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Properties of Zip4 accumulation during zinc deficiency and its usefulness to evaluate zinc status: A study of the effects of zinc deficiency during lactation

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AUTHOR CONTRIBUTIONS

A.H., T.K., and M.K. conceived of and designed the research study; A.H., M.N., N.T., S.M., K.K., T.G., Y.K., A.M., H.S., H.N., T.K., and M.K. performed experiments; A.H., M.N., N.T., S.M., T.G., T.K., and M.K. analyzed data; A.H., M.N., N.T., T.G., T.K., and M.K. interpreted the results of experiments; A.H., M.N., N.T., T.G., H.S., T.K., and M.K. prepared figures; A.H., T.K., and M.K. drafted the manuscript.

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ABSTRACT

Systemic and cellular zinc homeostasis is elaborately controlled by ZIP and ZnT zinc transporters. Therefore, detailed characterization of their expression properties is of importance. Of these transporter proteins, Zip4 functions as the primarily important transporter to control systemic zinc homeostasis because of its indispensable function of zinc absorption in the small intestine. In this study, we closely investigated Zip4 protein accumulation in the rat small intestine in response to zinc status using an anti-Zip4 monoclonal antibody that we generated, and contrasted this with the zinc-responsive activity of the membrane-bound alkaline phosphatase (ALP). We found that Zip4 accumulation is more rapid in response to zinc deficiency than previously thought. Accumulation increased in the jejunum as early as 1 day following a zinc-deficient diet. In the small intestine, Zip4 protein expression was higher in the jejunum than in the duodenum and was accompanied by reduction of ALP activity, suggesting that the jejunum can become zinc deficient more easily. Furthermore, by monitoring Zip4 accumulation levels and ALP activity in the duodenum and jejunum, we reasserted that zinc deficiency during lactation may transiently alter plasma glucose levels in the offspring in a sex-specific manner, without affecting homeostatic control of zinc metabolism. This confirms that zinc nutrition during lactation is extremely important for the health of the offspring. These results reveal that rapid Zip4 accumulation provides a significant conceptual advance in understanding of the molecular basis of systemic zinc homeostatic control, and that properties of Zip4 protein accumulation are useful to evaluate zinc status closely.

Keywords: zinc deficiency; Zip4 processing; small intestine; alkaline phosphatase; glucose homeostasis.

INTRODUCTION

Zinc plays a great variety of roles in diverse biological processes (35, 61), and therefore is crucial as an essential micronutrient for human health. Zinc deficiency causes a broad range of defects including diarrhea, alopecia, skin lesions, immune system and neuronal dysfunction, and taste disorders (13, 21, 35, 55), and is a clinical risk factor for a number of diseases in older people (20, 50). Because zinc deficiency also impairs optimal growth and development in infants, extremely low levels of zinc in breast milk, which is rarely caused by genetic diseases, results in severe health consequences in breast-fed infants (5, 26, 41, 47, 49). Moreover, maternal zinc deficiency during lactation, which results in low secretion of zinc (7), is shown to affect physiological responses in offspring in animal studies, such as effects on glucose metabolism (28), immune competence (56), and arterial blood pressure and renal functions (60). However, molecular investigations have not yet been fully performed.

Cellular and systemic zinc homeostasis is elaborately regulated by two zinc transporter families, ZIP and ZnT (6, 15, 36, 43). Among these, Zip4 is the most important transporter for the regulation of systemic zinc homeostasis because it plays an essential role in dietary zinc absorption in the small intestine (17). Additionally, it was identified as the gene responsible for acrodermatitis enteropathica (1, 33, 40, 63). Zip4 protein expression is dynamically regulated in response to zinc deficiency; Zip4 protein accumulates on the apical membrane by escaping from endocytosis and degradation, and undergoes processing by removal of the long extracellular amino-terminal region via proteolytic cleavage during prolonged zinc deficiency (9, 31, 37, 48, 64). Molecular and physiological properties of Zip4 protein expression and function have been characterized (2-4, 10, 11, 17, 45, 46, 62), but further investigation is needed for complete understanding. In the small intestine, membrane-bound alkaline phosphatase (ALP) activity is significantly decreased during

71 zinc-deficient conditions (32), and, thus, increased Zip4 expression and decreased membrane-bound ALP
72 activity are considered to accurately reflect zinc status.

73 Zip4 accumulation in the small intestine during zinc deficiency has not yet been extensively
74 investigated. In the present study, we examined it using an anti-Zip4 monoclonal antibody that we recently
75 generated (22). The antibody clearly showed more rapid accumulation of Zip4 protein in the jejunum in
76 response to a zinc-deficient diet than previously thought. Furthermore, in intimate monitoring of Zip4
77 expression levels detected by this antibody and the membrane-bound ALP activity, we examined the effects
78 of zinc deficiency during lactation on glucose tolerance in the offspring, which has been reported to be
79 affected (28). Evaluation of zinc status based on this perspective has not yet been conducted, and, therefore,
80 would give novel insights into zinc physiology.
81
82 **MATERIALS AND METHODS**

83 *Rat care, use, and dietary zinc manipulation.* Four-week-old male Sprague-Dawley (SD) rats
84 were purchased from Japan SLC (Hamamatsu, Japan), and acclimated for 3 d (fed a zinc-sufficient [Zn-Suf]
85 diet). After 3 d of acclimation, rats were divided into three groups and fed one of the following diets for 4 d:
86 zinc-deficient (Zn-Def), low-zinc (Zn-Low), and Zn-Suf diets, which contained 2.2, 4.1, and 33.7 mg
87 zinc/kg, respectively, as described previously (38). In experiments of the effects of zinc-deficient dams on
88 offspring, pregnant SD rats (16 d of pregnancy, d1=vaginal plug) were purchased from Japan SLC, and fed a
89 MB-3 diet (Funabashi Farms, Funabashi, Japan) until parturition. On the day of parturition (considered
90 lactation day 0), dams were divided into two groups and fed a Zn-Suf or Zn-Low diet until weaning day (3
91 weeks). After 3 weeks, nursing neonatal pups were weaned and fed a Zn-Suf diet and drinking water with
92 free access. In all experiments, rats were housed in individual stainless steel cages under controlled

93 conditions (temperature, 23 ± 2°C; humidity, 50 ± 10%; 12-h light–dark cycle, 08:00 am to 08:00 pm), and
94 were decapitated after being food deprived for 5 h. The blood and tissues, including small intestines, were
95 collected at the time points specified in each experiment from the dams and pups, quickly frozen in liquid
96 nitrogen, and used for experiments, such as membrane proteins preparation, as described below. Zinc
97 concentrations were measured in plasma. Food intakes and body weights were measured each day. The
98 experimental plan for the present study was approved by the Animal Research-Animal Care Committee of
99 Tohoku University and Kyoto University. The entire experiment was carried out in accordance with the
100 guidelines issued by this committee and Japanese governmental legislation (2005). The same committee
101 supervised the care and use of the rats used in this study.
102
103 *Generation of an anti-ZnT4 monoclonal antibody.* Fused proteins consisting of the
104 amino-terminal cytosolic region of human ZnT4 (112 amino acid residues from Met^{1} to Ala^{112}) and maltose
105 binding protein were used as a ZnT4 antigen. The same peptide fused to glutathione S transferase protein
106 was used as a solid-phase antigen for hybridoma screening as described previously (26, 34). The hybridoma
107 that produces the anti-ZnT4 antibody was subcloned by the limiting dilution method. An ascites was
108 generated by injection of 1×10^{7} hybridoma cells into pristine-primed mice. Membrane proteins (20 μg)
109 prepared from DT40 cells stably expressing carboxyl-terminally HA-tagged human ZnT4 (14) were used for
110 examining the specificity of the antibody.
111
112 *Preparation of membrane proteins from the small intestines and mammary glands*. The small
113 intestines, which were dissected and divided 0.5–2.5 cm distal from the pylorus for the duodenum and
114 5.5–8.5 cm for the jejunum in some experiments, were collected from male rats fed a Zn-Def, Zn-Low, or

