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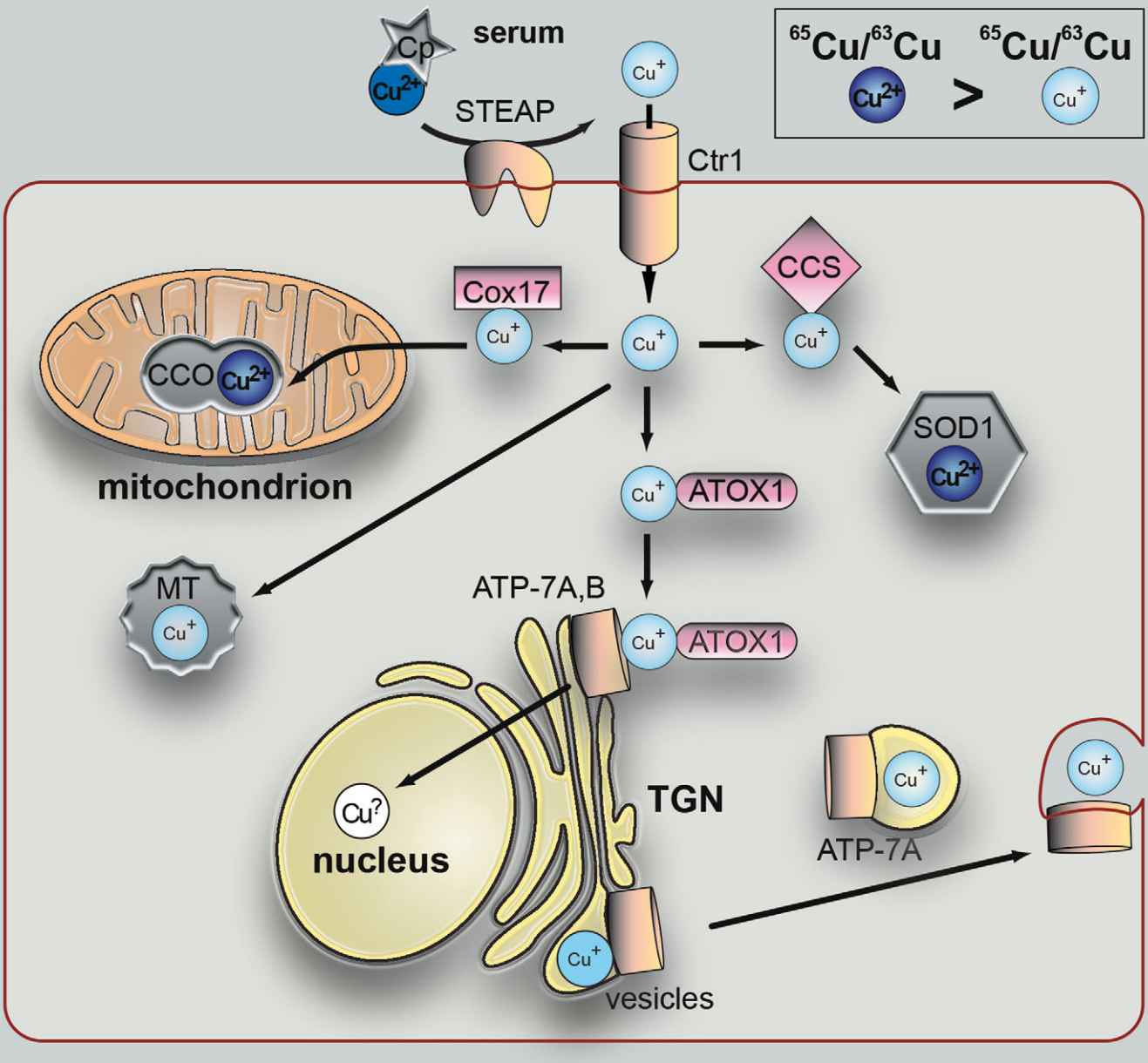

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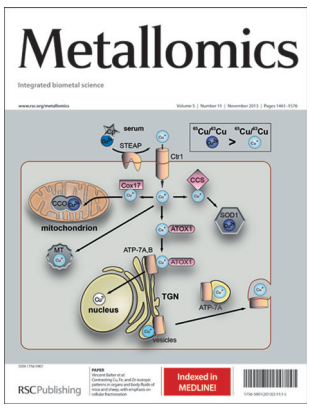

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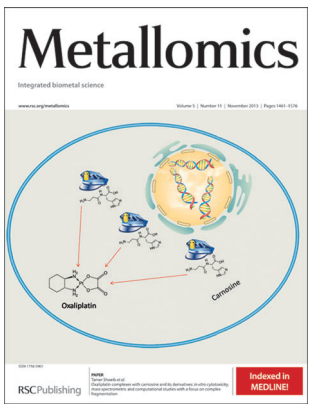

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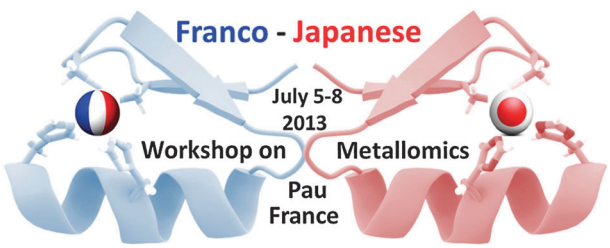

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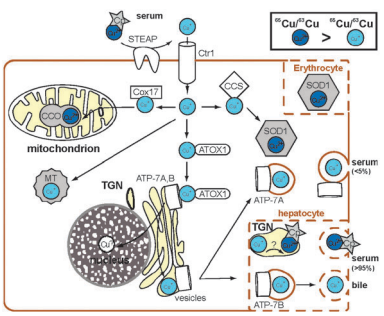

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Contrasting Cu, Fe, and Zn isotopic patterns in organs and body fluids of mice and sheep, with emphasis on cellular fractionation

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We report Cu, Fe, and Zn natural isotope compositions in organs, body fluids, diets and feces of mice and sheep. Large and systematic isotope variability is observed, notably in the δ^{66}Zn in liver and δ^{65}Cu in kidneys, but significant differences exist between mice, sheep and humans, especially in the δ^{66}Zn value of blood. The results are interpreted with reference to current knowledge of metal trafficking and redox conditions in cells. In general, the light isotopes preferentially fractionate into 'softer' bonds involving sulfur such as cysteine and glutathione, whereas heavy isotopes fractionate into 'harder' bonds involving nitrogen (histidine) and even more oxygen, notably hydroxides, phosphates, and carbonates. Bonds involving the reduced forms Cu^{+} and Fe^{2+} are enriched in the light isotopes relative to bonds involving the oxidized Cu^{2+} and Fe^{3+} forms. Differences in blood Zn isotope abundances between mice, sheep and humans may reflect a different prevalence of Zn ZIP transporters. The isotopically heavy Cu in the kidneys may reflect isotope fractionation during redox processes and may be relevant to ascorbate degradation into oxalate.

