Aging amplifies multiple phenotypic defects in mice with zinc transporter *Zip14* (Slc39a14) deletion

Tolunay Beker Aydemir^a, Catalina Troche^a, Jinhee Kim^a, Min-Hyun Kim^a, Oriana Y. Teran^a,
Christiaan Leeuwenburgh^b, Robert J. Cousins^a

^aFood Science and Human Nutrition Department

Center for Nutritional Sciences

College of Agriculture and Life Sciences

University of Florida

Gainesville, Florida

^bDepartment of Aging and Geriatric Research

and Institute on Aging

College of Medicine

University of Florida

Gainesville, Florida

Corresponding Author:

Dr. Robert J. Cousins

352-392-2133

cousins@ufl.edu

Keywords:

Inflammation, Sarcopenia, Signaling pathways, Interleukin-6, bone, growth

Abstract

Inflammation and zinc dyshomeostasis are two common hallmarks of aging. A major zinc transporter ZIP14 (slc39a14) is upregulated by proinflammatory stimuli, e.g. interleukin-6. We have evaluated the influence of age on the *Zip14* KO phenotype using wild-type (WT) and *Zip14* knockout (KO) mice. Aging produced a major increase in serum IL-6 concentrations that was dramatically augmented in the *Zip14* KO mice. In keeping with enhanced serum IL-6 concentrations, aging produced tissue-specific increases in zinc concentration of skeletal muscle and white adipose tissue. Metabolic endotoxemia produced by *Zip14* ablation is maintained in aged KO mice. Muscle non-heme iron (NHI) was increased in aged WT mice but not in aged *Zip14* KO mice demonstrating NHI uptake by muscle is ZIP14-dependent and increases with age. NF- κ B and STAT3 activation was greater in aged mice, but was tissue specific and inversely related to tissue zinc. Micro-CT analysis revealed that *Zip14* KO mice had markedly reduced trabecular bone that was greatly amplified with aging. These results demonstrate that the inflammation-responsive zinc transporter ZIP14 has phenotypic effects that are amplified with aging.

1. Introduction

Many mediators of the aging process are associated with inflammation and oxidative stress (Vasto et al., 2007; Frazzini et al., 2006). These consequences may be augmented if there is a concurrent low intake of zinc (Marcellini et al., 2006; Pepersack et al., 2001; Tudor et al., 2005). Proinflammatory conditions have been shown to influence zinc metabolism through cellular transport and intracellular zinc processing (Cousins et al., 2006). Many of the same mediators may influence aging. For example, it has been proposed that the proinflammatory cytokine IL-6 is a key factor in human age-associated diseases (Bennermo et al., 2004; Olivieri et al., 2006). A single nucleotide polymorphism (SNP) in the *IL-6* gene is associated with an increased inflammatory response (Bennermo et al., 2004; Mocchegiani et al., 2008). IL-6 has been shown to regulate components of the homeostatic mechanisms for zinc. Specifically, IL-6 has been shown to influence metallothionein expression and cellular zinc accumulation (Schroeder and Cousins, 1990).

We have established that the zinc transporter ZIP14 (slc39a14), a zinc importer usually located at the cell surface, is regulated by proinflammatory cytokines, particularly IL-6 (Liuzzi et al., 2005). The regulation of *Zip14* by IL-6 suggests ZIP14 expression could be influenced by aging. Mice with a knockout (KO) of *Zip14* have a phenotype that includes metabolic endotoxemia and altered glucose regulation (Aydemir et al., 2012; Guthrie et al., 2015; Troche et al., 2016). Such phenotypes are associated with aging. Here we present the first evidence on the influence of aging in mice with an ablation of *Zip14*. The aged mice were 22-24 months old thus placing them in an age comparable to humans between 56 and 69 years of age (Flurkey et al., 2007). The data shows that aging increases ZIP14 expression in muscle, that proinflammatory signaling pathways are influenced by aging and ZIP14 expression and that aging drastically reduced femoral trabecular bone with *Zip14* ablation.

98 2. Materials and Methods

99 2.1 Mice

100 *Zip14* heterozygous mice of a C57BL/6J and 129SF1 mixed background were used as
101 a breeding colony at the University of Florida (Aydemir et al., 2012). Both WT (*Zip14^{+/+}*) and
102 KO (*Zip14^{-/-}*) male mice were used for experiments. Young mice were 8 to 16 weeks of age
103 and aged mice were 22-24 months old. Based on experience with the *Zip14* KO strain in terms
104 of their proinflammatory status and their lower than normal body size, we decided to study the
105 mice at 24 months of age. Mice were provided with commercial chow diet (Harlan 7912) and
106 tap water ad libitum. Mice were anesthetized using isoflurane (Baxter, Deerfield, IL, USA) and
107 euthanasia was via cardiac puncture and exsanguination. All murine protocols were approved
108 by the University of Florida Institutional Animal Care and Use Committee.

