Electronic Supplementary Material (ESI) for Metallomics.

A liver-targeting Cu(I) chelator relocates Cu in hepatocytes and promotes Cu excretion in a murine model of Wilson's disease.

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Electronic Supplementary Information

Experimental Section.

Chel2 and Chel2*.

Chel2 and Chel2* were synthesized as previously described,[1,2] and stock solutions prepared in ultrapure laboratory grade MilliQ water (resistivity 18 MΩcm).

Cell culture.

WIF-B9 cells were grown in Coon's modified F12 medium (Sigma) supplemented with HAT (10^{-5} M hypoxanthine, 4×10^{-8} M aminopterin, 1.6×10^{-4} M thymidine), 5% FBS (Invitrogen), 2 mM glutamine and an anti-biotic and -fungal solution containing 100 µg/mL streptomycin, 100 U/mL penicillin and 0.25 µg/mL amphotericine B. Cells were cultured at 37 °C in a humidified atmosphere with 7 % CO_2. They were plated at 2 x 10^4 cells/cm^2 on plastic tissue culture dishes (Falcon), or on round (12 mm) autoclaved glass cover slips (Esco) in 6, or 12-well plates for fluorescence studies or else at 1.6 x10^5 cells/cm^2 on square (3 mm) silicon nitride membranes (Silson, UK) in 4-well plates for XRF studies. The culture medium was renewed every 2-3 days. Experiments were performed 5-7 days later, once bile canaliculi were clearly seen under the microscope (Fig. 1).

Trafficking and immunofluorescence.

WIF-B9 cells on glass cover slips were incubated at 37 °C for 2 h in the presence of 1 µM Cu, added as $CuCl_2$. Then Chel2* was added to the medium for various times, from 15 min to 3 h, to observe its trafficking. At the chosen times, the cells were rinsed 3 times, permeabilized with methanol for 4 min at -20 °C, rehydrated in PBS and incubated at 37 °C for 45 min with mouse primary antibody EEA1 for early endosomes (1/200 BD Biosciences), or rabbit primary antibody RAB7 for late endosomes (1/200 Santa Cruz), or rabbit primary polyclonal antibody LAMP1 for late endosomes/lysosomes (1/200, ab19294 Abcam) or P58K for the trans-Golgi network (1/200 ab5820 Abcam). After 3 rinses with PBS, cells were incubated for 20 min at 37 °C with a second antibody, a goat antirabbit or antimouse conjugated with Alexa 488 (1/1000 Molecular Probes). For lysosomes, we also used a direct marker LysoTracker™ Green DND-26 (L7526 Molecular Probes) that was added prior to Chel2* for 30 min to 2 h. Cells were then rinsed 3 times with PBS and the coverslips were mounted on glass slides with a mounting medium (Sigma, M-1289). Each slide was first observed with a Zeiss Axioskop microscope using a Plan-Neofluar 40 x objective lens (NA 1.30 oil immersion) plus optovar 1.25 and the following filters: red λ_{ex} 545/25 nm, λ_{em} 607.5/65 nm and green λ_{ex} 470/40 nm, λ_{em} 540/50 nm.

Then confocal images were obtained with a Zeiss LSM-700 laser scanning confocal microscope equipped with an Axiovert 200 microscope and a 40 x 1.3 NA plan-Neofluar oil differential interference

contrast objective lens. Fluorescent markers and Cyanine 5 were excited with laser diodes at a 488 and 639 nm, respectively. The wavelength for the emission were 518 nm and 660 nm. The pixel sizes were 0.10 x 0.10 µm^2 and pinholes were set to one Airy unit (*i.e.* 32 and 40 µm). The co-localization analysis was performed on a pixel by pixel basis with the co-localization module of the Zen software (Zeiss). The crosshairs were centred in the scatterplot at the same position for all markers. For easier visualization of the pixels exhibiting co-localization we assigned a yellow colour to pixels from quadrant 3 (false colour).