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Introduction

The role of metals in biochemistry is widely recognized, but our knowledge of their biological controls and pathways often remains patchy. Thousands of metalloproteins have been recognized and the processes by which they control specific biological processes are occasionally known in remarkable detail, yet an integrated understanding of metal pathways in organisms bridging the gap between physiology and molecular biochemistry is missing. By the sensitivity of isotope fractionation to coordination and bond energy, the isotopic variability of metals in organs and body fluids offers a new opportunity for a non-invasive investigation of the structural biology of metal trafficking in organisms and of multiple specific biological mechanisms. Because vibrational frequencies decrease with mass, heavy isotopes tend to be enriched in the strongest bonds. Evidence for substantial biological fractionation of stable isotopes of metals has been presented for Fe in the human body by Walczyk and von Blanckenburg,[1] for Zn among the different organs of sheep,[2] and for Cu and Fe in human erythropoiesis.[3] Van Heghe *et al.*[4] identified a diet effect with Zn in whole blood being isotopically heavier for vegetarian relative to omnivorous humans. Isotopic patterns can also be perturbed by pathologies. So far, fractionation of Fe isotopes in blood from hemochromatosis patients[5] and of Cu isotopes in Wilson disease patients[6] has been demonstrated. Likewise, the expression of a single Cu binding protein, the prion protein (PrP), was shown to alter the isotope ratios of Cu and Zn in mouse brain.[7] Moynier *et al.*[8] reported Zn isotope analyses of various organs and body fluids of mice from different genetic lines and observed that Zn is isotopically heavy in red blood cells and bones relative to serum. They also concluded that the differences in the gender and genetic background did not appear to affect the isotopic distribution of Zn.

A pre-requisite for understanding the mechanisms of isotope fractionation specific to particular biological environments is the knowledge of isotopic variability among different organs and body fluids, ideally in humans, but as a first step, in animal models. In the present work, we first report Cu, Fe and Zn isotope compositions in organs and body fluids of mice.

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In addition, sheep and other ruminants are known to be devoid of strong Cu regulation[9] resulting from their impaired ability to excrete Cu in bile.[10] As this homeostatic deficiency in turn affects erythropoiesis and other vital biological processes, we also extended Balter *et al.*'s[2] work on Zn in sheep organs to Cu and Fe isotopes.

Samples

Ten mice were euthanized by cervical dislocation and immediately dissected to collect blood and various tissues samples. All mice (5 females labeled 1–5, 5 males labeled 6–10) were 5 months old. Mice were TCR-transgenic (F5 strain) meaning that their CD8T cells are specific for the NP68 epitope from influenza A virus nucleoprotein. F5 mice were backcrossed on the C57BL/6 background as described recently.[11] Mice were bred under specific pathogen free conditions and maintained at the institute's animal facility (Anira PBES, Lyon, France). The design of the feeding experiment for sheep is described in Balter *et al.*[2] Briefly, four Suffolk cross lambs (2 males and 2 females), born between March and April 2006, were raised on artificial milk for 6 weeks and weaned from the artificial milk and introduced to a commercial diet (pre-experimental diet). The animals were maintained on the pre-experimental diet until the start of the experiment in September 2006. At the beginning of the experiment, the animals were switched to the experimental diet and assigned to two groups of one male and one female animal at either a low-energy allowance (LEA) or a high-energy allowance (HEA) of the experimental diet. Feed allowances were adjusted regularly throughout the experiment to ensure a constant weight gain of 50 g day^{-1} for animals receiving the LEA (#9125 and #9169) and of 150 g day^{-1} for animals receiving the HEA (#9351 and #9646). The animals were kept on the experimental diet for 231 days. Upon completion of the experiment (3 April, 2007), they were transported to the Teagasc, Food Research Centre, Ashtown, Dublin, Ireland, where their organs were excised and immediately freeze-dried. All procedures employed in this study were in compliance with EU Regulations concerning animal welfare and use. The sheep experiment was carried out with the approval of Teagasc, the Irish Agriculture and Food Development Authority.

Analytical techniques

Samples were dissolved in a 1 : 1 mixture of sub-boiled distilled concentrated HNO_3 and 30% H_2O_2 (analytical grade), evaporated to dryness, and re-dissolved in 1 mL of 7N HCl 0.001% H_2O_2. A 50 μL aliquot was taken for elemental concentration measurements and the remaining sample solution was processed for isotope analysis. Copper, iron and zinc were purified by anion-exchange chromatography using procedures adapted from Maréchal *et al.*[12] and Moynier *et al.*[13] Briefly, prior to each elution, 1.6 mL of macroporous resin AGMP-1 (100–200 mesh) was cleaned three times with 0.5N HNO_3 alternating with H_2O, and conditioned by 6 mL of 7N HCl 0.001% H_2O_2. Matrix and Cu were removed using 30 mL of 7N HCl 0.001% H_2O_2. Iron was removed using 10 mL of 2N HCl, and Zn was finally eluted with 10 mL of 0.5 HNO_3. This solution was evaporated to dryness and re-dissolved in 1 mL of 1.5N HBr. Zinc was further purified on 0.5 mL of AG-1x8 resin (200–400 mesh) using 3 mL of 0.5N HNO_3.

The mass spectrometry techniques have been described elsewhere.[2,3,14] Copper and zinc stable isotope compositions were determined by multi-collector inductively coupled plasma mass spectrometry (MC-ICPMS), using a Nu plasma 500 HR double-focusing mass spectrometer (Nu Instrument, Wrexham, UK) equipped with 12 Faraday detectors and with a mass resolution of 1000. The operating conditions were: rf power (1300 W), the plasma gas flow rate (13 L min^{-1}) and the auxiliary gas flow rate (0.9 L min^{-1}). Iron was run on a large-radius high-resolution Nu-1700 MC-ICPMS equipped with 16 Faraday detectors using dry plasma after desolvation in a Nu DSN and a resolution of 3 000. The samples were introduced by free aspiration in 0.05N distilled HNO_3. Instrumental mass fractionation on Zn and Cu was corrected using Cu-doping (Cu SRM 976, National Institute of Standards and Technology, Bethesda, MD, USA) and standard-sample bracketing (Zn JMC 3-0749L, Johnson Matthey, Royston, UK) following the recommendations provided in Maréchal *et al.*[12] and Albarède and Beard.[15] Sample measurement solutions were diluted to match the concentration of the standard mixture (Zn 0.5 ppm–Cu 0.5 ppm). The reproducibility of Cu, Zn, and Fe isotopic ratios was <0.05 per mil. Samples were randomized during analysis and duplicates were measured to check for systematic errors.

Copper, iron and zinc concentrations were measured by quadrupole inductively coupled plasma mass spectrometry (Q-ICPMS) at ENS Lyon using a 7500 CX quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The following operating parameters of Q-ICPMS were optimized: rf power (1550 W), the plasma gas flow rate (15 L min^{-1}), the auxiliary gas flow rate (2 L min^{-1}), the carrier gas flow rate (0.9 L min^{-1}), the makeup gas flow rate (0.15 L min^{-1}). The performance of the Q-ICPMS instrument was optimized and checked daily by testing a standard mixture containing 1 ng mL^{-1} Li, Y, Co, Ce, and Tl in 3% HNO_3. Indium at 1 ng mL^{-1} was used as an internal standard to correct for any long-term instrumental drift.

Results

The results from mice are listed in Table 1. Remarkable results are the following:

1. As previously observed by Moynier *et al.*,[8] Zn is isotopically heavy in blood with respect to food, much more so than in humans,[3] and to a lesser extent in bones (Fig. 1). Zinc in the liver and the brain is isotopically lighter than food. In general, the Zn isotope compositions measured on C57BL/6 mice in this work and by Moynier *et al.*[8] for different organs and body fluids are fully consistent (Fig. 2).

2. Cu and Fe in blood are consistent with literature values from humans[1,3,5,16] (Fig. 1).

3. Copper is isotopically heavy in kidneys (Fig. 1).

4. For the brain, δ^{65}Cu is much heavier in the transgenic F5 mice investigated in this work (+0.72 to +0.91‰) than in the four lines of wild mice investigated by Büchl *et al.*[7] (−0.12 to 0.78‰).

