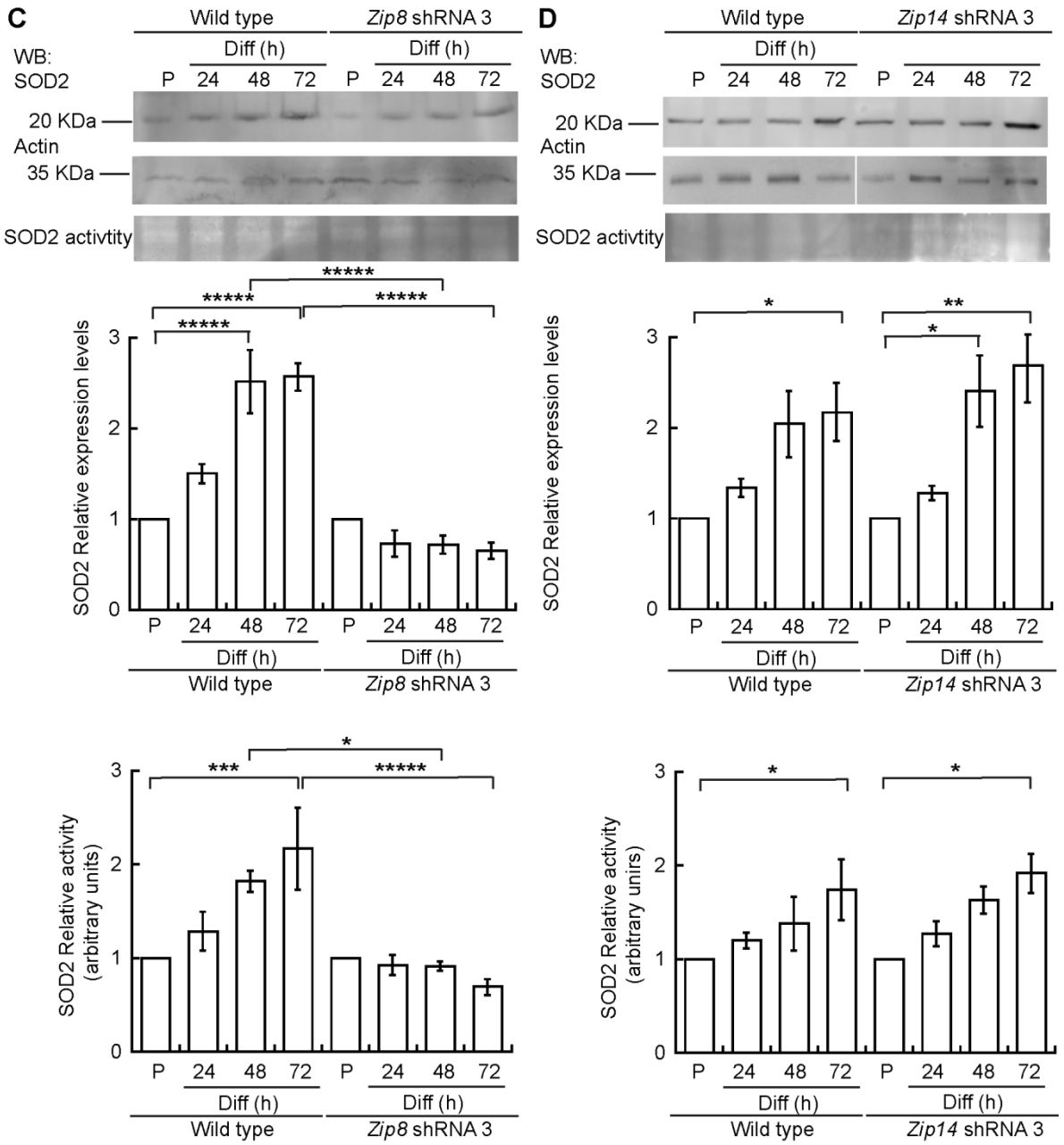


Supplementary figure 6. Mn and Fe content in *Zip8* and *Zip14* knockdown myoblasts. ICP-OES analysis of additional metals in wild type, *scr*, *Zip8* and *Zip14* knockdown cells. Whole cell Mn content comparing wild type and *scr* transduced myoblasts to *Zip8* **(A)** and Zip14 **(B)** knockdown myoblasts. Whole cell Fe content comparing wild type and *scr* transduced myoblasts to *Zip8* **(C)** and *Zip14* **(D)** knockdown myoblasts. Whole cell Zn content comparing wild type and *scr* transduced myoblasts to *Zip8* **(E)** and Zip14 **(F)** knockdown myoblasts. Whole cell Ca content comparing wild type and *scr* transduced myoblasts to *Zip8* **(G)** and *Zip14* **(H)** knockdown myoblasts. Statistical analyses showed significant differences in metal accumulation in differentiating myoblasts when compared to control myoblasts. All data were measured using ICP-OES and normalized to total protein. Shown is mean ± SE for three biological replicates. ****P<0.001, ***P < 0.005, **P < 0.01, *P ≤ 0.05

Supplementary Figure 7 (part A)



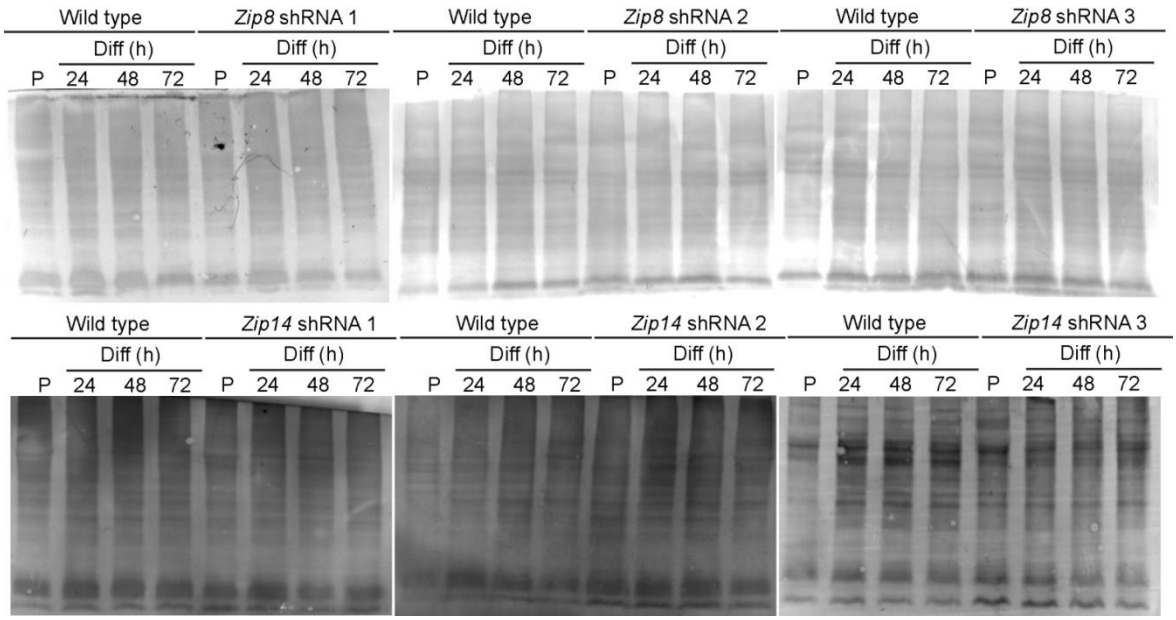
Supplementary Figure 7 (part B)



Supplementary Figure 7. SOD2 expression and activity is decreased in additional *Zip8* but not *Zip14* knockdown primary myoblasts. Additional immunoblots and activity gels analyses for SOD2 in proliferating and differentiating myoblasts expressing alternate shRNAs. **(A)** Representative Western blots and activity gels (top panels) and quantification of SOD levels and activity (bottom panels) in wild type and cells expressing an alternate *Zip8* shRNA (shRNA-1 *Zip8*). **(B)** Representative Western blots and activity gels (top panels) and quantification of SOD levels and activity (bottom panels) in wild type and cells expressing an alternate *Zip14* shRNA (shRNA-2 *Zip14*). **(C)** Representative Western blots and activity gels (top panels) and quantification of SOD levels and activity (bottom panels) in wild type and cells expressing an alternate *Zip8* shRNA (shRNA-3 *Zip8*). **(D)** Representative Western blots and activity gels (top panels) and quantification of SOD levels and activity (bottom panels) in wild type and cells expressing an alternate *Zip14* shRNA (shRNA-3 *Zip14*). For all samples, blots against actin and Coomasie-stained membranes were used as loading controls (Supp. Figure 8). Shown is mean ± standard