Nano-XRF : *WIF-B9 cell preparation, acquisition, data treatment and quantification*

Trace select water (95305) and methanol (42105) from Sigma were used for the XRF experiments. WIF-B9 cells on silicon nitride membranes were incubated at 37 °C for 2-5 h in the presence of 0, 1 or 15 µM added Cu to observe the metal distribution in the cells. In some cases, after for 2 h in 1 µM added Cu, the cells were further incubated with 50 µM Chel2. Then, the cells were washed thoroughly with PBS, immersed into methanol for 4 min at -20 °C for chemical cryofixation, air-dried overnight and reported to yield very clean samples.[3,4]

X-ray fluorescence analysis was performed at the nano-imaging station ID22NI,[5] using the intrinsic monochromaticity of the undulators of about $\delta\lambda/\lambda \simeq 0.01$. The experimental station is located at a distance of 63 m from the undulator source and at 37 m from the high power slits used as secondary source in the horizontal direction (25 µm slit opening). The synchrotron radiation is focused by an X-ray optical device consisting of two elliptically shaped mirrors acting in two orthogonal planes using the so-called Kirkpatrick-Baez geometry.[6] The mirrors are coated with a graded multi-layer. No other monochromator is used in the setup, resulting in a very high and unique X-ray flux (up to 10^{12} photons/s) at energies between 15 and 29 keV. In this work, the energy of the pink photon beam was set to 17.5 keV for all experiments. The focused beam was characterized by translation of a gold stripe of a nano-fabricated test pattern (Xradia, USA) recording both the transmitted intensity by a diode and the fluorescence emission line by a silicon drift detector (vortex-EX, SII NanoTechnology Inc., USA). The X-ray spot size was measured to be 86 nm horizontally by 94 nm vertically (FWHM). The samples were positioned in the focal plane of the KB system and translated by piezo-stages in the directions perpendicular to the beam. The X-ray fluorescence spectra of several samples were collected by the silicon drift energy dispersive detector positioned in the horizontal plane at 75° of the incoming X-ray beam. Based on the X-ray fluorescence energy spectrum, the areal mass (A_{mass}) of the element can be calculated through a fitting procedure. The samples being scanned were deposited on 3 × 3 mm^2 membranes made of 500 nm thick silicon nitride (Si_3N_4) (Silson, Blisworth, UK). A step-size of 150 nm was used and the dwell time of 200 ms. The collected spectra were fitted using PyMCA software[7] and calibrated using the NIST SRM 1577c bovine liver standard (NIST, Gaithersburg, USA) to

obtain different elemental distributions and concentrations (areal mass density, µg/cm^2) as previously published.[8-10]

Atomic Force Microscopy allows imaging the surface morphology at nanoscale resolution and measuring the force at nanoNewton scale. AFM can investigate thin and thick film coatings, synthetic and biological membranes, metals, polymers, semiconductors and study locally the electric, magnetic or mechanical properties of the sample.[11] Thanks to its versatility, AFM became a tool of choice in biophysics.[12] The WIF-B9 thickness was measured using Atomic force microscopy in Tapping mode under ambient conditions on an Asylum MFP-3D AFM instrument equipped with acoustic box. In this MFP-3D AFM the planar scan relies on an (X, Y) scanner in closed loop while an independent piezoelectric element drives the Z displacement of the probe according to the open feedback loop control. A standard Tapping probe was used with a measured resonant frequency of 325 kHz and a cantilever stiffness of about 45 N/m (average value provided by the manufacturer data sheet). The AFM topographic images were recorded with a numerical resolution of 256 × 256 pixels. The Tapping mode is implemented in ambient air by exciting the cantilever assembly near the cantilevers resonant frequency using a dedicated piezoelectric crystal. Thanks to this mechanical excitation, the cantilever oscillates with amplitude of typically few tens of nanometers; this amplitude is modulated when the tip comes into contact with the surface. The AFM measurement was performed on the fixed cells that were further measured by X-ray fluorescence. An average thickness of 0.9±0.1 µm (N=18) was obtained at the Surface Science Laboratory of the ESRF.

^{65}Cu solution for injection.

A 1 mg/mL ^{65}Cu solution was prepared by adding ^{65}CuO (CULM-4577-PK, Eurisotop) to a 0.1N HCl solution and stirred overnight. It was further used to prepare a 20 µg/mL ^{65}Cu solution in a sterile 1.4% $NaHCO_3$ solution. The mice were injected 50 µL of the 20 µg/mL ^{65}Cu solution in their caudal vein. For control experiments, *i.e.* without ^{65}Cu, the same volume of 1.4% $NaHCO_3$ solution was injected.