109 2.2 Serum and Tissue analyses

110 Blood was collected in clot activator tubes (Capiject, Terumo Medical, Somerset, NJ,
111 USA). Serum was obtained by centrifugation and stored at -80°C. Harvested tissues were
112 snap-frozen in liquid nitrogen and stored at -80°C. Zinc concentrations were measured by
113 flame atomic absorption spectrometry (AAS). Serum was diluted 1:5 in Milli-Q® water for AAS.
114 Tissues were weighed and digested in HNO₃ (90°C for 3 hours) and diluted (1:1 to 1:5) with
115 Milli-Q® water for AAS (Troche et al., 2016).

116 Tissue aliquots were homogenized in TRIzol reagent (Life Technologies, Thermo Fisher
117 Scientific, Waltham, MA, USA) with a Bullet blender (Next Advance, Averill Park, NY, USA)
118 using either zirconium oxide or stainless steel beads. Quantitative real-time PCR (qPCR) was
119 performed using Express One-Step Superscriptmix (Invitrogen, Carlsbad, CA, USA) and a
120 StepOnePlus Real-Time PCR detection system (Applied Biosystems, Foster City, CA, USA).

121 *TBP* mRNA and *GAPDH* mRNA (muscle) were used to normalize qPCR data (Aydemir et al.,
122 2012).

123 Tissue aliquots were homogenized as above in RIPA or non-denaturing lysis buffer
124 (Santa Cruz Biotechnology, Dallas, TX, USA) containing a protease/phosphatase inhibitor mix
125 (Thermo Fisher Scientific, Waltham, MA, USA) and phenylmethanesulfonyl fluoride (Sigma
126 Aldrich, St. Louis, MO, USA). Proteins were separated using 10% SDS-PAGE and transferred
127 to nitrocellulose with transfer verification through Ponceau Red staining. Protein abundance
128 was visualized by chemiluminescence (Super Signal West Pico, Thermo Fisher, Waltham, MA,
129 USA) and digital imaging (Protein Simple, San Jose, CA, USA). Tubulin was used as the gel
130 loading control. ZIP14 and ZIP8 polyclonal antibodies were produced in-house as previously
131 described (Ryu et al., 2008; Liuzzi et al., 2005). NF- κ B, phosphorylated NF- κ B, STAT3,
132 phosphorylated STAT3, ERK1,2, and phosphorylated ERK1,2 antibodies were purchased from
133 Cell Signaling Technology (Boston, MA, USA). Tissue non-heme iron was measured using
134 tissue homogenates by the ferrozine method (Rebouche, et al., 2004).

135 *2.3 Evaluation of bone structure with micro-CT*

136 Femurs from the left leg were removed and separated from adherent tissue and was
137 stored at -80°C. Thawed bones were individually scanned using a Scano μ CT scanner (μ CT-
138 40; Scano Medical AG, Bassersdorf, Switzerland) as described in detail by Cao and Picklo
139 (2013). Cortical and trabecular bone of the mid-femur was analyzed.

140 *2.4 Statistical Analysis*

141 Reported data are as means $\pm$ SE. Comparison of WT vs. KO genotype was by
142 Student's *t*-test. Genotype and age designation were treated as independent effects. ANOVA
143 analyses were performed using the PROC MIXED procedures of SAS version 9.2 (SAS

Institute, Cary, NC, USA) and Prism InStat 3. Multiple treatment comparisons were analyzed using the Turkey-Kramer means separation, and significance was established at $P \leq 0.05$.

3. Results

3.1 Zinc concentrations of specific tissues are altered in aged mice

Zinc concentrations in spleens of WT mice were significantly decreased with age ($p < 0.01$). In contrast, zinc concentrations in WAT and skeletal muscle were significantly increased ($p < 0.05$) in aged mice (Table 1). Brain zinc concentrations were also greater ($p < 0.03$) in the aged mice. The concentrations in other tissues including bone showed no significant differences, indicating tissue specificity in zinc accumulation with aging. Because of the potential for inflammation to influence spleen and muscle, we focused on these tissues using liver as the control.

3.2 Aging influenced serum zinc, endotoxin, IL-6 concentrations and spleen IL-6 mRNA

The *Zip14* ablation reduced growth in both young and aged mice compared to WT mice as shown by body length (Supporting Fig. 1) and body weight (Fig. 1A). Of note is that *Zip14* KO mice maintained body weight in contrast to WT mice. Muscle to body weight ratios of young male mice were less in the KO genotype, but were not measured in the aged mice. Serum endotoxin was significantly greater in the *Zip14* KO mice. This signature of metabolic endotoxemia was maintained in the aged KO mice (Fig. 1B). Measurement of serum IL-6 at steady state, using a high sensitivity ELISA, revealed that the WT aged mice had greater levels than young WT mice. Aging greatly amplified the differential in serum IL-6 in *Zip14* KO mice (Fig. 1C). The exact cellular source(s) of the elevated serum IL-6 is not known. It is of note that IL-6 mRNA levels in the spleen of the aged KO mice are double those of the other groups indicating splenic macrophages could be one site of origin of the elevated IL-6 (Fig. 1D).

3.3 Activation of pro-inflammatory signaling pathways

169 Activation of NF- κ B, STAT3 and ERK1/2 was examined in spleen, muscle and liver of
170 young and aged WT and KO mice. Aging increased p-NF- κ B in all tissues (Fig. 1E). This effect
171 was markedly less in muscle and liver of KO mice while it was amplified in spleen which may
172 lead to higher IL-6 expression. Aging also increased p-STAT3 in spleen and muscle, but
173 activation was not influenced by genotype. p-ERK1/2 was less in liver of aged mice (Fig. 1E),
174 but was not changed in the other tissues. Insulin receptor phosphorylation was less in aged
175 mice.