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Table 1 Isotope compositions for Cu, Fe and Zn in mice. Copper, iron and zinc concentrations in the digestive tract are reported expressed as *x*/Mg ratios

	Mouse ID	δ^{65}Cu	δ^{56}Fe	δ^{57}Fe	δ^{66}Zn	δ^{67}Zn	Zn/Mg	Cu/Mg	Fe/Mg
Blood	1	0.02	−2.51	−3.76	0.85	1.72			
	2	0.09	−2.63	−3.94	0.86	1.76			
	3	0.20	−2.59	−3.85	0.87	1.71			
	4	−0.14	−2.46	−3.72	0.96	1.83			
	5	0.06			0.95	1.82			
	6	−0.01	−2.54	−3.84	0.92	1.91			
	7	−0.19	−2.45	−3.67	0.89	1.76			
	8	−0.04	−2.43	−3.57	0.88	1.68			
	10	−0.09	−2.54	−3.72	0.79	1.67			
Liver	1	0.12	−0.63	−0.95	−0.21	−0.35			
	2	0.33	−0.98	−1.41	−0.27	−0.55			
	3	0.24	−0.90	−1.33	−0.18	−0.42			
	4	0.05	−0.71	−1.04	−0.05	−0.21			
	5	0.44	−0.89	−1.26	−0.14	−0.27			
	6	0.31	−0.49	−0.74	−0.18	−0.38			
	7	0.33	−0.78	−1.17	−0.21	−0.40			
	8	0.27	−0.74	−1.06	−0.15	−0.33			
	9	0.71	−0.59	−0.90	−0.19	−0.42			
	10	0.79	−0.82	−1.26	−0.25	−0.54			
Brain	1	0.80	−1.86	−2.84	−0.05	−0.27			
	2	0.91	−2.01	−2.98	−0.27	−0.49			
	6	0.78	−3.13	−4.70	0.06	−0.01			
	7	0.72	−1.95	−3.05	−0.06	−0.15			
Muscle	1	0.01	−2.34	−3.62	0.28	0.60			
	2								
	6	−0.20	−1.63	−2.36	0.17	0.26			
	7	−0.14	−1.50	−2.24	0.18	0.35			
Skin	1	0.76	−2.54	−3.92	0.24	0.43			
	2	0.94	−3.32	−5.01	0.21	0.50			
	6	0.89	−0.73	−1.18	0.01	0.15			
	7	1.12	−0.65	−1.03	0.36	0.64			
Bone	1	0.06	−0.83	−1.24	0.61	1.14			
	2		−1.03	−1.59	0.51	1.09			
	6		−0.59	−0.89	0.61	1.17			
	7	0.29	−0.78	−1.17	0.65	1.24			
Kidney	1	1.50	−1.92	−2.72	0.18	0.32			
	2	1.67	−1.96	−3.04	0.19	0.32			
	6	1.37	−2.03	−2.98	0.15	0.32			
Pancreas	1	−0.01	−1.86	−2.79	0.23	0.44			
	2	−0.01	−1.88	−2.77	0.26	0.41			
	6	0.01	−1.77	−2.63	0.23	0.47			
	7	0.06	−1.72	−2.55	0.24	0.36			
Bone marrow	1		−1.89	−2.92	0.58	0.99			
	2		−1.70	−2.58	0.27	0.65			
	7		−0.35	−0.50	0.38	0.83			
Spleen	1		−0.92	−1.32	0.19	0.21			
	2	0.20	−0.94	−1.46	0.02	0.05			
	6	0.00	−1.07	−1.61	0.01	0.10			
	7	0.09	−1.01	−1.47	0.03	0.14			
Intestine	1	0.84	−2.53	−3.77	−0.04	0.06			
	2	0.50	−2.68	−4.10	−0.17	−0.23			
	6	0.55	−1.91	−2.82	−0.13	−0.26			
	7	0.56	−2.26	−3.25	−0.06	−0.07			
Node	1		−1.55	−2.42	−0.07	−0.01			
	2		−1.68	−2.55					
Seminal	6	0.30	−1.61	−2.43	0.01	0.07			
	7		−3.26	−4.92	0.00	0.23			

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Table 1 (*continued*)

	Mouse ID	δ^{65}Cu	δ^{56}Fe	δ^{57}Fe	δ^{66}Zn	δ^{67}Zn	Zn/Mg	Cu/Mg	Fe/Mg
Stomach content	2	0.21	−0.12	−0.11	0.04	0.05	0.08	0.05	0.31
	7	−0.07	−0.47	−0.74	0.48	1.05	0.07	0.05	0.13
Intestine content	1	0.61	−2.09	−3.07	−0.01	−0.04	0.09	0.01	0.12
	2	0.84	−1.80	−2.66	−0.13	−0.23	0.10	0.01	0.19
Caecal content	2	0.45	−0.08	−0.13	0.33	0.81	0.06	0.02	0.25
	6	0.48	−0.08	−0.15	0.37	0.75	0.06	0.01	0.22
Diet		0.33	0.07	0.11	0.35	0.77	0.03	0.01	0.12

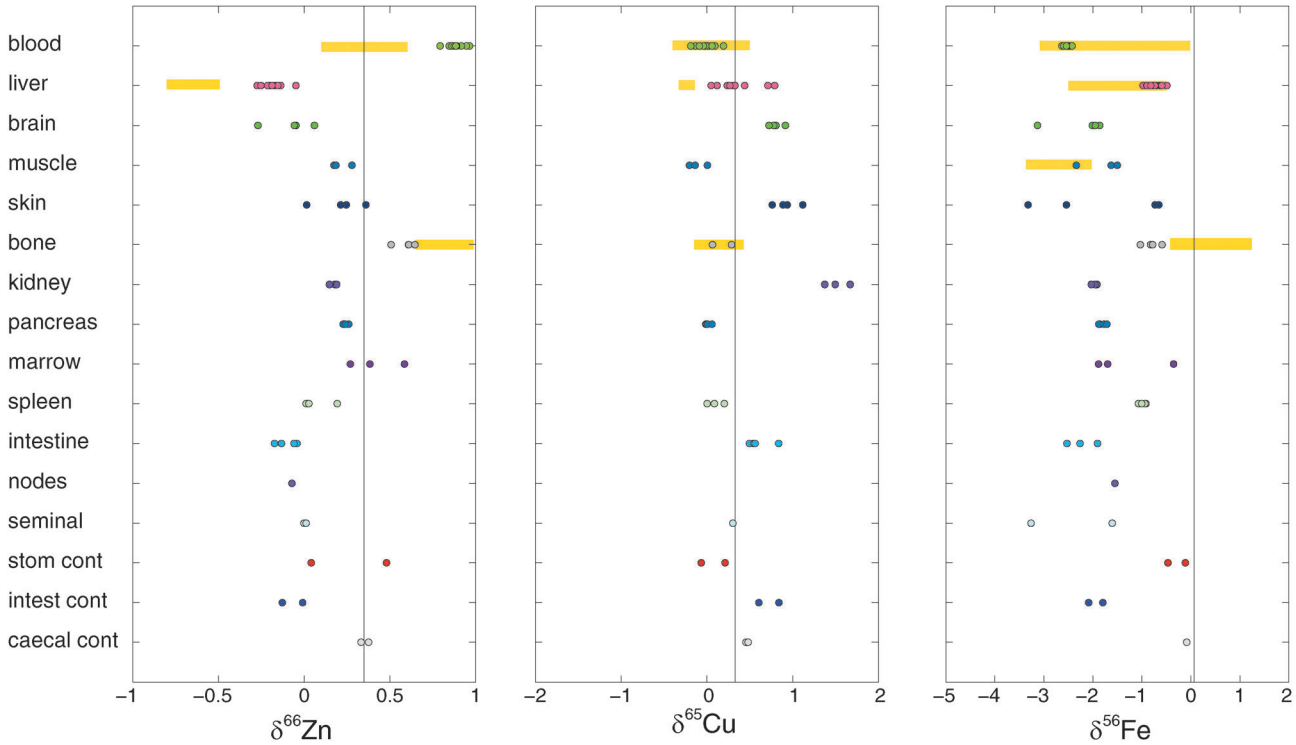

Fig. 1 Zinc, copper, and iron isotope variability among organs, bones, body fluids, and intestinal content of mice reported in delta units per mil. The range of variations for humans is shown in orange.[1,3,5,14b,16,39] Typical uncertainties are ±0.05‰ (2-sigma error).