115 Zn-Suf diet or lactating dams fed a Zn-Suf or Zn-Low diet and pups fostered by the dams on the indicated
116 days. The mammary gland was simultaneously harvested from the dams. Tissue samples including the
117 duodenum, jejunum, or mammary grand were homogenized using a homogenizer (Disperser T 10 basic,
118 IKA, Tokyo, Japan) or multi-beads shocker (Yasui Kikai, Osaka, Japan), and membranes were recovered
119 from the post-nuclear supernatant by centrifugation (24,000 × *g* for 30 min at 4°C). The membrane pellet
120 was washed once with phosphate buffered saline (PBS) and suspended in alkaline phosphatase buffer (10
121 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, and 0.1% Triton X-100) and frozen at −80°C until assayed as
122 described previously (32). The protein concentrations were determined using Protein Assay CBB Solution
123 (Nacalai Tesque, Kyoto, Japan). Extraction of membrane proteins by Na_2CO_3 was performed as described
124 previously (31). Briefly, membranes prepared from the small intestine of rats fed a Zn-Def diet for several
125 days were resuspended in 0.05 mM Na_2CO_3, and incubated on ice for 15 min before centrifugation for 30
126 min at 24,000 × *g* at 4°C. The supernatant fraction (peripheral membrane proteins) and pellet fraction
127 (integral membrane proteins) were recovered.
128
129 *Immunoblot blot analyses.* Membrane proteins (5 or 10 μg, if not indicated) were lysed in 6 ×
130 SDS-sample buffer at 37°C for 30 min, separated by electrophoresis through 7.5% SDS polyacrylamide gels
131 and transferred to polyvinylidene difluoride membranes (Pall Corporation, East Hills, NY). The blot was
132 blocked with blocking solution (1% skim milk and 0.1% Tween 20 in PBS) or SuperBlock blocking buffer
133 (Thermo Fisher Scientific, Rockford, IL) supplemented with 0.1% Tween 20 for detection of rat Zip4, and
134 then incubated with an anti-Zip4 monoclonal antibody (1:1000 dilution) (22), anti-ZIP4 polyclonal
135 antiserum (1:600) (64), anti-ZnT2 (1:2000) (26), anti-ZnT4 (1:1000), anti-GPR39 (Abcam, Cambridge, UK,
136 1:1500), and anti-GRP78 (ABR, Golden, CO, 1:1000). Horseradish peroxidase-conjugated anti-mouse or

anti-rabbit secondary antibodies (GE Healthcare, Waukesha, WI) were used at a 1:3000 dilution for detection. Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP Substrates (Millipore, Billerica, MA) or Chemi-Lumi One L (Nacalai Tesque). The fluoroimage was obtained using a LAS1000 plus image analyzer (Fujifilm, Tokyo, Japan) and LAS500 (GE Healthcare).

Membrane-bound alkaline phosphatase assay. Membrane proteins (1–3 μg) were assayed for alkaline phosphatase activity with 2 mg/mL *p*-nitrophenyl phosphate in 1 M diethanolamine buffer, pH 9.8, containing 0.5 mM $MgCl_2$ as described previously (16, 32). *p*-Nitrophenol release was measured by the absorbance at 405 nm. Calf intestine alkaline phosphatase (Promega, Madison, WI) was used as the standard (14).

Measurement of plasma glucose and insulin levels. Plasma glucose levels were measured by enzymatic colorimetric methods using the Glucose C-II test kit (Wako Pure Chemical Co., Osaka, Japan) after 5 h of starvation at 4, 7, 9, 11, 13, and 15 weeks. Plasma insulin levels were measured using a rat insulin ELISA kit (Morinaga Institute of Biological Science, Inc. Yokohama, Japan).

Statistical analyses. Values for animal tissues are expressed as means ± standard error of the mean. Statistical comparisons between each group were performed using one-way analysis of variance followed by Tukey's multiple comparisons *post hoc* test. $P<0.05$ was considered significant. The experimental data of biochemical measurements including ALP activity are depicted as mean ± standard deviation, where statistical significance was determined by the Student's *t*-test and accepted as significant at $P<0.05$.

159

160

161 **RESULTS**

162 ***Intestinal Zip4 expression is rapidly increased in the rat small intestine.*** Expression of Zip4 in the small
163 intestine is highly induced in mice fed a zinc-deficient diet (9, 10, 32, 45, 64), but that has not been well
164 investigated in other species, including rats. The anti-Zip4 monoclonal antibody, which we have recently
165 reported (22), clearly detected Zip4 as two bands (~75 kDa and ~38 kDa) in the membrane fractions
166 prepared from the small intestine of rats fed the Zn-Def diet (Fig. 1A, left panel). The monoclonal antibody
167 was generated against the extracellular amino terminal portion (ectodomain) of Zip4 (22), and, thus, the two
168 bands correspond to the full-length (~75 kDa) and the cleaved ectodomain (~38 kDa). This was confirmed
169 by similar results obtained using ZIP4 polyclonal antiserum (Fig. 1A, right panel), which was directed
170 against the large intracellular loop between Zip4 transmembrane domains (TMDs) III and IV. Thus, the
171 antibody detected both the full-length and the processed Zip4 corresponding to Zip4 with eight TMDs
172 without the ectodomain (31). In our previous report (31), we calculated the size of the processed Zip4 as ~37
173 kDa, but the band was detected as ~40 kDa in the present study because of the use of different protein size
174 markers. The cleaved ectodomain (~38 kDa) of rat Zip4 was prepared in the membrane fractions as a
175 peripheral membrane protein loosely associated with the membrane fractions, as in the case of mouse Zip4
176 (31) because it was extracted in the supernatant with Na_2CO_3, while the full-length Zip4 and Znt4 (both
177 integral membrane proteins) were not (Fig. 1B).

178 The clear detection of Zip4 by our monoclonal antibody enabled us to investigate more closely
179 Zip4 expression regulation in adult male rats fed the Zn-Def diet. First, we examined the response of Zip4
180 expression to zinc-deficient diets. We divided rats in three groups fed Zn-Def, Zn-Low, or Zn-Suf diets and

181 monitored Zip4 expression in the small intestine by immunoblot analysis (Fig. 2A). Both the full-length
182 form and the cleaved ectodomain of Zip4 were faintly detected after 1 d, and the cleaved ectodomain was
183 clearly detected after 2 d. The membrane-bound ALP activity, which reflects zinc-deficient status (16, 32),
184 decreased dose-dependently in response to the zinc contents of the diets (e.g., 3.8 mU/μg protein in the
185 Zn-Suf group, 2.0 mU/μg protein in the Zn-Low group, and 1.2 mU/μg protein in the Zn-Def group after 1
186 d) (Fig. 2B). Moreover, rat plasma zinc concentrations were reduced by about 50% after 1 d when fed the
187 Zn-Low or Zn-Def diet compared with the Zn-Suf diet (Fig. 2C). Thus, Zip4 protein accumulation and
188 processing events likely occurred following the reduction of plasma zinc concentrations and
189 membrane-bound ALP activity. However, the processing event may be sophisticatedly controlled by the
190 degree of severity of zinc deficiency because the ectodomain of Zip4 was more enhanced in the Zn-Def
191 group than in the Zn-Low group compared with differences in plasma zinc concentrations and ALP activity
192 on days 1 and 2 (Fig. 2A, B and C).