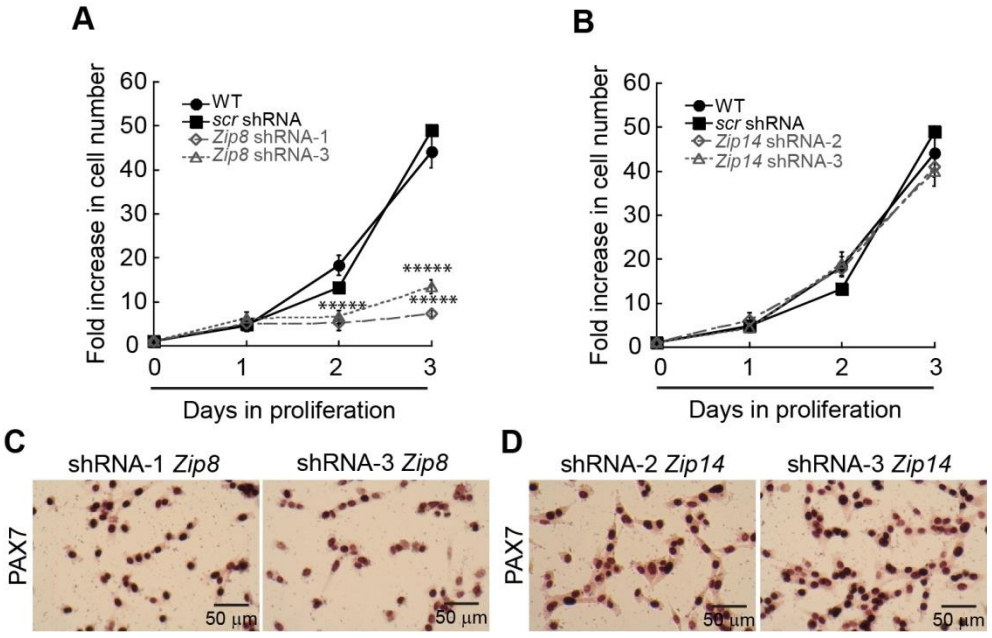
error for three biological replicates. For wild type differentiating myoblasts, statistical analyses showed significant differences when compared to proliferating cells. Statistical analyses for *Zip8*-knockdown cells showed significant differences when compared to control cells at the corresponding time points. Statistical analyses for *Zip14*-knockdown cells showed significant differences in SOD2 levels and activity when compared to proliferating *Zip14*-mutant myoblasts. *****P<0.0001 ****P<0.001, ***P < 0.005, **P < 0.01, *P ≤ 0.05

Supplementary Figure 8.