The difference in δ^{66}Zn is much less −0.27 to + 0.06‰ for the F5 *vs.* −0.25 to 0.42‰ for Büchl *et al.*'s[7] wild mice.

5. Iron isotopes are systematically lighter in organs and body fluids than in food, as previously observed by Walczyk and von Blanckenburg[1] (Fig. 1).

6. Using isotopic abundances and X/Mg ratios (where X stands for Cu, Zn, or Fe) and assuming that Mg is not very significantly depleted or enriched by digestion, the stomach seems to receive, in addition to food, an input of isotopically heavy Cu (Fig. 3).

7. The upper part of the intestine is the locus of absorption of isotopically light Cu and excretion of isotopically light Zn and Fe (Fig. 3).

8. As expected, the lower part of the intestine down to the caecum brings back concentrations and isotope compositions to values near those of the intake: excretion of isotopically heavy Cu and Fe and reabsorption of light Zn (Fig. 3).

The results from sheep (Fig. 4) are not as detailed (organ by organ) as those from mice (Table 2). In particular, no detail is available on the food transit. Noticeable observations are:

1. Zinc in sheep blood is not as isotopically heavy as in mice. In stark contrast with humans,[3] Zn in Red Blood Cells (RBC) is isotopically lighter than in serum.

2. As for mice, Cu is isotopically heavy in kidneys.

3. As for humans (Jaouen *et al.*[14b]), Zn and Cu are isotopically light in livers.

4. As for mice, Fe isotopes are systematically lighter in organs than in food.

5. A *t*-test of differences between feces and diet δ^{56}Fe (p = 0.01) suggests that Fe is approximately conserved during the digestive process. In contrast, δ^{65}Cu (p = 0.035) and δ^{66}Zn (p = 0.69) and the Cu/Zn ratio (p = 0.18) indicate that Cu and Zn are not at the steady-state (see Balter *et al.*[2]).

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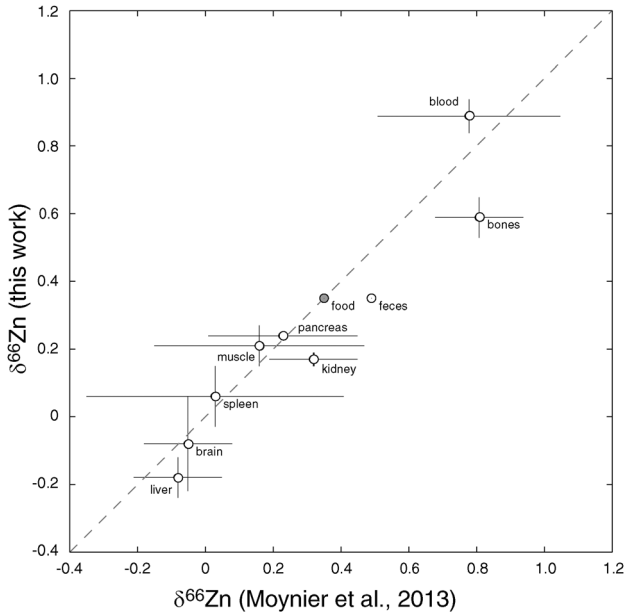

Fig. 2 Comparison of the δ^{66}Zn values measured in this work and by Moynier *et al.*[8] of C57BL/6 mice organs, bones, muscle, and feces. Bars indicate the ±1-sigma dispersion.

Discussion

A remarkable outcome of the present study is that the isotopic patterns of Cu, Fe and Zn with respect to diet are very different between mice and sheep, and as much as fragmentary data for humans allow us to speculate, they are also different from humans. Even among mice, the δ^{65}Cu and δ^{66}Zn results for brain obtained by Büchl *et al.*[7] in wild mice show a very large range of Cu and Zn isotopes from line to line, but, since these authors did not report δ^{65}Cu and δ^{66}Zn in the food, these values cannot be directly compared with those in the present F5 mice. The range of δ^{65}Cu (±0.32‰), δ^{66}Zn (±0.37‰), and δ^{56}Fe (±0.38‰) values in total blood among large groups of humans[3] can be largely accounted for by the diet as vegetarians and omnivorous individuals differ,[4] but Büchl *et al.*'s[7] mice from each line received the same diet. The large discrepancies between sheep and mice, however, raise some important questions about the complexity of metal homeostasis and the role of metalloproteins. Given the similar breeding conditions and diet, it is unlikely that the extreme isotopic variability among and between sheep and mice reflects an individual variability of digestive uptake. It rather betrays differences in isotope fractionation patterns during cellular import or export. Such differences may be caused either by widely different prevalent ligands and coordination (*e.g.*, different isoforms of the ubiquitous carbonic anhydrase (CA), superoxide dismutase (SOD1), ceruloplasmin (Cp), or for instance cytochrome *c* oxidase (Cco)).

Coordination and inferred Cu, Fe and Zn isotope fractionation

Each body compartment, organ or body fluid, can be considered a 'reservoir' for each metal regulated by uptake and excretion. Each compartment can be considered at the steady-state, *i.e.*, assuming that uptake = excretion, or in transience, typically when juveniles are still growing. The steady-state is a convenient reference for organs that do not irreversibly accumulate metals. For instance, ^{66}Zn/^{64}Zn fractionation during Zn uptake by an organ from the blood can be shown that, to a good approximation by:

$$\delta^{66}\mathrm{Zn}_{\mathrm{organ}} \approx \delta^{66}\mathrm{Zn}_{\mathrm{blood}} + 1000(\alpha^{66}_{\mathrm{upt}} - \alpha^{66}_{\mathrm{excr}}),$$

in which α is a measure of the isotope fractionation of isotope ^{66}Zn with respect to the reference isotope ^{64}Zn upon transfer