193 We then explored region-specific differences in Zip4 expression. We dissected and divided the
194 small intestine into two pieces, the duodenum (0.5–2.5 cm distal from the pylorus) and jejunum (5.5–8.5
195 cm), and examined Zip4 expression (Fig. 2D). Zip4 expression increased more intensely and rapidly in the
196 jejunum than in the duodenum. This indicates zinc sensitivity was different between the duodenum and
197 jejunum. These close investigations clearly indicate Zip4 expression is sensitively responsive to zinc
198 deficiency in the rat small intestine.

199

200 ***The effects of zinc deficiency on lactating dams.*** To investigate the effects of zinc deficiency during
201 lactation on pups, dams were fed Zn-Low or Zn-Suf diets from birth until weaning (for 3 weeks). As
202 described in previous reports (18, 53, 58), the amount of food intake decreased in dams fed the Zn-Low diet

(Fig. 3A). Anorexia from zinc deficiency was observed by a typical cyclical 3- to 4-d pattern of decreased food intake in dams fed the Zn-Low diet. This was accompanied by decreases in body weights (Fig. 3B) and reduced plasma zinc concentrations (about 80% lower) (Fig. 3C) compared with those fed the Zn-Suf diet. Membrane proteins were prepared from the duodenum and jejunum of dams fed both Zn-Suf and Zn-Low diets at 3 weeks and analyzed by immunoblot. Zip4 of the full-length and the ectodomain, the latter of which was much higher, was detected in the duodenal and jejunal membranes prepared from the Zn-Low dams (Fig. 4A) but not in those of the Zn-Suf dams. Zip4 expression was somewhat higher in the jejunum than duodenum, although less significant compared with that in male rats fed a Zn-Def diet for a shorter period (Fig. 2D). Consistent with the result, the reduction rate of the membrane-bound ALP activity was more significant in the jejunum than in the duodenum of dams fed a Zn-Low diet, both of which were significantly reduced (Fig. 4B). Because Znt4 and the zinc receptor GPR39 are known to be highly expressed in the small intestine (19, 44, 51, 52), expression levels of both proteins were also examined. Compared with that of Zip4, the expression was not significantly affected by the Zn-Low diet (Fig. 4A). Moreover, because we have antibodies against both Znt2 and Znt4, both of which are known to be responsible for supplying zinc into the breast milk in humans (5, 26, 41, 47, 49) and in mice (24, 42), we investigated the effects of zinc deficiency on the expression of both proteins in mammary glands. The expression was not significantly changed between dams fed Zn-Low and Zn-Suf diets compared with Zip4 expression in the jejunum and duodenum (Fig. 4C). However, the membrane-bound ALP activity in the mammary glands was significantly reduced in dams fed a Zn-Low diet (Fig. 4D).

The effects of zinc deficiency during the lactating period on pre-weanling pups. We next examined the effects of Zn-Low diet feeding of dams on their pups. The body weights of pups fostered by dams fed a

225 Zn-Low diet were reduced at 1 week after birth and more significantly reduced at weaning (3 weeks after
226 delivery) compared with those fed a Zn-Suf diet (Fig. 5A). At weaning (3 weeks), plasma zinc
227 concentrations were about 60% lower in pups fostered by dams fed a Zn-Low diet than those fed a Zn-Suf
228 diet (Fig. 5B), indicating that the pups were severely zinc deficient. This was clearly confirmed by the fact
229 that Zip4 expression was increased in both the duodenum and jejunum of pups fostered with dams fed a
230 Zn-Low diet (Fig. 5C). Moreover, membrane-bound ALP activity was severely decreased in both tissues
231 (Fig. 5D). Under our experimental conditions, the effects of Zn-Low diet feeding of dams were obvious on
232 Zip4 expression and ALP activity in pups. Znt4 and GPR39 expression levels were not significantly changed
233 between the two groups (Fig. 5C), as with the dams (Fig. 4A).
234
235 ***The effects of zinc deficiency during lactation on plasma glucose levels in offspring.*** The weaned pups
236 fostered by dams fed Zn-Suf or Zn-Low diets were fed a Zn-Suf diet. Because the amount of food intake and
237 the body weight gain became almost the same within 1 week, we isolated the duodenum and jejunum from
238 pups in both groups at 4 weeks and examined the same items as those measured at 3 weeks. Plasma zinc
239 concentrations were not significantly different between both groups (Fig. 6A), and Zip4 expression was not
240 detected at the same exposure time as used in Fig. 5C in both the duodenum and jejunum of pups fostered by
241 dams fed a Zn-Low diet until weaning (Fig. 6B). Consistent with this, membrane-bound ALP activity in
242 both tissues was almost equivalent between the two groups (Fig. 6C). These results confirmed that pups
243 fostered by dams fed a Zn-Low diet until weaning were not apparently zinc deficient and maintain normal
244 homeostatic control of zinc metabolism. Znt4 and GPR39 expression levels were not significantly changed
245 between the two groups (Fig. 6B), consistent with observations in the pups at weaning (3 weeks).
246 Last, we investigated the effects of zinc deficiency during lactation on plasma glucose

247 homeostasis in the offspring because maternal zinc deficiency during this period is shown to induce
248 long-term changes related to abnormal glucose tolerance in the offspring (28). Plasma glucose levels were
249 higher in male offspring fostered by dams fed a Zn-Low diet at 11 and 13 weeks (Fig. 7A); however, plasma
250 insulin levels were not significantly changed (Fig. 7B). We did not observe similar changes in plasma
251 glucose levels in the female offspring fostered by dams fed a Zn-Low diet (Fig. 7C). Taken together, these
252 results suggest that zinc deficiency during lactation may transiently alter glucose homeostasis in the
253 offspring in a sex-specific manner, even after the status and homeostatic control of zinc become normal.
254
255 **DISCUSSION**
256 Given that many zinc nutrition studies are performed using rats, clarification of zinc absorption
257 processes in the rat small intestine, including Zip4 expression regulation, is important. However, there are
258 few examinations of rat Zip4 expression. Moreover, the fundamental issue relating to rat Zip4 is that several
259 discrepancies have been identified between rat and mouse Zip4 detection by immunoblot analysis, despite
260 the high sequence identities (over 89%; similarity, 93%). Specifically, Zip4 protein accumulation during
261 zinc deficiency was drastically increased or not, and the immunoreactive bands corresponding to Zip4 were
262 detected at either 70–75 kDa or 35–40 kDa (25, 27, 31, 36, 45, 64). In this study, we revealed that Zip4 was
263 detected in several bands corresponding to the full-length, the processed or the cleaved ectodomain of Zip4.
264 Furthermore, Zip4 accumulation was dramatic in the cleaved ectodomain form (implying that the processed
265 Zip4 was also increased) but not so significant in the full-length form during zinc deficiency (Fig. 2A and D).
266 Thus, previous discrepancies in Zip4 detection likely resulted from differences in the detected
267 immunoreactive bands, which are altered by zinc deficiency duration. The inducibility of Zip4 accumulation
268 is different in the regions of the small intestine (e.g., Zip4 expression is higher in the jejunum than

duodenum), which may be an alternative reason why the discrepancies occurred in different immunoblots. Our results are important in clarifying these points and, moreover, revealed that Zip4 expression regulation in rats and mice is consistent. The rapid up-regulation of Zip4 expression in the small intestine during zinc-deficient conditions corroborates the important role of Zip4 in the systemic control of zinc homeostasis.