176 3.4 Aging and ZIP14 expression influences tissue zinc and non-heme iron concentrations

177 Our initial hypothesis was that since serum IL-6 levels increase with age, expression of
178 *Zip14*, which is induced by IL-6 (Liuzzi et al., 2005), may increase with age enhancing zinc
179 transport and retention in specific tissues. A comparison of steady state serum zinc
180 concentrations revealed that these were significantly greater ($p < 0.02$) in the *Zip14* KO mice
181 (Fig. 2A). There was no effect due to age. A comparison of tissue zinc levels in WT versus KO
182 mice revealed significant ($p < 0.01$) increases in muscle of aged WT mice (Fig. 2B and C).
183 These levels were increased further in muscle of aged *Zip14* KO mice (Fig. 2C). This suggests
184 that zinc transport kinetics are differentially altered in a tissue-specific fashion with aging. NHI
185 was not influenced by aging in liver. In contrast, however, NHI levels in muscle were influenced
186 by aging in the *Zip14* KO mice used for these experiments (Fig. 2D).

187 3.5 ZIP8 and ZIP14 are differentially expressed with aging

188 We examined ZIP14 and its closest homologue ZIP8 (Schmitt-Ulms et al., 2009) in
189 tissues of young and aged WT and KO mice. Protein levels for ZIP14 in liver of WT mice were
190 comparable (Fig. 3A). ZIP14 in muscle was much more abundant in aged mice (Fig. 3A). In
191 contrast, muscle ZIP8 expression was greatly increased with aging (Fig. 3B). Liver ZIP8 levels
192 were much greater in the *Zip14* KO mice. Aging increased ZIP8 levels in WT and KO mice with

aging. These data suggest multiple factors are involved regulation of these transporters with aging. Of note is that MT1 mRNA levels were not influenced in muscle or spleen by aging or genotype (Fig. 3C).

3.6 Growth, cortical bone volume/surface area, and trabecular bone are altered with aging in *Zip14* KO mice

Bone morphology was influenced by both age and *Zip14* expression. Specifically, cortical bone volume decreased in aged KO mice and bone surface area increased in aged KO mice (Fig. 4A and 4B). Both of these measurements indicated that *Zip14* ablation reduced bone mass. The striking loss of trabecular bone of the femur in mice with the null *Zip14* mutation is shown in the μ CT 3D representations (Fig. 5A). While not pronounced in young *Zip14* KO mice, the aged KO mice showed significant reductions in trabecular bone based on numerical indices of bone mineral density (Fig. 5B). In keeping with these results Ear Mesenchymal Stem Cells (EMSC) from *Zip14* KO mice and cultured in osteogenic medium were less mineralized than WT counterparts (Supporting Fig. 2). The effect of LPS while dramatic in WT, was not significant in KO EMSC. Further evaluation of these cells through western blots showed that pSmad1/5, a key transducer of the Bone morphogenetic protein (BMP) pathway, was downregulated with KO.

4. Discussion

There is currently great interest in the relationship of zinc transporter activity and disease. The range is extensive and has implicated many of the 24 ZnT/ZIP proteins that transport zinc. It has been proposed that the large number of zinc transporters is necessitated because of the plethora of intracellular functions in which this essential micronutrient participates (Lichten and Cousins, 2009a). Examples for the ZIP transporters/disease relationship include adipose inflammation and ZIP14 (Troche et al., 2016), ZIP8 and ZIP14 and

217 response to sepsis (Knoell et al., 2009; Wessels and Cousins, 2015), ZIP13 and Ehlers-Danlos
218 syndrome (Jeong et al., 2012), ZIP14 and metabolic endotoxemia (Guthrie et al., 2015), ZIP8
219 and osteoarthritis (Kim et al., 2014) and mutant *Zip14* and parkinsonism/dystonia (Tuschi et
220 al., 2016). One report that relates ZIP transporter function to aging was an evaluation of *Zip6*
221 mRNA expression in immune cells (Wong et al., 2013). The findings reported here are the first
222 to specifically target the phenotypes of a ZIP transporter that are accentuated with aging.

223 Among the tissues most frequently associated with disorders of human aging are
224 skeletal muscle and bone/ connective tissues. In experiments reported here we compared the
225 response of muscle to other tissues with respect to zinc homeostasis, proinflammatory
226 signaling pathways and bone development. The animals in this study were at steady state and
227 only males were compared. The aged mice used were 22-24 months old and are comparable
228 to humans between 56 and 69 years of age (Flurkey et al., 2007). This age range is
229 appropriate because potential therapeutic intervention studies to combat age-related disease
230 routinely use mice that are about 22 months old to test effectiveness (Camporez et al., 2016).
231 Of the original cohort of WT and KO mice reserved for this study, no mortality was observed
232 during the 22 month comparison period. Gross examination would however classify the KO
233 mice as low-functioning compared to the WT mice.