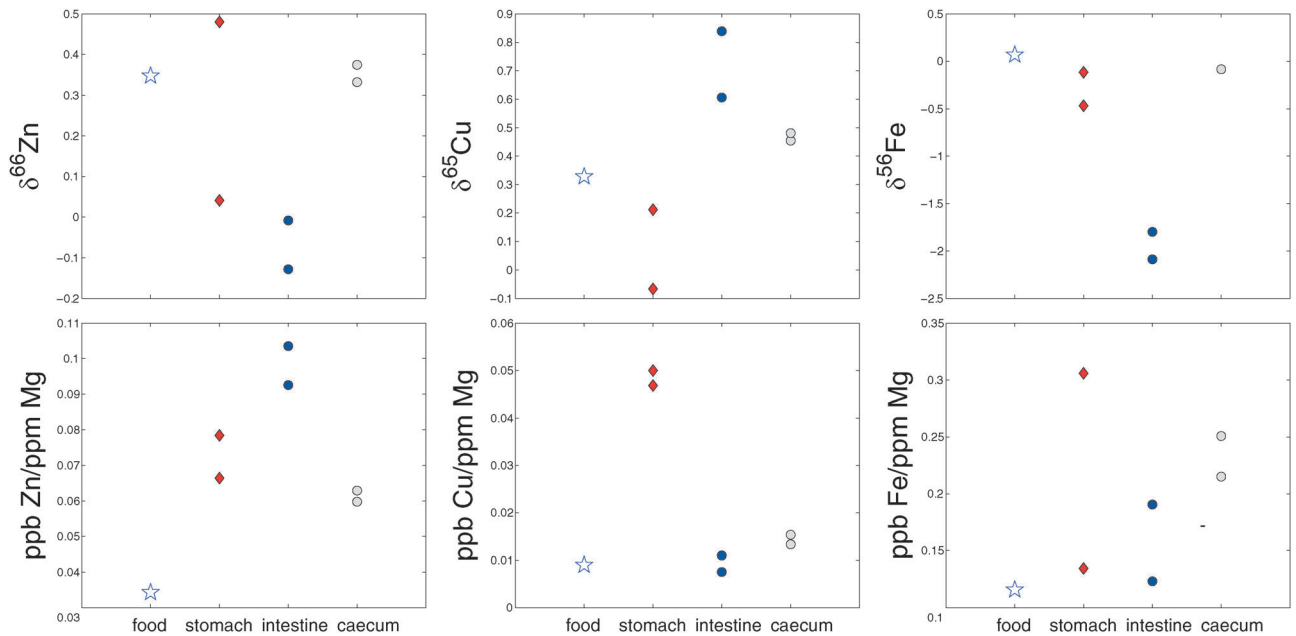

Fig. 3 Zinc, copper, and iron isotopes (top) and X/Mg (X = Zn, Cu, and Fe) (bottom) variability down the digestive tract. The choice of Mg for normalization was motivated by the abundance of this element in food and its relatively minor assimilation during digestion.

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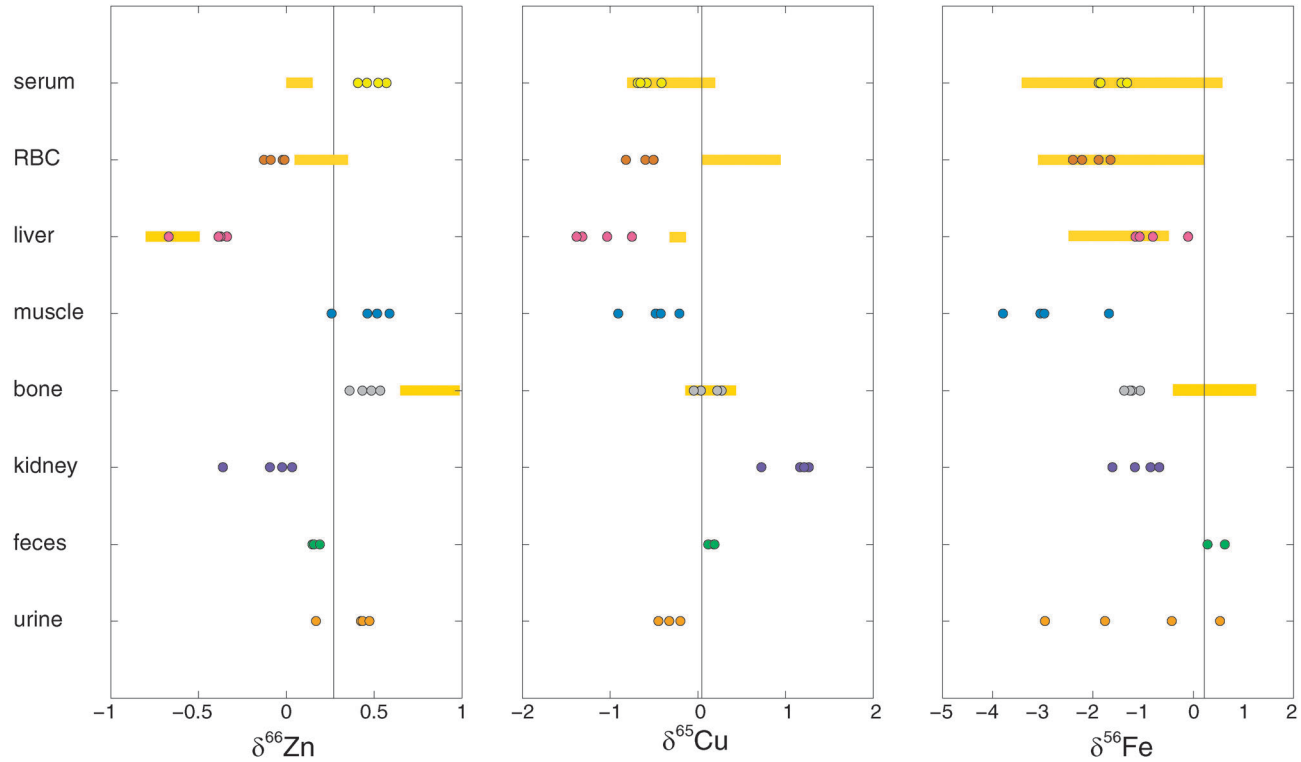

Fig. 4 Zinc, copper, and iron isotope variability among organs, bones, body fluids, and intestinal content of four sheep reported in delta units per mil. Zinc isotope data are from Balter *et al.*[2] The range of variations for humans is shown in orange.[1,3,5,14b,16,39] Typical uncertainties are ±0.05‰ (2-sigma error).

from one environment to the next. The parameter of an exchange reaction α is determined by quantum mechanics *ab initio* calculations from the ratio of the reduced partition functions β of isotopologues (*e.g.*, ^{66}ZnS–X and ^{64}ZnS–X) in the particular molecules involved in the exchange. The dominant cause of isotope fractionation is the difference in zero-point vibrational energy between heavy and light isotopes: this difference arises from the uncertainty principle, which predicts that the length and the vibration frequency of a bond cannot both be known with an infinite precision.

Heavy isotopes tend to concentrate in the tight bonds, in particular those involving the oxidized form (Cu^{2+} over Cu^{+} and Fe^{3+} over Fe^{2+}) and to bind preferentially with ligands with the stronger electronegativity (O > N > S).[8] Available *ab initio* calculated fractionation factors for Zn (Fig. 8a),[8,17] Cu (Fig. 8b)[18] and Fe[19] show that these general trends are closely obeyed, with oxidized forms being isotopically heavier than reduced forms. Likewise phosphates, hydroxides and carbonates along with histidine compounds concentrate heavy Zn isotopes, whereas sulfides, along with glutathione and cysteine compounds are enriched in light Zn isotopes. Results have not been plotted for Fe because we do not have the same detailed calculations as for Zn and Cu. It can therefore be anticipated that bonds with cysteine, as within metallothionein (MT) and within the Cu chaperone (CCS) chaperone delivering copper to Cu, Zn-SOD1,[20] will favor the light isotopes, whereas those with O-bearing compounds as in CA and phosphatic bones, will favor the heavy isotopes.

The redox chemistry of Cu and Fe alter their coordination and the nature of the prevalent ligands. The coordination of Cu^{2+} 'blue' copper proteins (plastocyanine, Cp) involves two Cu–N bonds with histidine and one Cu–S bond with cysteine. Ceruloplasmin comprises three mononuclear sites with Cu bound to histidine and cysteine and one trinuclear domain in which Cu^{2+} binds to histidine and dioxygen.[21] Human CTR1 stably binds two Cu^{+} as cysteine or methionine Cu–S bonds.[22] Coordination in ATP7A depends on the Cu oxidation state, four histidine residues for Cu^{2+} and histidine + methionine for Cu^{+}.[23] In ATP7B, all Cu^{+} ions appear to be bound by two cysteine sulfur residues in a distorted linear arrangement S–Cu–Cu–S.[24] Copper binds to the four N atoms of the tetra-pyrrole ring in bilirubin, a product of heme degradation excreted by the liver into the bile[25] and is reduced to Cu^{+} upon complexation.[26] Cu isotope fractionation by mixed-residue proteins such as Cp and blue copper proteins is difficult to predict. Because ATP7A, ATP7B, and bilirubin involve Cu^{+}–S and Cu^{+}–N bonds, they may be expected to concentrate the light isotopes.