Another interesting aspect in this study on Zip4 expression regulation is that the processing of Zip4 clearly occurred by 2 d at the latest after feeding with the Zn-Def and Zn-Low diets. The cleaved ectodomain was faintly detected after 1 d in the jejunum (Fig. 2D, right panel). This is in contrast to the previous thought that Zip4 processing needs a prolonged amount of time during zinc-deficient conditions, which was derived from an *in vitro* study using Hepa cells or transfected cells (31). The appearance timing of the cleaved ectodomain of Zip4 preceded the first significant suppression of food intake, which began at 3 d after feeding the same Zn-Low diet in our experimental conditions (53) (Fig. 3A). An orexigenic signal by dietary zinc, which could be derived from the intestine to the brain via vagus nerve signal transduction, occurs during oral but not intraperitoneal administration (53). Thus, it is an intriguing hypothesis that the processing of Zip4 (or the processed or the cleaved ectodomain of Zip4) in enterocytes may be involved in the suppression of food intake, as well as controlling zinc transport activity as discussed below.

The association of the cleaved Zip4 ectodomain with the cellular membrane after processing, which was proposed in an *in vitro* study (31), was confirmed in the rat small intestine (see Fig. 1B). The processing event in Zip4 or the cleaved ectodomain of Zip4 itself can be considered physiologically relevant because acrodermatitis enteropathica-causing mutations inhibit the processing of Zip4 (31). Considering these and the abundance of histidine and cysteine residues in the ectodomain (15, 35), the ectodomain may capture dietary zinc and control its transfer to the pore formed by eight TMDs of Zip4 (3). Alternatively, the ectodomain itself may control pore opening or closing through its releasing (or inhibiting its release), as

291 previously speculated (59). Processing may be an important, conserved regulatory mechanism in some ZIP
292 transporters because similar ectodomain cleavage has been found in ZIP6 and ZIP10 (12, 23), both of which
293 possess the long extracellular amino-terminal portion abundant in histidine and cysteine residues like ZIP4
294 (15, 35). Interestingly, the ectodomain of the transporter involved in copper absorption is proteolytically
295 cleaved, which is thought to control copper uptake (54). Thus, the processing event in metal transporters
296 may be one of the important regulatory mechanisms to control metal uptake and absorption rates from the
297 extracellular milieu.

298 In vertebrates, several ALP isozymes (tissue non-specific, intestine and germ cell specific, and
299 placenta specific in humans) are expressed. Each of these is probably zinc dependent, although zinc
300 dependence of the germ cell-specific ALP has not been molecularly examined (29, 30). In this study, we
301 used membrane-bound ALP activity as a marker reflecting zinc status in examined tissues as in our previous
302 report (36). Membrane-bound ALP activity in the small intestine was dependent on dietary zinc contents,
303 which was inversely associated with Zip4 expression levels (see Fig. 2A and B). Along with more enhanced
304 Zip4 expression (both the full length and the cleaved ectodomain) in the jejunum than the duodenum (Figs.
305 2D, 4A and 5C), the reduction rate of the membrane-bound ALP activity, which is considered by comparing
306 that in Zn-Low with that in Zn-Suf, was more significant in the jejunum than the duodenum (Figs. 4B and
307 5D). These results indicate that the jejunum likely becomes more zinc deficient than the duodenum during
308 zinc deficiency, and may suggest that a distal part of the intestine from the pylorus may become more easily
309 zinc deficient. This hypothesis should probably be extensively explored because one of representative
310 features of zinc deficiency is diarrhea. Moreover, membrane-bound ALP activity in mammary glands was
311 significantly decreased in the dams fed a Zn-Low diet compared with those fed a Zn-Suf diet. Znt2 and Znt4
312 protein expression levels were not significantly changed between both groups (see Fig. 4C and 4D),

313 indicating both Znt2 and Znt4 protein expression are less sensitive to zinc deficiency in mammary glands.
314 Thus, membrane-bound ALP activity would be useful to evaluate apparent zinc status and to investigate
315 zinc-responsive protein expression including ZIP and ZnT transporters in tissues.

316 Lastly, the quite important aspect of this study is that we reasserted the effects of zinc deficiency
317 during lactation on plasma glucose homeostasis in male offspring, even if zinc status and homeostatic
318 control were normally regulated. Similar results, including the sex-specific effects of blood glucose levels,
319 were previously reported by Jou et al. (28); however, some discrepancies are present between our results and
320 previous reports. This may be because dams were fed a zinc-deficient diet (7 mg/kg) and control diet (25
321 mg/kg) for 6 weeks from 3 weeks preconception to 21 d post-parturition in the previous study (28), while
322 dams were fed Zn-Low (4.1 mg/kg) and Zn-Suf (33.7 mg/kg) diets in this study. Importantly, the molecular
323 mechanism of how plasma glucose levels are affected by zinc deficiency during lactation has not yet been
324 clarified, although epigenetic changes may occur on the promoter of molecules involved in glucose
325 homeostasis during zinc deficiency, like in the case of the epigenetic alteration in the MT2 promoter region
326 during zinc deficiency *in utero* (39). These points should be more intensely investigated in a future study.
327 About 80% of pregnant and lactating women are estimated to be at risk for marginal zinc deficiency (8, 57).
328 Thus, zinc levels during this period should receive much attention from viewpoints of optimal growth and
329 development, as well as future good health in offspring.
330

331 **PERSPECTIVES AND SIGNIFICANCE**

332 Zip4 accumulation in the small intestine rapidly occurred during zinc deficiency. The processing
333 of Zip4 also rapidly occurred during zinc deficiency. These properties of Zip4 may be useful as a biomarker
334 reflecting zinc deficient levels in the body, as well as for evaluating bioavailable zinc levels. In fact, the

potential efficacy of detecting Zip4 expression as a marker was described in the study of zinc deficiency during lactation, contrasting this with the zinc-responsive activity of ALP. Moreover, the rapid processing of Zip4 during zinc deficiency suggests its physiological importance in the regulation to control (or activate) its zinc transport function. The result would lead to new directions in the research field of zinc transporters because the processing is found in other ZIP transporters. Zinc deficiency puts human health at risk and is still a worldwide problem (65, 66). Thus, comprehensive understanding of the expression properties of ZIP4 is of critical importance, given its primary importance to control systemic zinc homeostasis. The present study may provide useful information to establish new strategies to prevent zinc deficiency.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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542
543