234 The *Zip14* KO genotype clearly handles aspects of aging differently than WT mice. We
235 interpret the increases of serum zinc levels in both young and aged *Zip14* KO mice as
236 indicative of a decrease in ZIP14-mediated uptake of systemic zinc. Tissue zinc levels in
237 muscle are greater in aged mice, however. The finding that increased circulating IL-6, a
238 signature of aging (Oliveri et al., 2006), is substantially increased in *Zip14* KO mice suggests
239 that the loss of ZIP14 leads to overexpression of IL-6 production, most likely through loss of a
240 feedback loop. As mentioned earlier, the spleen could be a source of circulating IL-6 as *IL-6*

241 mRNA is increased. Previously we have shown that ZIP14 regulates inflammatory signaling in
242 adipose tissue (Troche et al., 2016). Adipose is a major source of the increased IL-6
243 associated with aging (Starr et al., 2009). Hence in the *Zip14* KO mice, adipose tissue may be
244 a contributor to increased systemic IL-6 observed with aging in our experiments here as this
245 genotype has increased body fat (Aydemir et al., 2012).

246 Elevated muscle zinc concentrations are in agreement with the increased ZIP14 in
247 muscle of WT mice. Greater levels of zinc in muscle of *Zip14* KO mice suggest a
248 compensatory zinc transport function has occurred. The upregulation of muscle ZIP8 could
249 contribute to this increase. Aging clearly increased ZIP8 expression in muscle and also liver.
250 Most research on ZIP8 expression/function has focused on immune cells, starting with its
251 identification via inducibility by lipopolysaccharide (Begum et al., 2002). We identified that
252 activation of human primary T-cells dramatically increased ZIP8 and influenced intracellular
253 zinc and inhibition of calcineurin phosphatase activity (Aydemir et al., 2009). Migration of ZIP8
254 as distinct bands of 75KDa and 150KDa in western analysis were noted at that time. Multiple
255 molecular weights were noted here for splenic, muscle and liver ZIP8 (Fig 3D). The difference
256 in molecular weight of ZIP8 could be related to shedding of the ectodomains (Ehsani et al,
257 2011) or formation of a homo- or hetero-dimer. To our knowledge, the current experiments are
258 the first to report ZIP8 expression in skeletal muscle and the first to show up-regulation with
259 aging.

260 Muscle was selected as a focus for these studies because of the influence of aging and
261 sarcopenia and our previous profiling of Zip transporter mRNAs that respond to
262 proinflammatory stimuli. Following an LPS injection (18 hr) *Zip14* mRNA was increased 18-fold
263 in skeletal muscle (Aydemir et al., 2012) and *Zip8* mRNA with a 4-fold increase was the only
264 other *Zip* mRNA showing a major response to LPS treatment (unpublished data). This is of

265 interest since ZIP14 and ZIP8 have the closest homology among the ZIP transporter family
266 and comprise one branch of the LIV-1 subfamily (Schmitt-Ulms et al., 2009). ZIP14 has been
267 localized to the plasma membrane and endosomes (Liuzzi et al., 2005; Guthrie et al., 2015
268 Troche et al., 2016) and ZIP8 has been detected at the plasma membrane, mitochondria and
269 lysosomes (He et al., 2006; Besecker et al., 2008; Aydemir et al., 2009). Under in vitro
270 conditions these ZIP transporters may facilitate the movement of zinc and in some cases iron,
271 manganese and cadmium (Lichten and Cousins, 2009). The magnitude of these metal fluxes is
272 likely dependent upon the available pool of free metal ion to serve as a substrate for transport.

273 Our new findings merge with a concept where NHI accumulates in muscle and may
274 contribute to muscle decline-of-function and atrophy (Xu et al., 2010). We demonstrate here
275 that NHI does not accumulate in muscle of male *Zip14* KO mice as it does in male WT mice
276 (Fig. 2D). This suggests ZIP14 is responsible for the NHI accumulation. Aging male mice show
277 augmented ZIP14 and ZIP8 in muscle as shown in this report (Fig 3 A, B). ZIP8 can also
278 transport iron in vitro, albeit to a more limited extent than zinc (Wang et al., 2012). The
279 prevention of muscle NHI accumulation with *Zip14* ablation suggests ZIP8 is not a factor in
280 muscle NHI accumulation. In contrast, previously we observed that ablation of *Zip14* increased
281 NHI in liver of young *Zip14* KO female mice (Aydemir et al., 2012). Collectively these findings
282 suggest that accumulation of NHI in muscle and liver in mice is influenced by both gender and
283 age. The data support the concept that gender differences in iron metabolism occur in murine
284 models.

285 Recent studies have clearly established a link between murine osteoarthritis, zinc and
286 ZIP8 (Kim et al., 2014). In this scenario, zinc increases MTF-1 activation and thereby
287 upregulated expression of specific metalloproteases involved in bone matrix degradation. Most
288 likely a comparable situation is not involved in the current experiments as *Mt-1* mRNA

289 expression did not differ as a function of age in mice of the *Zip14* KO genotype. *Mt-1* mRNA is
290 a sentinel for MTF-1 activation. We have focused here on muscle and spleen. Aging may
291 produce changes in labile zinc pools in specific cells, e.g. leukocytes, Approaches to address
292 such issues directly are beyond the scope of this report.