The coordination of Fe can only be briefly covered here. Heme iron involves the Fe^{2+}–N bonds with pyrrolic groups. Ferritin is constituted of FeOOH ferrihydrite. Upon uptake by DMT1, Fe first bounds to asparagine (Zn–N) and aspartate (Zn–O).[27]

Aspects of Cu, Fe and Zn controls in generalized mammalian cells

Zinc. Zn trafficking in mammalian cells has been recently reviewed by Hirano *et al.*,[28] Lichten and Cousins,[29] Colvin *et al.*,[30] and Fukada *et al.*[31] (Fig. 5). Multiple aspects of the coordination

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Table 2 Isotope compositions for Cu and Fe in sheep

	Sheep ID	δ^{65}Cu	δ^{56}Fe	δ^{57}Fe
Serum	9125	−0.58	−1.88	−2.76
	9169	−0.68	−1.29	−1.98
	9351	−0.65	−1.32	−1.96
	9646	−0.41	−1.85	−2.76
RBC	9125	−0.50	−2.22	−3.24
	9169	−0.60	−2.38	−3.45
	9351	−0.82	−1.88	−2.86
	9646	−0.51	−1.65	−2.40
Bone	9125	0.04	−1.06	−1.44
	9169	0.27	−1.22	−1.75
	9351	−0.05	−1.25	−1.91
	9646	0.22	−1.37	−2.02
Muscle	9125	−0.48	−3.05	−4.50
	9169	−0.91	−3.79	−5.69
	9351	−0.42	−2.97	−4.39
	9646	−0.21	−1.74	−2.58
Kidney	9125	1.17	−1.16	−1.68
	9169	0.72	−0.85	−1.36
	9351	1.27	−1.61	−2.39
	9646	1.21	−0.68	−0.95
Liver	9125	−1.32	−1.15	−1.71
	9169	−1.38	−1.07	−1.54
	9351	−1.03	−0.80	−1.21
	9646	−0.75	−0.10	−0.22
Feces	9125	0.18	0.60	0.90
	9169	0.12	0.03	0.01
	9646	0.19	0.28	0.40
Urine	9172	−0.20	−1.76	−2.66
	9414	−0.33	−2.96	−4.32
	9443	−0.45	−0.43	−0.47
Diet	12/12M	0.04	0.35	0.53
	01/08M	−0.02	0.19	0.33
	28/08M	0.12	0.13	0.30

chemistry of the non-redox Zn in proteins have also been covered.[32] Zinc can coordinate to four amino acids, cysteine (Zn–S), histidine (Zn–N), glutamate and aspartate (Zn–O). Cysteine can interact with multiple Zn ions as in MT. In human CA, Zn is coordinated to three histidine residues and a hydroxide group (water or hydrogenocarbonate).[33] Cu, Zn-SOD1, responsible for destroying free superoxide radicals in the body, is characterized by a disulfide bond with metals. In cells, multiple transmembrane transporters (ZIP*x*; x = 1 to 14 for humans, x = 1 to 5 for mice) introduce Zn into the cytosol.[29,34] The cytosolic pool of free and loosely MT-bound Zn is less than the total cellular Zn.[35] In the cytosol, Zn is largely present as SOD1 and in vesicles, while in erythrocytes (RBC) Zn hosted in CA (CAII) may be prevalent. As Zn uptake by ZIP does not seem to be controlled by cysteine residues,[36] it is likely that Zn isotope fractionation takes place between SOD1, MT, or CA, and the cytosol. Zinc is also concentrated at the sites of calcification.[37] It stimulates osteoblast differentiation, proliferation, and through alkaline phosphatase, which generates free phosphate groups for uptake and use, mineralization.[38]

The present data agree well with the previous assumptions that Zn isotopes fractionate according to the nature of the ligands the metal bonds with. In accord with the prediction of *ab initio* calculations,[17c] Zn bonding with phosphates in bones results in positive δ^{66}Zn values, which can reach +1‰ for humans (Fig. 1). Zinc in liver is isotopically light, with δ^{66}Zn $\sim$ −0.7‰ for humans. This likely reflects that Zn fractionation must involve cysteine-rich bonds such as those present in MT and SOD1. This must also hold true, although to a lesser extent, for kidneys and brain.

The situation for blood is still enigmatic. A striking result of the work of Moynier *et al.*[8] confirmed by the present work is the high δ^{66}Zn in the blood of mice compared to sheep and to humans.[3,39] Erythrocytes (RBC), which account for $\sim$90 percent of the total Zn present in human blood[3] and are free of organelles, contain SOD1 and CA. As discussed above, the former is likely to be depleted and the latter enriched in Zn heavy isotopes. Isotopically heavy Zn in mouse erythroid cells suggests that metal is bound to a ligand with a strong electronegativity. Three explanations can be suggested to explain the observations.

1. δ^{66}Zn in RBC is controlled by the CA/SOD1 ratio. This ratio should be reflective of the Cu/Zn ratio of the total blood, Cu being present in SOD1 only. Although the difference between the Cu/Zn ratio in the blood of humans (0.17 ± 0.12)[3] and mice (0.115 ± 0.015) is significant, it is clearly too marginal to account for the large difference in Zn isotope compositions observed between the blood of mice on the one hand and that of sheep and humans on the other hand.

2. A strong bond of Zn with carbonates would favor isotopically heavy Zn.[17b] It was actually observed that human ZIP2 activity is stimulated by HCO_3^- suggesting a joint transport mechanism of Zn^{2+} and HCO_3^-.[40] It is not clear, however, if HCO_3 concentration should be different among blood from mice, sheep and humans.

3. CAII in erythrocytes specifically binds to the cytoplasmic Cl–HCO_3 exchanger AE1 and the complex acts as a HCO_3^- transporter across cells and epithelial layers.[41] Docking of AE1 to CAII may affect the environment of the Zn ion in CAII in a way unique to mice.

Overall, it is likely that the difference in Zn isotopes in blood signals a different cycle of murine CA with respect to sheep and humans.[8]

The δ^{66}Zn value is higher in mouse blood relative to bone, whereas the opposite is true in sheep and humans. Given that phosphates preferentially fractionate heavy Zn isotopes,[17c] this observation suggests that Zn bonding with PO_4^{3-} may not be sufficient to make this metal in the blood of mice isotopically heavy.

Copper. Copper trafficking in mammalian cells has been reviewed many times[42] (Fig. 6). Under ambient redox conditions, Cu is naturally present in solutions as Cu^{2+}. Before uptake in the digestive tract, Cu is reduced to Cu^+ by ferrireductases of the Steap family, which stimulate cellular uptake of both iron and copper *in vitro*.[43] Copper I is taken up into the cytosol by the transmembrane transporter Ctr1.[44] Because Cu^+

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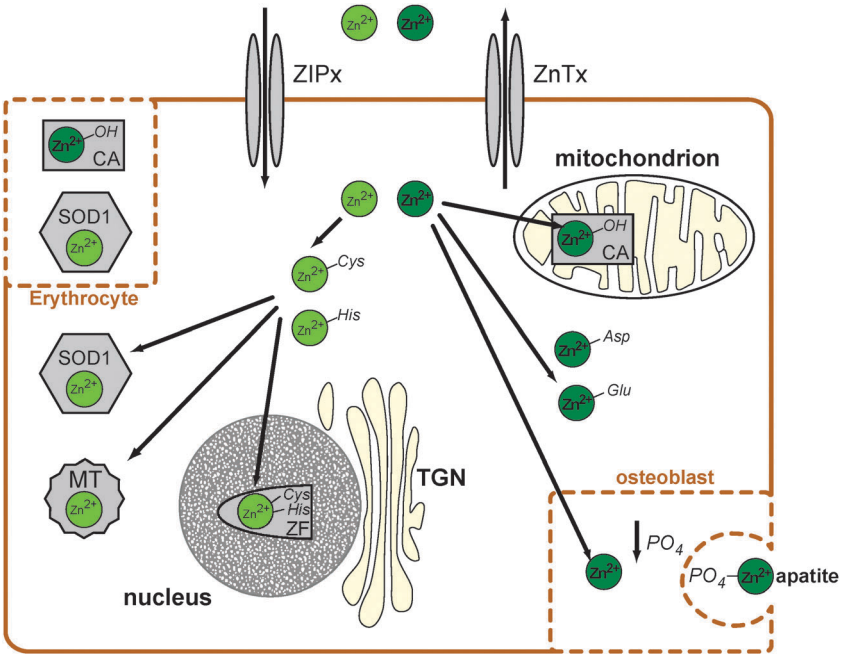