544 **FIGURE LEGENDS**

545 Figure 1. Anti-Zip4 monoclonal antibody detects rat Zip4. *A*, The anti-Zip4 monoclonal antibody detected
546 Zip4 in the rat small intestine. Membrane proteins prepared from the small intestine of rats fed a Zn-Low (L)
547 or Zn-Suf (S) diet for 3 weeks were subject to immunoblot analysis. Zip4 protein was detected as both the
548 full-length (~75 kDa) and the cleaved ectodomain (Ecto) (~38 kDa) only in membranes prepared from rats
549 fed a Zn-Low diet by the anti-Zip4 monoclonal antibody (*left* panel). Note that Zip4 protein
550 immunoreactivity detected by the anti-ZIP4 polyclonal antiserum (*right* panel) is similar, but the antiserum
551 detects both the full-length (~75 kDa) and the processed Zip4 (corresponding to the remaining
552 carboxyl-terminal region with eight TMDs lacking the ectodomain, ~40 kDa). The same membrane proteins
553 were run on separate blots, and the blotted membrane was used for detection by either anti-Zip4 monoclonal
554 antibody or anti-ZIP4 polyclonal antiserum. GRP78 was used as a loading control. *B*, The cleaved
555 ectodomain of Zip4 was detected on membrane fractions after processing. Membrane proteins prepared from
556 the small intestine of rats fed a Zn-Def diet were extracted in Na_2CO_3, and the pellet (Mem) and extract
557 (Sup) were analyzed by immunoblot analysis. Note that the cleaved ectodomain of Zip4 was detected in Sup,
558 but the full-length Zip4 and Znt4, both of which are integral membrane proteins, were detected in Mem. The
559 Sup and Mem fractions extracted by PBS from the same membrane proteins were used as a control. The
560 noncontiguous boxed lanes in the PBS and Na_2CO_3 panels were derived from the same blot. The specificity
561 of the anti-ZnT4 monoclonal antibody was confirmed by verifying that the anti-ZnT4 antibody and
562 commercial anti-HA antibodies (HA) detected the same bands in the immunoblot analysis using membrane
563 proteins (20 μg) prepared from DT40 cells stably expressing HA-tagged human ZnT4 (+) and those not
564 expressing (−) (*right* panels). The same blotted membrane was used for detection by each antibody after
565 stripping. Calnexin was used as a loading control. In *A* and *B*, the representative results are displayed.

566

567 Figure 2. Rapid accumulation of Zip4 protein in the rat small intestine. *A*, Zip4 is expressed in the rat small
568 intestine in response to zinc deficiency. Membrane proteins prepared from the small intestine of rats fed a
569 Zn-Def (D), Zn-Low (L), or Zn-Suf (S) diet for up to 4 d were subject to immunoblot analysis. Znt4
570 expression was not affected by dietary zinc levels, which was used as a loading control in *D*. CBB staining
571 is also displayed as a loading control. *B*, ALP activity in the membrane proteins used in *A*. Data are
572 expressed as means ± SD (n = 5, *P<0.05, **P<0.01). *C*, Plasma zinc concentrations at 1–4 d. Values are
573 means ± SEM (n = 5, **P<0.01). *D*, Accumulation of Zip4 in response to zinc deficiency is higher in the
574 jejunum than in the duodenum. Membrane proteins prepared from the small intestine of rats fed a Zn-Def
575 (D) or Zn-Suf (S) diet for up to 4 days were subject to immunoblot analysis. Znt4 was used as a loading
576 control. In *A* and *D*, the lanes of day 0 are shown as "S" because rats were fed a Zn-Suf diet before being
577 divided into three groups. The representative results are displayed.

578

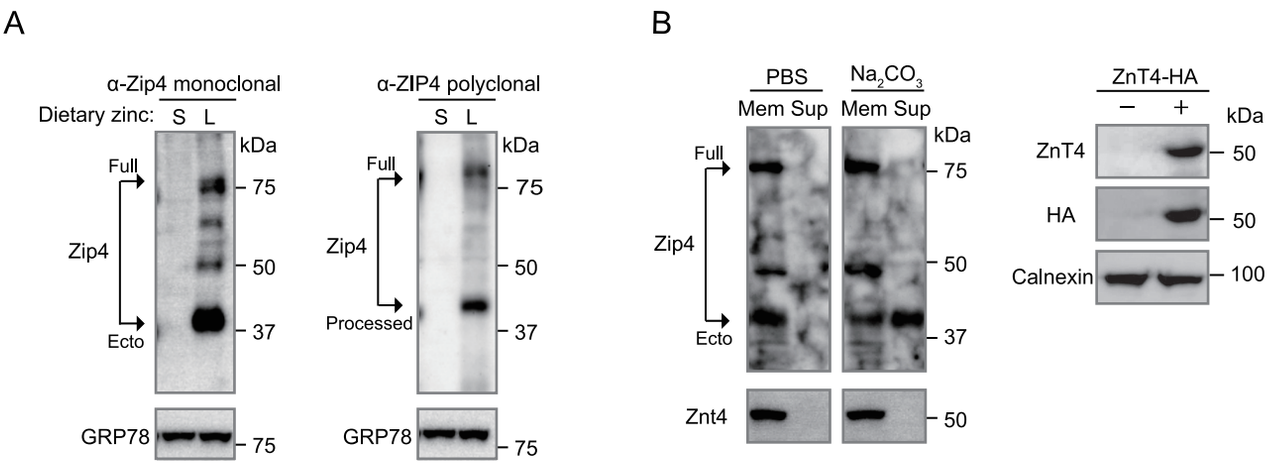
579 Figure 3. Effects of dietary zinc deficiency on lactating dams. The dams were fed a Zn-Suf or Zn-Low diet
580 from parturition (day 0 = parturition) until weaning (for 3 weeks). *A*, The amount of daily food intake for 3
581 weeks. *B*, Body weights at 16 d gestation and each week after parturition. Values are means ± SEM (n = 5,
582 *P<0.05). *C*, Plasma zinc concentrations at 1 d after weaning their pups. Suf, Zn-Suf group; Low, Zn-Low
583 group. Values are means ± SEM (n = 5, **P<0.01).

584

585 Figure 4. Effects of dietary zinc deficiency on zinc transporter and zinc receptor expression on lactating
586 dams. *A*, Immunoblot analysis of Zip4, Znt4, and GPR39 in the duodenum and jejunum. Membrane proteins
587 were prepared from the duodenum or jejunum of lactating dams fed a Zn-Suf (S) or Zn-Low (L) diet for 3

588 weeks (at 1 d after weaning their pups) as shown in Figure 2. Two representative results of five are
589 displayed. The noncontiguous boxed lanes were derived from the same blot in the duodenum and the
590 jejunum. GRP78 was used as a loading control. *B*, ALP activity in the membrane proteins used in *A*. Data
591 are expressed as means ± SD (n = 5, $^{**}P<0.01$) in the duodenum (*left*) or jejunum (*right*). *C*, Immunoblot
592 analyses of Znt4 and Znt2 in membrane proteins prepared from mammary glands from the same rats used in
593 *A*. Two representative results of five are displayed. *D*, ALP activity of the membrane proteins used in *C*.
594 Data are expressed as means ± SD (n = 5, $^{*}P<0.05$). In *A* and *C*, GRP78 was used as a loading control.
595
596 Figure 5. Effects of zinc deficiency during lactation on pre-weanling pups. *A*, Change in body weights for 3
597 weeks. The weights of pups fostered by dams fed a Zn-Suf or Zn-Low diet were measured each week. *B*,
598 Plasma zinc concentrations at the day of weaning (at 3 weeks). Suf, Zn-Suf group; Low, Zn-Low group. In *A*
599 and *B*, values are means ± SEM (n = 5, $^{*}P<0.05$). *C*, Immunoblot analysis of Zip4, Znt4, and GPR39 in the
600 duodenum or jejunum of the pups. Membrane proteins were prepared from the duodenum or jejunum of the
601 pups. The noncontiguous boxed lanes were derived from the same blot in the duodenum and the jejunum.
602 GRP78 was used as a loading control. Two representative results of five are displayed. *D*, ALP activity in
603 the membrane proteins used in *C*. Data are expressed as means ± SD (n = 5, $^{**}P<0.01$) in the duodenum
604 (*left*) or jejunum (*right*)..
605
606 Figure 6. Effects of zinc deficiency during lactation on pups after weaning. *A*, Plasma zinc concentrations at
607 1 week after weaning (at 4 weeks). Values are means ± SEM (n = 5). *B*, Immunoblot analyses of Zip4, Znt4,
608 and GPR39 in the duodenum or jejunum of the pups. Membrane proteins were prepared from the duodenum
609 or jejunum of the pups of two groups. The noncontiguous boxed lanes were derived from the same blot in