293 The influence of aging on femoral bone density in the aged mice observed in our
294 studies by several indices using micro-CT is in line with age-related bone loss in humans
295 (Khosla, 2013). The mice in this study were males eliminated from our breeding colony and
296 allowed to age. At 22-24 months of age they are comparable to humans aged 56-69 years
297 (Flurkey et al., 2007). Of major emphasis is the marked reduction in trabecular bone in only the
298 aged KO mice. The mechanism responsible is not currently established. ZIP14 could influence
299 bone accretion or resorption through zinc regulated signaling systems. This could include a
300 role for IL-6. Our preliminary finding with osteogenic differentiation of EMSC from WT and
301 *Zip14* KO mice would support that concept (Supporting Fig. 2). Upregulation of NF- κ B
302 activation in stem cells derived from the *Zip14* KO mice supports that motion. We cannot rule
303 out the involvement of another ZIP14-regulated process in the loss of trabecular bone in the
304 aged *Zip14* KO mice. It has been postulated, based on *in vitro* transfection studies and data
305 from zebrafish, that the major role for ZIP14 is to transport Mn (Tuschl et al., 2016). If that
306 hypothesis is correct, ZIP14 dysfunction could reduce biliary Mn elimination, causing Mn
307 retention in the brain leading to neurological disorders. Nutritional Mn deficiency has been
308 shown to produce skeletal abnormalities in animals however excess Mn has not been shown
309 to influence bone (Underwood, 1977). Hence Mn deficiency or toxicity is not a likely factor in
310 the loss of trabecular bone observed in the aged *Zip14* KO mice as described in our report.

311 **5. Conclusion**

312 In summary, the data presented in this report show that aging influences zinc
313 metabolism particularly in muscle (Fig. 6). This suggests some influence of proinflammatory
314 cytokines and related signaling pathways. Activation of NF- κ B and STAT3 pathways in muscle
315 is most likely involved. Ablation of ZIP14 accentuates the elevated circulating levels of
316 proinflammatory IL-6 and produces metabolic endotoxemia. Increases in ZIP8 expression
317 suggest this transporter is also dysregulated in aging. ZIP14 ablation eliminates the NHI
318 accumulation in muscle that occurs in aging. Proper functioning of ZIP14 in bone is essential
319 during aging to maintain trabecular and cortical bone density. ZIP14 may influence diseases of
320 aging including sarcopenia and osteoporosis.

321

322 **Acknowledgments**

323 The authors are grateful to Dr. Jay Cao of the USDA Grand Forks Human Nutrition
324 Research Center for performing the micro CT scans of the bone and the resulting analyses.
325 We also thank Matthew P. Beke for help with the manuscript.

326 **Funding**

327 This project was supported by the National Institute of Diabetes and Digestive and
328 Kidney Diseases (Grant No R01-DK094244) and Boston Family Endowment Funds of the
329 University of Florida Foundation to RJC.

330 **Author contributions**

331 T.B.A., C.T. and R.J.C. designed the experiments. T.B.A., C.T., J.K., M.H.K., and O.T.
332 performed the experiments; T.B.A., C.T., C.L. and R.J.C. contributed to data interpretation and
333 T.B.A. and R.J.C. wrote the paper. All authors critically reviewed and gave final approval to this
334 version of the manuscript.

335 **Conflict of Interest**

336 The authors have no conflict of interest.

337

338

339

340

341

342

343

344 **References**

- 345 Aydemir TB, Chang SM, Guthrie GJ, Maki AB, Ryu MS, Karabiyik A, Cousins RJ (2012). Zinc
346 transporter ZIP14 functions in hepatic zinc, iron and glucose homeostasis during the
347 innate immune response (endotoxemia). *PloS one*. 7, e48679.
- 348 Aydemir TB, Liuzzi JP, McClellan S, Cousins RJ (2009). Zinc transporter ZIP8 (SLC39A8) and
349 zinc influence IFN- γ expression in activated human T cells. *J Leuk Biol*. 86, 337-348.
- 350 Begum NA, Kobayashi M, Moriwaki Y, Matsumoto M, Toyoshima K, Seya T (2002).
351 Mycobacterium bovis BCG cell wall and lipopolysaccharide induce a novel gene,
352 BIGM103, encoding a 7-TM protein: identification of a new protein family having Zn-
353 transporter and Zn-metalloprotease signatures. *Genomics*. 80, 630-645.
- 354 Bennermo M, Held C, Stemme S, Ericsson CG, Silveira A, Green F, Tornvall P (2004). Genetic
355 predisposition of the interleukin-6 response to inflammation: implications for a variety of
356 major diseases? *Clin chem*. 50, 2136-2140.
- 357 Besecker B, Bao S, Bohacova B, Papp A, Sadee W, Knoell DL (2008). The human zinc
358 transporter SLC39A8 (Zip8) is critical in zinc-mediated cytoprotection in lung epithelia.
359 *Am J Physiol Lung Cell Mol Physiol*. 294, L1127-1136.
- 360 Camporez JP, Petersen MC, Abudukadier A, Moreira GV, Jurczak MJ, Friedman G, Haqq CM,
361 Petersen KF, Shulman GI (2016). Anti-myostatin antibody increases muscle mass and
362 strength and improves insulin sensitivity in old mice. *Proc Natl Acad Sci U S A*. 113,
363 2212-2217.
- 364 Cao JJ, Picklo MJ (2014). N-acetylcysteine supplementation decreases osteoclast
365 differentiation and increases bone mass in mice fed a high-fat diet. *J Nutr*. 144, 289-
366 296.