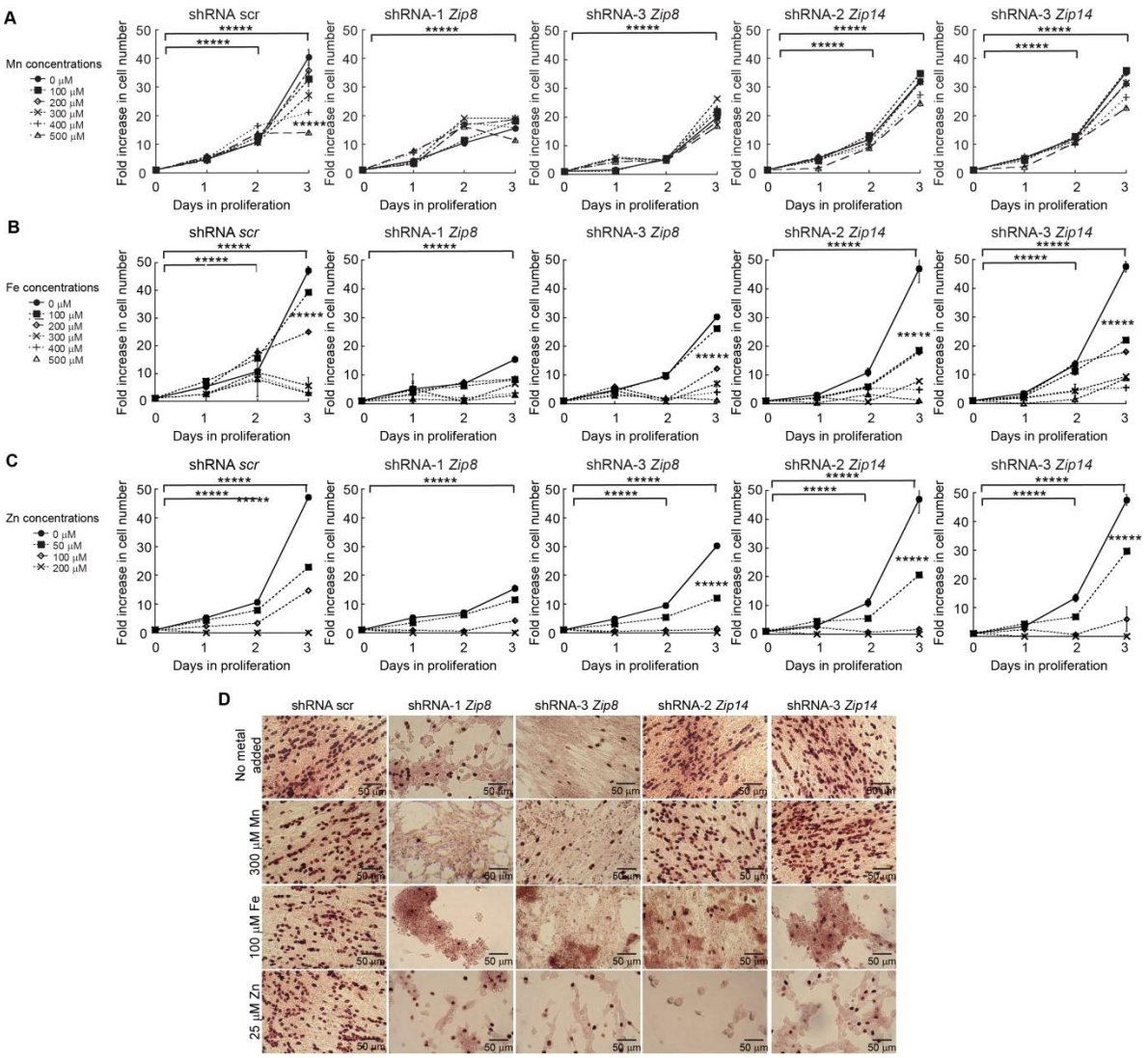
Supplementary Figure 8. Coomassie-stained immunoblot membrane controls. Representative Coomasie-stained membranes demonstrating total protein load. These membranes were utilized as a loading control for in-gel SOD2 activity assays shown in Fig. 3 and Supp. Figs. 3, 7.

Supplementary figure 9



Supplementary figure 9. Partial depletion of *Zip8*, but not *Zip14*, impairs growth of primary myoblasts. Cell counting assay of proliferating myoblasts. Data for wild type and *scr* cells is the same as in Fig. 7. Myoblasts transduced with shRNA against *Zip8* (**A**) or *Zip14* shRNAs (**B**). Data represent the mean ± SE for three independent experiments. *****P<0.0001. Representative light micrographs of *Zip8* (**C**) and *Zip14* (**D**) knockdown proliferating myoblasts immunostained with an anti-Pax7 antibody.

Supplementary figure 10.



Supplementary figure 10. Manganese supplementation partially rescues the proliferation defect of additional *Zip8* knockdown myoblasts, but not the differentiation phenotype. Cell counting assays performed in myoblasts expressing alternate 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 and to the wild type time point at 72h. For all experiments, data represent mean ± 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.

SUPPLEMENTARY TABLE

Supplementary table 1. Sequences of shRNA used.