Chel2 solution for injection.

A 10 mg/mL Chel2 solution was prepared in a sterile NaCl 0.9% solution. For IV treatment, a 30 mg/kg dose was intravenously injected in the caudal vein after weighing each animal. For control experiments, i.e. without Chel2, a volume of NaCl 0.9% was injected that was equivalent to 3 times the weight of the animal. For SC treatment, a 50 mg/kg dose was subcutaneously injected in the back of the mice at the neck level.

Mice, animal care, and experimental procedure.

The *Atp7b*$^{-/-}$ mice have been described earlier;[13] they were kindly given to us by Prof. Svetlana Lutsenko. All mice were housed under the same conditions at the animal facilities of the Commissariat à l'Énergie Atomique (CEA, Grenoble, France) that has accreditation from the French authorities under the reference D3818510001. Animal breeding, housing and experiments were performed in strict accordance with the French animal testing regulation. The mice were kept on a 12 h/12 h light/dark cycle and fed with maintenance dry food #3469 (Kliba Nafag CH), containing 14 mg/kg copper. For ^{65}Cu experiments, 29 adult *Atp7b*$^{-/-}$ mice (9 to 18 weeks of age) were fed with food containing only 0.367 mg/kg copper (C1041 Altromin dry food) for 3-5 weeks, a long enough period of time to lower and stabilize the copper content in their faeces. Food and water were provided ad libitum. Altogether, 29 *Atp7b*$^{-/-}$ mice were included in the study and separated into 4 groups. In 2 Chel2 groups, the mice received one ^{65}Cu dose by IV injection followed by 4 or 8 Chel2 doses. The treatment was either intravenously or subcutaneously injected, once a day for 4 days in a row, during 1 or 2 weeks. In the control groups, 4 mice received either one 1.4% $NaHCO_3$ solution injection followed by 4 Chel2 doses (once a day for 4 days in a row) or one ^{65}Cu dose followed by 4 NaCl 0.9% solution injections (once a day for 4 days in a row). Every other day, half of the mice were transferred to metabolism cages to allow the collection of their 24h-hour faeces. This protocol was approved by the French Research Ministry under the reference APAFIS #926-20 1506251 0212698 v4. One mouse died during the experiment.

Animals were euthanized by CO_2 inhalation the day after the last injection. Blood was immediately collected by cardiac exsanguination puncture in trace element dedicated tubes (Becton Dickinson Vacutainer, #369032, Le Pont-de-Claix, France) for copper measurements. The serum was collected after a 10-min centrifugation at 3000 rpm; it was kept at −20 °C for further analysis. Livers were removed rapidly after exsanguination, immediately frozen in liquid nitrogen and kept at −80 °C. Faeces and livers were weighted (wet weight) and sent to the Laboratoire de Géologie for further analysis.

Sample preparation for ^{65}Cu measurement.

Livers and faeces samples were predigested at room temperature in Savillex® PFA beakers using approximately 5 mL 14 N HNO_3 during 24 hours. Then vials were heated at 110 °C for minimum 3 days; 0.5 mL Suprapur 30 % H_2O_2 was then added on cooled samples. After 2 h at room temperature vials were heated slowly and gradually until 100 °C on hotplate for at least 2 days and evaporated to dryness. Samples were then dissolved in 3 mL 7 N HCl + H_2O_2 0.001 %. A 30 µL aliquot was taken for elemental concentration measurements and a 1 mL solution processed for copper isotope analysis according to the technique of Maréchal et al.[14]

Instrumentation and measurement protocols.

Copper isotopic compositions were measured by MC-ICP-MS (Nu 1700 HR). The copper concentrations were adjusted to 300 µg.L^{-1} in 0.05 N sub-boiled distilled HNO_3. Instrumental mass discrimination was corrected using Zn-doping (Zn JMC-Lyon) and standard sample bracketing.[14] Wet plasma was used to avoid differential isotopic fractionations that occur in the membrane of the desolvating systems. During the whole analysis, the Cu isotopic standard NIST SRM 976 was used to measure r_{ref}, the $^{65}Cu/^{63}Cu$ standard ratio.