367 Cousins RJ, Liuzzi JP , Lichten LA (2006). Mammalian zinc transport, trafficking, and signals.
368 *J Biol Chem.* 281, 24085-24089.

369 Ehsani S, Huo H, Salehzadeh A, Pocanschi CL, Watts JC, Wille H, Westaway D, Rogaeva E,
370 St George-Hyslop PH, Schmitt-Ulms G (2011). Family reunion--the ZIP/prion gene
371 family. *Prog Neurobiol.* 93, 405-420.

372 Fagiolo U, Cossarizza A, Scala E, Fanales-Belasio E, Ortolani C, Cozzi E, Monti D, Franceschi
373 C, Paganelli R (1993). Increased cytokine production in mononuclear cells of healthy
374 elderly people. *Eur J Immunol.* 23, 2375-2378.

375 Flurkey K, M. Currer J, Harrison DE (2007). Chapter 20 - Mouse Models in Aging Research
376 A2 – Fox JG. In *The Mouse in Biomedical Research (Second Edition)*. (MT Davisson,
377 FW Quimby, SW Barthold, CE Newcomer , AL Smith, eds). Burlington: Academic
378 Press, pp. 637-672.

379 Frazzini V, Rockabrand E, Mocchegiani E, Sensi SL (2006). Oxidative stress and brain aging:
380 is zinc the link? *Biogerontology.* 7, 307-314.

381 Guthrie GJ, Aydemir TB, Troche C, Martin AB, Chang SM, Cousins RJ (2015). Influence of
382 ZIP14 (slc39A14) on intestinal zinc processing and barrier function. *Am J Physiol*
383 *Gastrointest Liver Physiol.* 308, G171-178.

384 He L, Girijashanker K, Dalton TP, Reed J, Li H, Soleimani M, Nebert DW (2006). ZIP8,
385 member of the solute-carrier-39 (SLC39) metal-transporter family: characterization of
386 transporter properties. *Mol Pharmacol.* 70, 171-180.

387 Ionescu AM, Schwarz EM, Vinson C, Puzas JE, Rosier R, Reynolds PR , O'Keefe RJ (2001).
388 PTHrP modulates chondrocyte differentiation through AP-1 and CREB signaling. *J Biol*
389 *Chem.* 276, 11639-11647.

390 Jeong J, Walker JM, Wang F, Park JG, Palmer AE, Giunta C, Rohrbach M, Steinmann B, Eide
 391 DJ (2012). Promotion of vesicular zinc efflux by ZIP13 and its implications for
 392 spondylocheiro dysplastic Ehlers-Danlos syndrome. *Proc Natl Acad Sci U S A.* 109,
 393 E3530-3538.

394 Khosla S (2013). Pathogenesis of age-related bone loss in humans. *J Gerontol A Biol Sci Med*
 395 *Sci.* 68, 1226-1235.

396 Kim JH, Jeon J, Shin M, Won Y, Lee M, Kwak JS, Lee G, Rhee J, Ryu JH, Chun CH , Chun JS
 397 (2014). Regulation of the catabolic cascade in osteoarthritis by the zinc-ZIP8-MTF1
 398 axis. *Cell.* 156, 730-743.

399 Knoell DL, Julian MW, Bao S, Besecker B, Macre JE, Leikauf GD, DiSilvestro RA , Crouser ED
 400 (2009). Zinc deficiency increases organ damage and mortality in a murine model of
 401 polymicrobial sepsis. *Crit Care Med.* 37, 1380-1388.

402 Lichten L , Cousins RJ (2009). Mammalian zinc transporters: nutritional and physiologic
 403 regulation. *Annu Rev Nutr.* 29, 153-176.

404 Lichten LA, Liuzzi JP, Cousins RJ (2009). Interleukin-1beta contributes via nitric oxide to the
 405 upregulation and functional activity of the zinc transporter Zip14 (Slc39a14) in murine
 406 hepatocytes. *Am J Physiol Gastrointest Liver Physiol.* 296, G860-867.

407 Liuzzi JP, Lichten LA, Rivera S, Blanchard RK, Aydemir TB, Knutson MD, Ganz T, Cousins RJ
 408 (2005). Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the
 409 hypozincemia of the acute-phase response. *Proc Natl Acad Sci U S A.* 102, 6843-6848.

410 Marcellini F, Giuli C, Papa R, Gagliardi C, Dedoussis G, Herbein G, Fulop T, Monti D, Rink L,
 411 Jajte J, Mocchegiani E (2006). Zinc status, psychological and nutritional assessment in
 412 old people recruited in five European countries: Zincage study. *Biogerontology.* 7, 339-
 413 345.

414 Mocchegiani E, Malavolta M (2008). Zinc-gene interaction related to inflammatory/immune
 415 response in ageing. *Genes Nutr.* 3, 61-75.