Fig. 5 A sketch of Zn trafficking in a generalized mammalian cell. Zinc in dark green and in light green stands for the Zn heavy isotope and the Zn light isotope, respectively. Abbreviations: Zn transporter family, ZnTx; Zn transporter ZIP family, ZIPx; metallothioneine, MT; Cu, Zn-superoxide dismutase, SOD1; Zn finger, ZF; carbonic anhydrase, CA; Cysteine, Cys; Histidine, His; Gluthatione, Glu; Trans-Golgi network, TGN.

is a very efficient reactive oxygen species that produces in cells harmful free radicals, it is readily chaperoned or scavenged. Within the cytosol, Cu^{+} is transported by chaperones, to the Cu-ATPases by Atox1,[45] to SOD1 by CCS, and to the cytochrome *c* oxidase (CCO) of the mitochondrion membrane by Cox17.[46] Metallothioneins also readily bind Cu^{+}, in addition to Zn^{2+}. Copper-ATPases ATP7A (Menkes) ubiquitous except in the liver, and ATP7B (Wilson) in the liver maintain the intracellular Cu concentration by transporting Cu into intracellular exocytic vesicles from the Golgi network.[47] While Cu is stored as Cu^{2+} in MT, it is incorporated into SOD1 and CCO as Cu^{+}.[20] In serum, Cu is largely accommodated by Cp and accounts for 70–95% of the total copper.[48]

The well-documented lack of Cu regulation in sheep[9,10] does not warrant a strong interpretation of the present values of δ^{65}Cu for this species. While the range of δ^{65}Cu values for humans overlaps,[3] the higher δ^{65}Cu in the serum of sheep with respect to humans suggests a different balance of Cp and bilirubin excretion by the liver.

Another major outcome of the present work is the remarkably high δ^{65}Cu in mouse and sheep kidneys. In the mouse kidney, the Cu chaperone Atox1 has been localized to the cortex around the glomeruli and adjacent tissues and in the medulla.[49] Potential explanations may be considered for the accumulation of isotopically heavy Cu in this organ:

1. The oxidative breakdown of ascorbic acid into oxalic acid[50,51] produces Cu-oxalate, which is isotopically heavy with respect to Cu ascorbate[18b] and in general to any other form of copper. More generally, the high δ^{65}Cu in mouse and sheep kidneys may indicate a redox-controlled isotope fractionation between the tissue and circulating blood.

2. As the kidneys act to regulate the pH and alkalinity of the blood, isotopically heavy Cu may preferentially bond to carbonate. It is unclear, however, if the bicarbonate concentration is sufficient to allow for the binding of a substantial fraction of the available Cu.[18b]

3. Upon reabsorption of Cu into the venous system after filtration in glomeruli, ^{63}Cu diffuses faster than ^{65}Cu, the latter therefore has a longer residence time in the kidneys than ^{63}Cu. This purely kinetic interpretation is not supported by the lack of large isotope fractionation for Zn and Fe in kidneys.

At this stage, we must admit that the processes and proteins accounting for the high δ^{65}Cu in the kidneys have not been identified.

Iron. As expected from its ubiquity and redox properties, Fe distribution is complex[52] (Fig. 6) and the extent to which it interacts with Cu homeostasis is unknown.[53] Nearly all absorption of dietary Fe occurs in the duodenum. Iron is reduced by duodenal ferric reductase Dcytb and the Steap family prior to the uptake of Fe^{2+} by the enterocyte through the divalent metal transporter 1 (DMT1). Iron is liberated into the blood stream through the transmembrane ferroportin, and then oxidized by ferroxidases, which can be either transmembrane hephaestin or serum Cp, into Fe^{3+}, which is itself transported by transferrin (Tf). Erythroid cells need to produce large quantities of heme Fe^{2+}. The Tf receptors allow Fe^{3+} into the endosome and upon further reduction by step 3 and transfer into the cytosol by DMT1, Fe^{2+} is either taken to the mitochondrion by chaperone frataxine to produce hemoglobin or stored in hollow ferritin (Ft) *via* chaperone PCBP1 (Fig. 7).[54]

Overall, the present observations are consistent with isotope fractionation being dominated by redox processes. The δ^{56}Fe in

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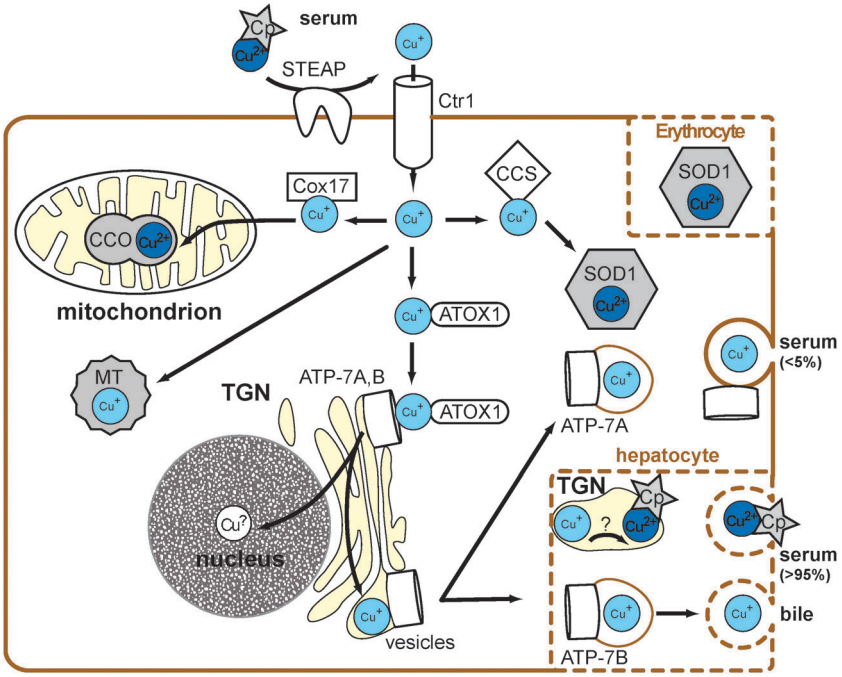

Fig. 6 A sketch of Cu trafficking in a generalized mammalian cell. Copper in dark blue and in light blue stands for the Cu heavy isotope and the Cu light isotope, respectively. Abbreviations: ceruloplasmin, Cp; Cu reductase, STEAP; cytochrome *c* oxidase, CCO; metallothionein, MT; Cu, Zn-superoxide dismutase, SOD1; Cu chaperone, CCS; Copper-transporting ATPase 1, ATP7A,B; Trans-Golgi network, TGN.

liver, in which most iron is stored as Fe^{3+} by Ft, is systematically higher than that of both whole blood (mice) and RBC (humans and sheep), in which Fe^{2+} is bound to hemoglobin (Hb). Likewise, for humans,[1] mice, and sheep (this work), muscle, in which heme Fe^{2+} is also bound to myoglobin (Mb), holds the isotopically lightest Fe in the entire body except for hair and skin.