610 the duodenum and the jejunum. GRP78 was used as a loading control. Two representative results of five are
611 displayed. *C*, ALP activity in the membrane proteins used in *B*. Data are expressed as means ± SD ($n = 5$) in
612 the duodenum (*left*) or jejunum (*right*).
613
614 Figure 7. Effects of zinc deficiency during lactation on plasma glucose homeostasis in the offspring. *A*,
615 Change in plasma glucose levels in male offspring of two groups from 4 weeks until 15 weeks ($n = 5$,
616 $*P<0.05$). *B*, Plasma insulin levels in the same male offspring at 11 and 13 weeks. *C*, Change in plasma
617 glucose levels in female offspring in the same period as in *A* ($n = 5$).
618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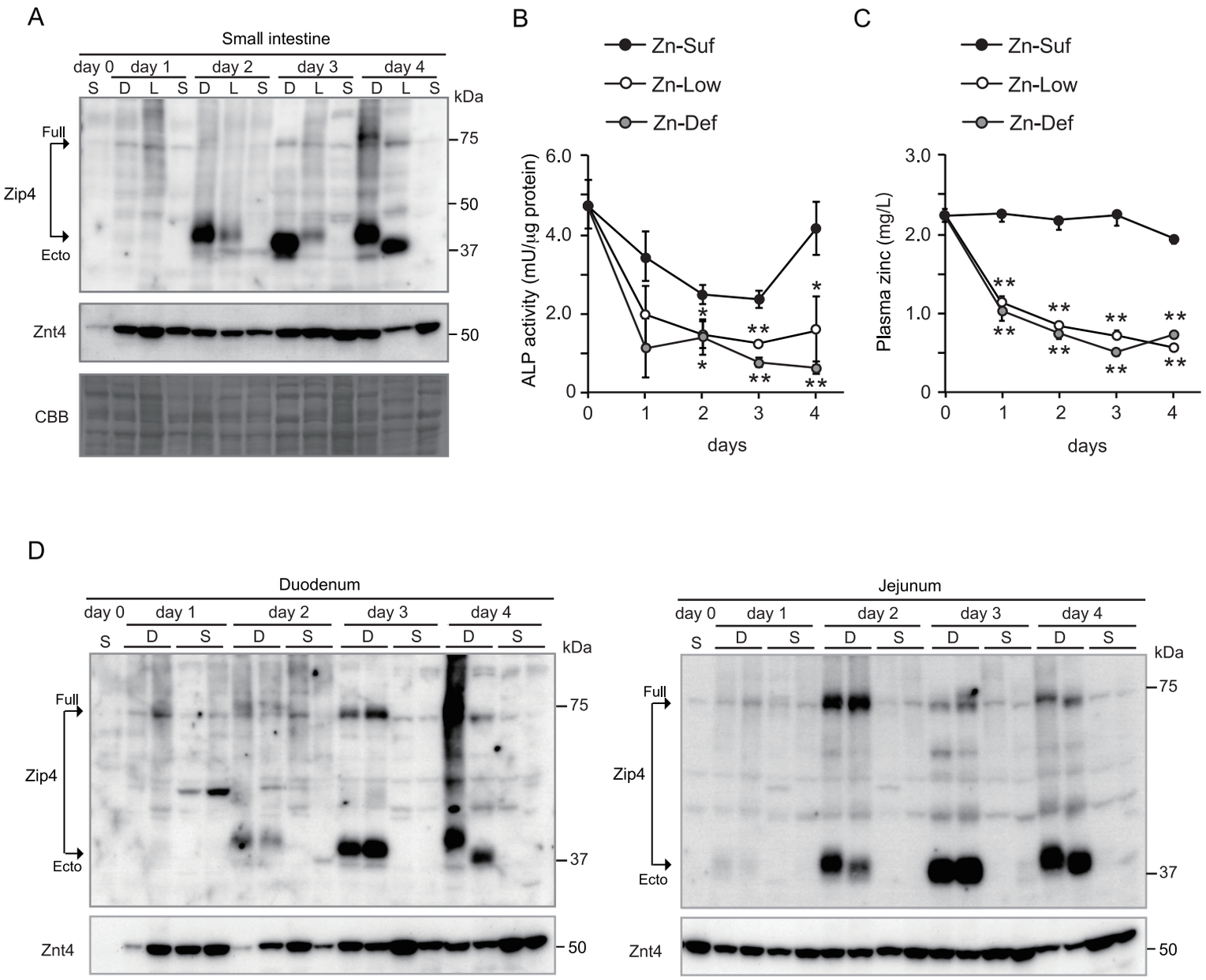
100

Fig. 1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day 1
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Full
Zip4
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Znt4
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Zn-Low
Zn-Def
ALP activity (mU/μg protein)
6.0
4.0
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Zn-Def
Plasma zinc (mg/L)
3.0
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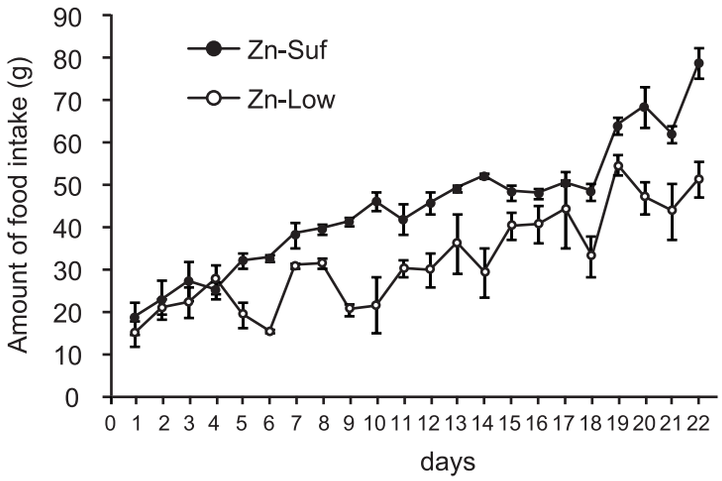
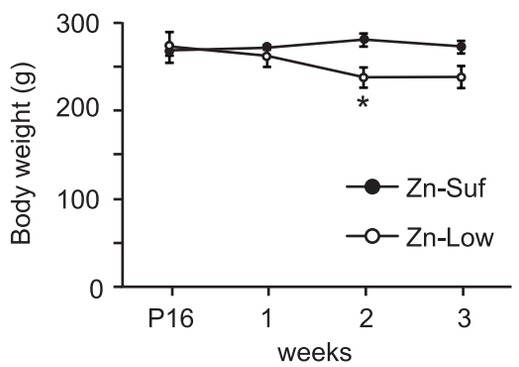
Fig. 2

A


Amount of food intake (g)
90
80
70
60
50
40
30
20
10
0
Zn-Suf
Zn-Low
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
days