416 Olivieri F, Antonicelli R, Cardelli M, Marchegiani F, Cavallone L, Mocchegiani E , Franceschi C
 417 (2006). Genetic polymorphisms of inflammatory cytokines and myocardial infarction in
 418 the elderly. *Mech Ageing Dev.* 127, 552-559.

419 Pepersack T, Rotsaert P, Benoit F, Willems D, Fuss M, Bourdoux P, Duchateau J (2001).
 420 Prevalence of zinc deficiency and its clinical relevance among hospitalised elderly. *Arch*
 421 *Gerontol Geriatr.* 33, 243-253.

422 Rebouche CJ, Wilcox CL , Widness JA (2004). Microanalysis of non-heme iron in animal
 423 tissues. *J Biochem Biophys Methods.* 58, 239-251.

424 Ryu MS, Lichten LA, Liuzzi JP, Cousins RJ (2008). Zinc transporters ZnT1 (Slc30a1), Zip8
 425 (Slc39a8), and Zip10 (Slc39a10) in mouse red blood cells are differentially regulated
 426 during erythroid development and by dietary zinc deficiency. *J Nutr.* 138, 2076-2083.

427 Schmitt-Ulms G, Ehsani S, Watts JC, Westaway D , Wille H (2009). Evolutionary descent of
 428 prion genes from the ZIP family of metal ion transporters. *PloS one.* 4, e7208.

429 Schroeder JJ, Cousins RJ (1990). Interleukin 6 regulates metallothionein gene expression and
 430 zinc metabolism in hepatocyte monolayer cultures. *Proc Natl Acad Sci U S A.* 87, 3137-
 431 3141.

432 Starr ME, Evers BM, Saito H (2009). Age-associated increase in cytokine production during
 433 systemic inflammation: adipose tissue as a major source of IL-6. *J Gerontol A Biol Sci*
 434 *Med Sci.* 64, 723-730.

435 Troche C, Beker Aydemir T, Cousins RJ (2016). Zinc transporter Slc39a14 regulates
 436 inflammatory signaling associated with hypertrophic adiposity. *Am J Physiol Endocrinol*
 437 *Metab.* 310, E258-268.

438 Tudor R, Zalewski PD, Ratnaike RN (2005). Zinc in health and chronic disease. *J Nutr Health*
439 *Aging*. 9, 45-51.

440 Tuschl K, Meyer E, Valdivia LE, Zhao N, Dadswell C, Abdul-Sada A, Hung CY, Simpson MA,
441 Chong WK, Jacques TS, Woltjer RL, Eaton S, Gregory A, Sanford L, Kara E, Houlden
442 H, Cuno SM , Prokisch H (2016). Mutations in SLC39A14 disrupt manganese
443 homeostasis and cause childhood-onset parkinsonism-dystonia. *Nat Commun* 7, 11601.

444 Underwood, ER (1977). Manganese in: Trace Elements in Human and Animal Nutrition, fourth
445 edition. New York: Academic Press.

446 Vasto S, Candore G, Balistreri CR, Caruso M, Colonna-Romano G, Grimaldi MP, Listi F,
447 Nuzzo D, Lio D , Caruso C (2007). Inflammatory networks in ageing, age-related
448 diseases and longevity. *Mech Ageing Dev*. 128, 83-91.

449 Wang CY, Jenkitkasemwong S, Duarte S, Sparkman BK, Shawki A, Mackenzie B , Knutson
450 MD (2012). ZIP8 is an iron and zinc transporter whose cell-surface expression is up-
451 regulated by cellular iron loading. *J Biol Chem*. 287, 34032-34043.

452 Wessels I, Cousins RJ (2015). Zinc dyshomeostasis during polymicrobial sepsis in mice
453 involves zinc transporter Zip14 and can be overcome by zinc supplementation. *Am J*
454 *Physiol Gastrointest Liver Physiol*. 309, G768-778.

455 Wong CP, Magnusson KR, Ho E (2013). Increased inflammatory response in aged mice is
456 associated with age-related zinc deficiency and zinc transporter dysregulation. *J Nutr*
457 *Biochem*. 24, 353-359.

458 Xu J, Marzetti E, Seo AY, Kim JS, Prolla TA , Leeuwenburgh C (2010). The emerging role of
459 iron dyshomeostasis in the mitochondrial decay of aging. *Mech Ageing Dev*. 131, 487-
460 493.

Supporting Information

Supporting Fig. 1. Influence of aging on body length of WT and Zip14 KO mice.

Supporting Fig. 2. Ear mesenchymal stem cells (EMSC) were collected from WT and KO mice (n=3 mice per genotype) and processed as described previously (Rim et al., 2005; Troche et al., 2016). Post-processing cells were plated and grown until confluence. Upon visual confirmation of confluence cells were treated with osteogenic differentiation medium (DMEM-F12 supplemented with 10% FBS, 200 μ M ascorbic acid 2-phosphate, 10nM dexamethasone, 7mM β -glycerophosphate, and 1% penicillin-streptomycin). Cells were maintained in culture for 18 days. For experiments that involved LPS, cells were maintained in osteogenic medium supplemented with 10ng/ml LPS throughout the entire course of the experiment; medium was changed every 3 days.