Copper, iron and zinc isotope fractionation during gastrointestinal transit

As pointed out by Walczyk and von Blanckenburg,[1] Fe is isotopically lighter in human organs and body fluids with respect to food. With the exception of kidneys, Cu is also isotopically lighter than food in sheep, while the difference is

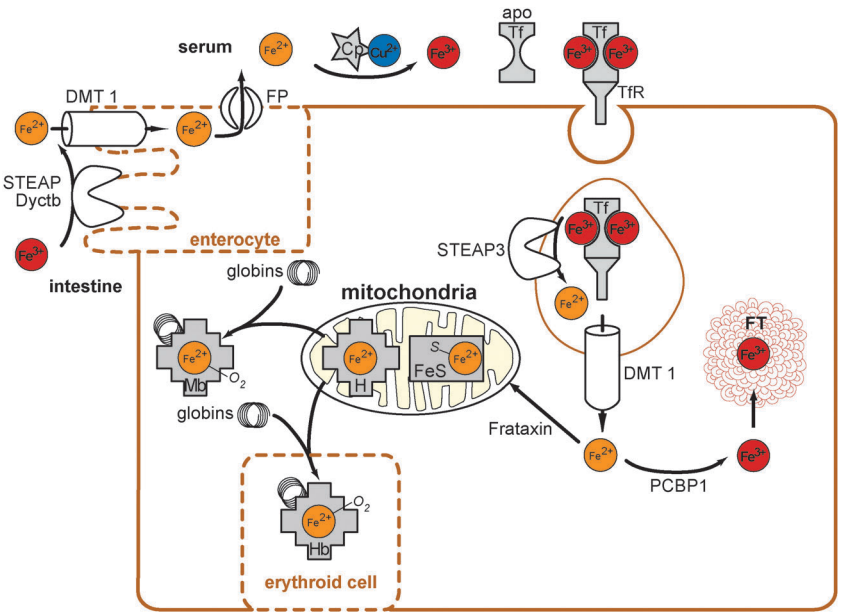

Fig. 7 A sketch of Fe trafficking in a generalized mammalian cell. Iron in dark red and in light red stands for the Fe heavy isotope and the Fe light isotope, respectively. Abbreviations: Ferrireductase, STEAP and Dyctb; Di-metal transporter 1, DMT1; ferroportin, FP; transferrin, Tf; Transferrin receptor, TfR; ceruloplasmin, Cp; hemoglobin, Hb; myoglobin, Mb; ferritin, FT.

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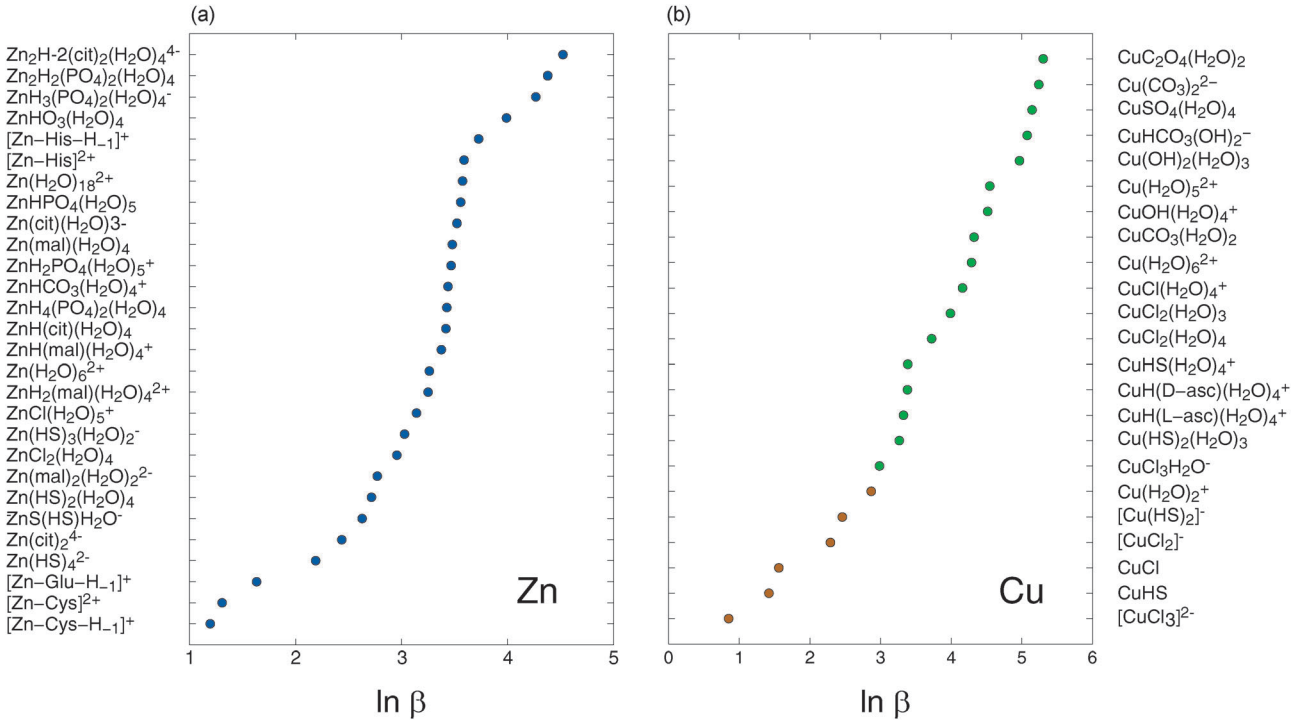

Fig. 8 Fractionation of Zn (a) and Cu (b) isotopes by different ligands. Green: Cu^{2+}, brown: Cu^{+}. Cit = citrate, mal = malate, asc = ascorbate, His = histidine, Cys = cysteine, Glu = glutathione. The parameter β is the ratio of partition functions of isotopologues (*e.g.*, ^{66}ZnS–X and ^{64}ZnS–X). The fractionation factor between two species A and B can be calculated as $\alpha_{AB} = \beta_A/\beta_B$. Data are from the following ref. 8, 17 (Zn) and 18 (Cu).

unclear for mice. Metal reduction upon intestinal uptake therefore seems to be the rule for sheep and humans but not necessarily for mice.

The higher X/Mg (with X = Zn, Cu, Fe) in the stomach content relative to food suggests that the pyloric sphincter opened post-mortem and that food may have been contaminated by bile and pancreatic juices. Insulin-secreting pancreatic beta cells are exceptionally rich in Zn.[55] This suggests that Zn from pancreatic juice and Cu from the bile may be both enriched in lighter isotopes, which is consistent with the trafficking of Cu^{+} by ATP7B into the bile[56] and with the soft bonding of Zn with the cysteine of MT particularly abundant in pancreatic secretions.[57] Zinc and copper excreted into the digestive tract at the duodenum seem to be largely reabsorbed in the lower parts of the gut, Cu earlier than Zn. Isotopes nevertheless show that the steady-state is not achieved for these elements down the digestive tract. The excess Fe with respect to Mg appearing in the caecum content demands non-steady state conditions which do not affect Fe isotope compositions and are not easily explained with the present data.

Conclusions

Large and systematic Cu, Fe and Zn isotope variability was observed in the organs of mice, sheep and humans, some being consistent among the three models, and others apparently more specific. Redox-induced and coordination-induced isotopic fractionation is invoked to account for the observed variations, with good agreement in some cases with experimental data. While the study aimed at describing the isotopic systematics of Zn, Cu and Fe in the body, this effort is yet insufficient to account for all the observed variability, notably because the metals occur at different redox and coordination states in cells. A promising direction for future studies will probably be to measure the isotopic compositions of Zn, Cu or Fe in purified cellular compartment and even, proteins.

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