B


Body weight (g)
300
200
100
0
*
Zn-Suf
Zn-Low
P16
1
2
3
weeks

C

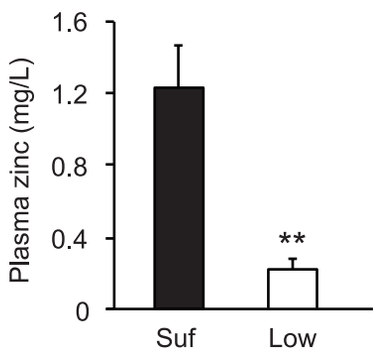

Plasma zinc (mg/L)
1.6
1.2
0.8
0.4
0
**
Suf
Low

Fig. 3

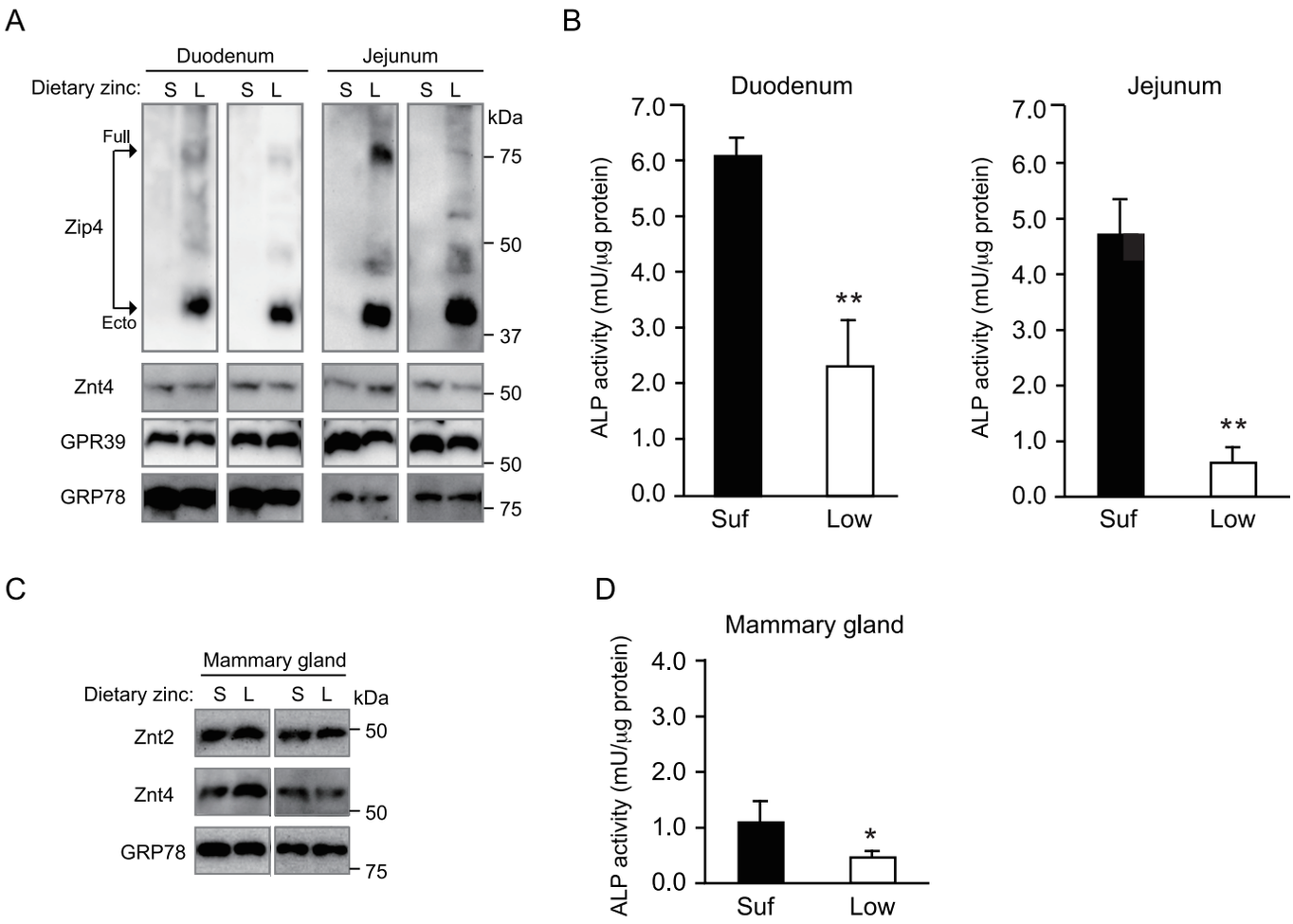

A
Duodenum
Jejunum
Dietary zinc:
S L S L
S L S L
kDa
Full
Zip4
Ecto
75
50
37
Znt4
50
GPR39
50
GRP78
75
B
Duodenum
Jejunum
ALP activity (mU/μg protein)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
Suf
Low
C
Mammary gland
Dietary zinc:
S L S L
kDa
Znt2
50
Znt4
50
GRP78
75
D
Mammary gland
ALP activity (mU/μg protein)
4.0
3.0
2.0
1.0
0.0
*
Suf
Low