Name	Sequence
Zip8 sh1	CCGGGCCAAGTTATCTCAGGAATTACTCGAGTAATTCCTGAGATAACTTGGCTTTTTG
Zip8 sh2	CCGGCAACGCGGGAAGGCATTTAATCTCGAGATTAAATGCCTTCCCGCGTTGTTTTTG
Zip8 sh3	CCGGTACGCAGGAGACATCGAATTGCTCGAGCAATTCGATGTCTCCTGCGTATTTTTG
Zip14 sh1	CCGGCCTCCCTTTCTCTTGGAAGAACTCGAGTTCTTCCAAGAGAAAGGGAGGTTTTTG
Zip14 sh2	CCGGCCTCTACTCCAACGCCCTCTTCTCGAGAAGAGGGCGTTGGAGTAGAGGTTTTTG
Zip14 sh3	CCGGTCCAGAATCTTGGCCTCCTAACTCGAGTTAGGAGGCCAAGATTCTGGATTTTTG

Supplementary table 2. Mn, Fe and Zn levels in proliferating and differentiating cells supplemented with either metal.

		Mn (pmol/mg protein)		Fe (nmol/mg protein)		Zn (nmol/mg protein)	
Cells	**Treatment**	**Proliferation**	**Differentiation**	**Proliferation**	**Differentiation**	**Proliferation**	**Differentiation**
Wild type	NT	60.96 ± 3.13	209.92 ± 26.93	0.40 ± 0.32	1.24 ± 0.28	3.70 ± 0.80	7.11 ± 1.84
	Mn			0.70 ± 0.23	0.78 ± 0.07	1.37 ± 0.10	1.73 ± 0.07
	Fe	52.91 ± 5.64	332.05 ± 78.73			0.74 ± 0.05	2.07 ± 0.27
	Zn	50.78 ± 1.09	158.47 ± 6.88	0.29 ± 0.03	1.48 ± 0.44		
***Zip8* shRNA-2**	NT	41.01 ± 3.03	79.44 ± 7.42	0.18 ± 0.11	0.42 ± 0.15	1.51 ± 0.15	2.72 ± 0.63
	Mn			0.31 ± 0.06	0.37 ± 0.10	1.18 ± 0.05	0.79 ± 0.05
	Fe	42.76 ± 2.71	80.29 ± 6.17			1.09 ± 0.24	1.18 ± 0.11
	Zn	31.54 ± 1.15	79.01 ± 7.11	0.33 ± 0.07	0.70 ± 0.15		
***Zip14* shRNA-1**	NT	29.75 ± 2.09	81.41 ± 5.85	0.22 ± 0.33	0.57 ± 0.26	2.32 ± 0.07	5.26 ± 1.23
	Mn			0.67 ± 0.26	0.41 ± 0.17	2.20 ± 0.14	1.50 ± 0.06
	Fe	33.99 ± 2.37	315.52 ± 18.41			0.95 ± 0.10	4.26 ± 0.32
	Zn	31.74 ± 1.38	61.64 ± 5.03	0.39 ± 0.02	0.79 ± 0.29		

SUPPLEMENTARY METHODS

Alignments

Sequences for ZIP8 and ZIP14 were obtained from UniProt and were aligned using EMBOSS Needle Pairwise Sequence Alignment software [1,2].

Membrane staining by Coomassie Brilliant Blue.

After developing the PVDF membranes with the appropriate antibodies, the membranes were rinse with H_2O for 10 min. Then, they were stain with 0.025% (w/v) Coomassie brilliant blue R-250 (Sigma) in 40% methanol/7% acetic acid (v/v) for 5 min. Membranes were de-stained by washing 3 times with 50% methanol/7% acetic acid for 10 min and rinsed with H_2O [3].

SUPPLEMENTARY REFERENCES

1. Needleman, S.B.; Wunsch, C.D. A general method applicable to the search for similarities in the amino acid sequence of two proteins. *Journal of molecular biology* **1970**, *48*, 443-453.
2. Kruskal, J.B. *An overview of squence comparison*. Addison Wesley.: 1983.
3. Goldman, A.; Harper, S.; Speicher, D.W. Detection of proteins on blot membranes. *Current protocols in protein science* **2016**, *86*, 10 18 11-10 18 11.