Mineralization, a marker of osteogenic differentiation, was evaluated through Alizarin Red S (ARS) a compound that binds ionic calcium. At 14 days, cells were fixed in 4% formaldehyde and stained with 40 mM ARS for 30 min. Cells were washed, and images were taken. Elution of Alizarin Red S was achieved through a protocol previously described by Gregory et al. (2004). Briefly, previously stained cells were incubated in 10% acetic acid for 30 min at room temperature. The monolayer was then scrapped from wells and heated at 85°C for 10 min. Cells were pelleted by centrifugation and the supernatant was transferred to a new tube where 10% ammonium hydroxide was used to stop the reaction. Absorbance was read at 405nm.

Supporting References

Rim J-S, Mynatt RL, Gawronska-Kozak B (2005). Mesenchymal stem cells from the outer ear: a novel adult stem cell model system for the study of adipogenesis. *FASEB J.* 9, 1205–1207.

Gregory CA, Gunn WG, Peister A, Prockop DJ (2004). An Alizarin red-based assay of

486 mineralization by adherent cells in culture: comparison with cetylpyridinium chloride
487 extraction. *Anal Biochem.* 329, 77-84.

488 **Table 1**
489 Zinc Concentrations of specific tissues of young (3-4 month) and aged
490 (22 month) WT mice.
491

Tissue	Age	$\mu\text{g Zn/mg tissue}$	P-Value
Spleen	Young	23.89 ± 0.26	0.01
	Aged	22.10 ± 0.47	
Adipose	Young	2.11 ± 0.13	0.04
	Aged	2.73 ± 0.16	
Muscle	Young	8.31 ± 1.27	0.05
	Aged	12.35 ± 1.27	
Brain	Young	5.52 ± 0.11	0.03
	Aged	5.97 ± 0.11	
Liver	Young	34.80 ± 1.36	0.4
	Aged	34.75 ± 1.13	
Heart	Young	13.95 ± 1.10	0.1
	Aged	16.63 ± 2.41	
Lung	Young	17.18 ± 1.45	0.4
	Aged	16.90 ± 1.34	
Pancreas	Young	29.89 ± 0.31	0.2
	Aged	27.71 ± 2.28	
Jejunum	Young	28.00 ± 0.99	0.1
	Aged	26.56 ± 1.00	
Cecum	Young	16.41 ± 1.16	0.3
	Aged	17.13 ± 0.86	
Colon	Young	23.65 ± 1.55	0.1
	Aged	21.05 ± 1.09	

492 Zinc was measured by AAS.
493 Results are means $\pm$ S.E.M. of samples from 4 mice per age group.
494
495
496
497

498 **Figure Legends**

499 Fig. 1. Influence of aging and *Zip14* ablation using young and aged WT and *Zip14* KO mice on
500 (A) body weight, (B) serum endotoxin, (C) serum IL-6, (D) splenic *IL-6* mRNA and (E)
501 inflammatory pathways in spleen, muscle and liver. (A)-(D) values are mean $\pm$ SE, n=4 per
502 group. P< values are shown.

503 Fig. 2. Influence of aging and *Zip14* ablation using young and aged WT and *Zip14* KO mice on
504 (A) serum zinc concentration, (B) splenic zinc concentration, (C) muscle and liver zinc
505 concentrations, (D) muscle and liver non-heme iron (NHI) concentrations. Values are mean $\pm$
506 SE, n=4 per group. P< values are shown.

507 Fig. 3. Influence of aging and *Zip14* ablation using young and aged WT and *Zip14* KO mice on
508 (A) muscle and liver ZIP14 using western blots normalized to tubulin, (B) spleen, muscle and
509 liver ZIP8 using western blots normalized to tubulin (C) MT1 mRNA in muscle and spleen.
510 Values are mean $\pm$ SE, n=4 per group.

511 Fig. 4. Cortical bone microstructure of midshaft femur of young and aged WT and *Zip14* KO
512 mice measured using micro-CT. (A) Representative 3D reconstructions of femur cross
513 sections. (B) Estimates of cortical bone volume/total volume (BV/TV), bone surface/bone
514 volume (BS/BV) and cortical thickness (TbTh). Values are mean $\pm$ SE, n=4 per group.

515 Fig. 5. Trabecular bone microstructure of distal femur of young and aged WT and *Zip14* KO
516 mice measured using micro-CT. (A) Representative 3D reconstructions of trabecular
517 architecture of the femur. (B) Estimates of trabecular bone volume/total volume (BV/TV),
518 connectivity density (ConnD), trabecular thickness mgHA/cm³ (TbTh) and bone mineral density
519 (BMD). Values are mean $\pm$ SE, n=4 per group.

520 Fig. 6. Summary of changes associated with aging and those associated with ablation of
521 *Zip14*. Increased circulating IL-6 is a signature of aging that is augmented with ZIP14 ablation.
522 Aging increases muscle zinc and non-heme iron and decreases bone mineral density. These
523 are influenced with ablation of ZIP14. Metabolic endotoxemia is a signature of ZIP14 ablation,
524 but is not changed in aging.

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

A

B

FIGURE 5

FIGURE 6

Supporting Figure1

Supporting Figure 2