Fig. 4

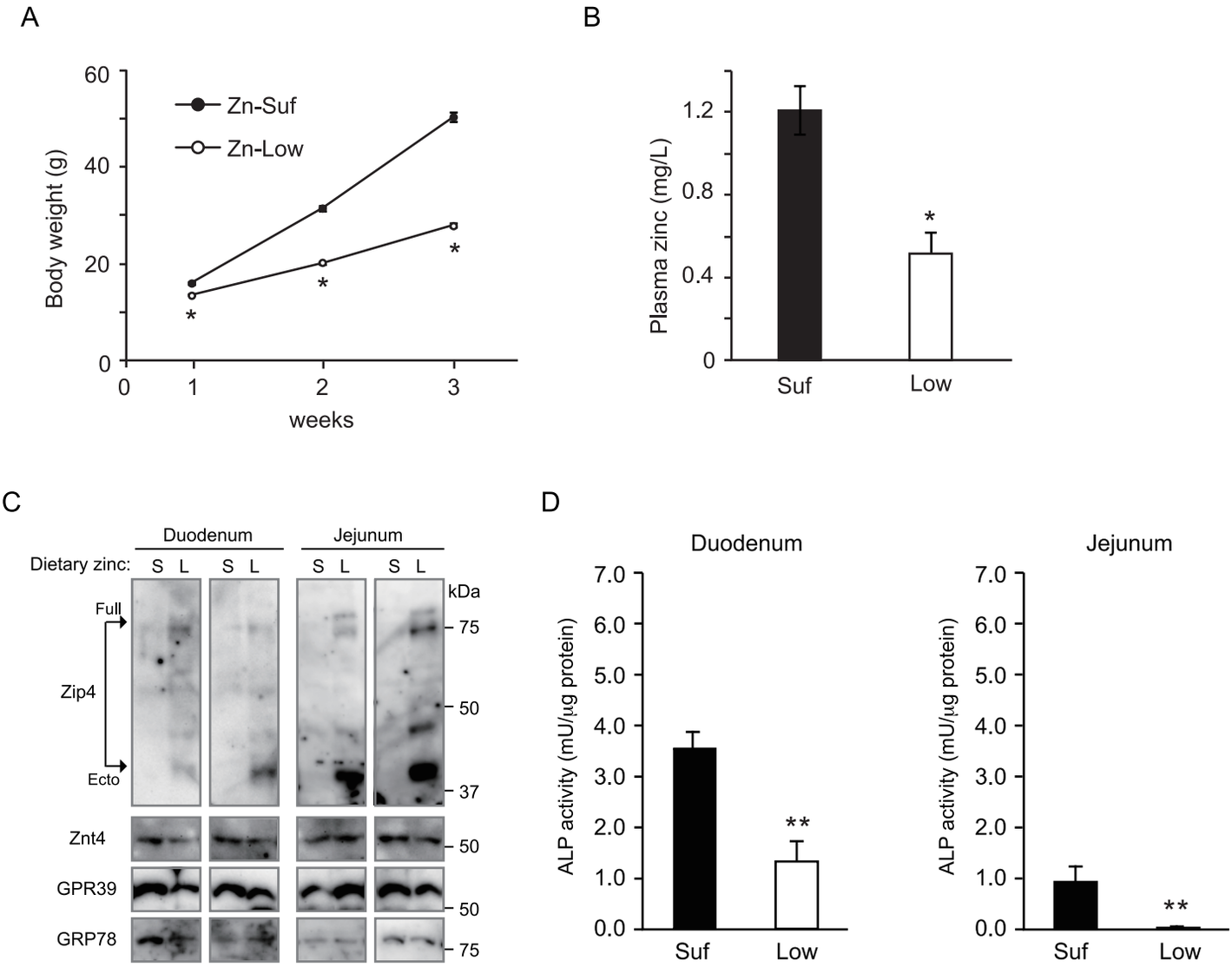
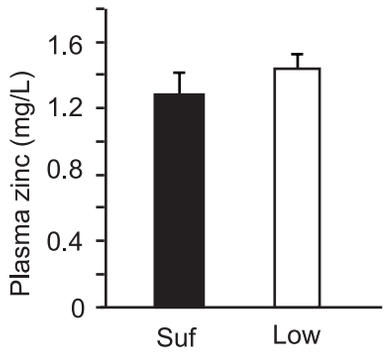
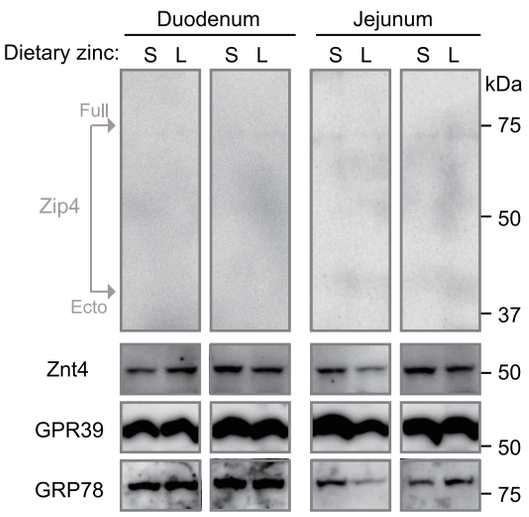

A
Body weight (g)
60
40
20
0
0
1
2
3
weeks
Zn-Suf
Zn-Low
*
*
*
B
Plasma zinc (mg/L)
1.2
0.8
0.4
0
*
Suf
Low
C
Duodenum
Jejunum
Dietary zinc:
S L S L S L S L
kDa
Full
Zip4
Ecto
75
50
37
Znt4
50
GPR39
50
GRP78
75
D
Duodenum
Jejunum
ALP activity (mU/μg protein)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
**
Suf
Low

Fig. 5

A


Plasma zinc (mg/L)
1.6
1.2
0.8
0.4
0
Suf
Low

B


Duodenum
Jejunum
Dietary zinc:
S L S L S L S L
kDa
Full
Zip4
Ecto
75
50
37
Znt4
50
GPR39
50
GRP78
75

C

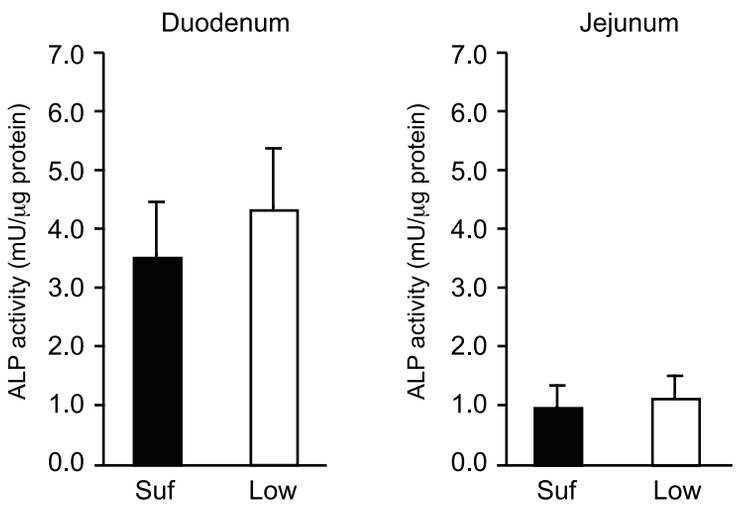
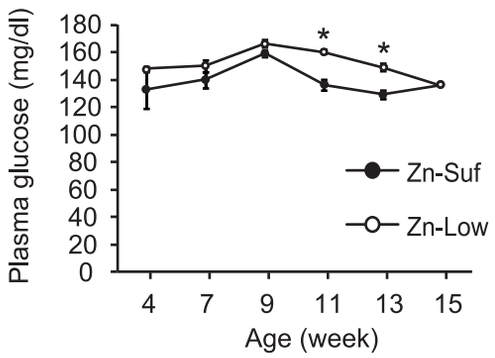
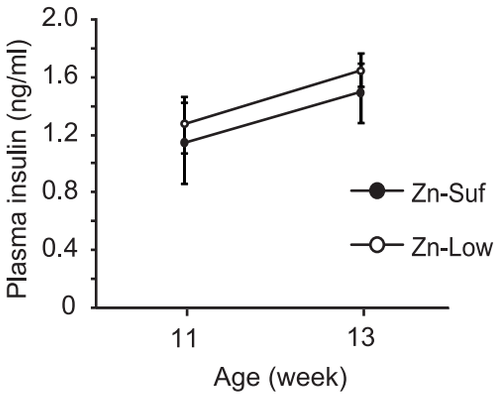

Duodenum
Jejunum
ALP activity (mU/μg protein)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Suf
Low

Fig. 6

A


Plasma glucose (mg/dl)
180
160
140
120
100
80
60
40
20
0
*
*
Zn-Suf
Zn-Low
4
7
9
11
13
15
Age (week)

B


Plasma insulin (ng/ml)
2.0
1.6
1.2
0.8
0.4
0
Zn-Suf
Zn-Low
11
13
Age (week)

C

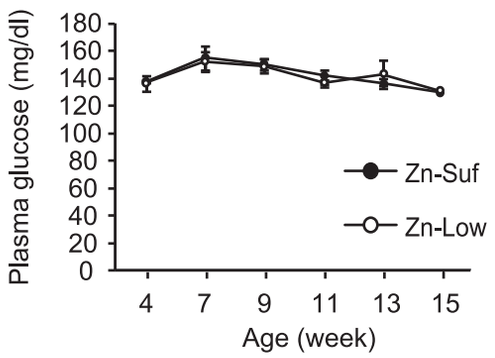

Plasma glucose (mg/dl)
180
160
140
120
100
80
60
40
20
0
Zn-Suf
Zn-Low
4
7
9
11
13
15
Age (week)

Fig. 7