Statistical analyses

To analyse the effect of Chel2 on the intracellular copper content and spatial distribution, we gathered 3 datasets from the nano-XRF experiments, basal condition, 1 µM Cu and 1 µM Cu + 50 µM Chel2 (Fig. 5). To analyse the effect of Chel2 on Cu excretion by mice, the ^{65}Cu experiments also produced 3 datasets, one for each treatment, mock, IV and SC (Fig. 6). The Kruskal-Wallis analysis of variance on ranks is a nonparametric test that is adapted to small size sampling and does not require assuming all the samples were drawn from normally distributed populations of equal variances. ANOVA on ranks was therefore performed, using the SigmaStat option of SigmaPlot Version 13 (Systat Software, San Jose, CA, USA). The result is given as an H statistic value and a P value, the latter being the probability of wrongly rejecting the null hypothesis based on the H value. We choose $P<0.05$ to conclude that the distributions are different. When this was the case, multiple comparisons were performed pairwise to identify the different populations (Student-Newman-Keuls Method or Dunn's Method when there is a control group).

Supplementary fluorescence images with Chel2*.

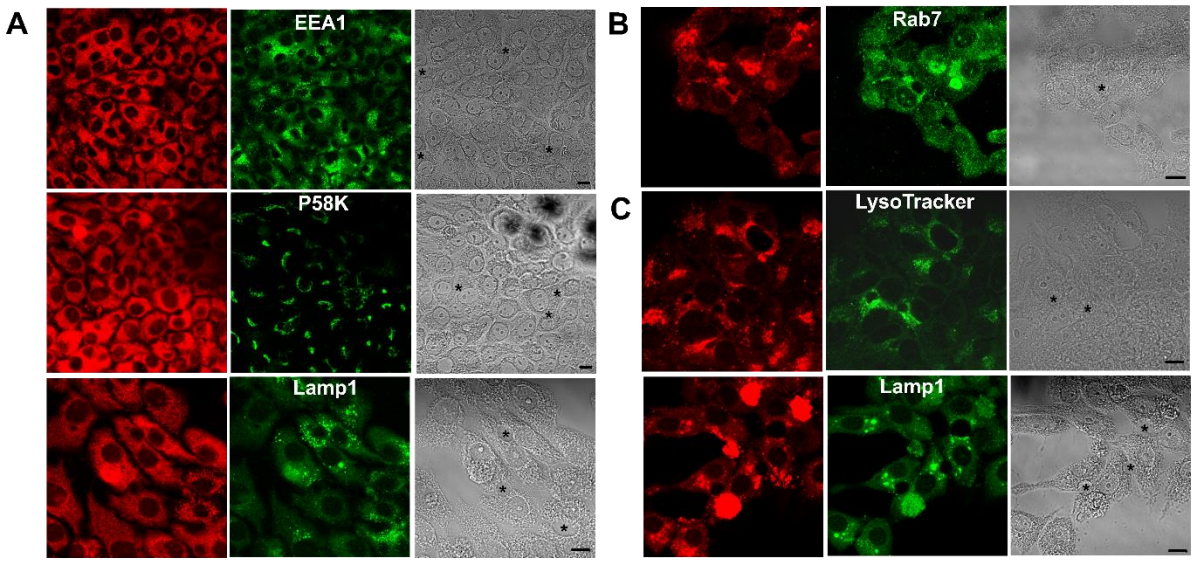

Fig. S1. Fluorescence images obtained by confocal microscopy of WIF-B9 epithelia forming canaliculi (*) and incubated for 2 h with 1 µM Cu and for (A) 30 min, (B) 1 h, (C) 2 h with 1 µM Chel2*. Left panels, red fluorescence of Chel2*; middle panels, green immunofluorescence of various protein markers or fluorescence of LysoTracker™ added 2 h before Chel2*; right panels, phase contrast image. EEA1: early endosomes, P58K: trans-Golgi network, LysoTracker™: lysosomes, Lamp1: late endosome/ lysosome, Rab7: late endosomes. (n) nucleus, scale bar 10 µm.

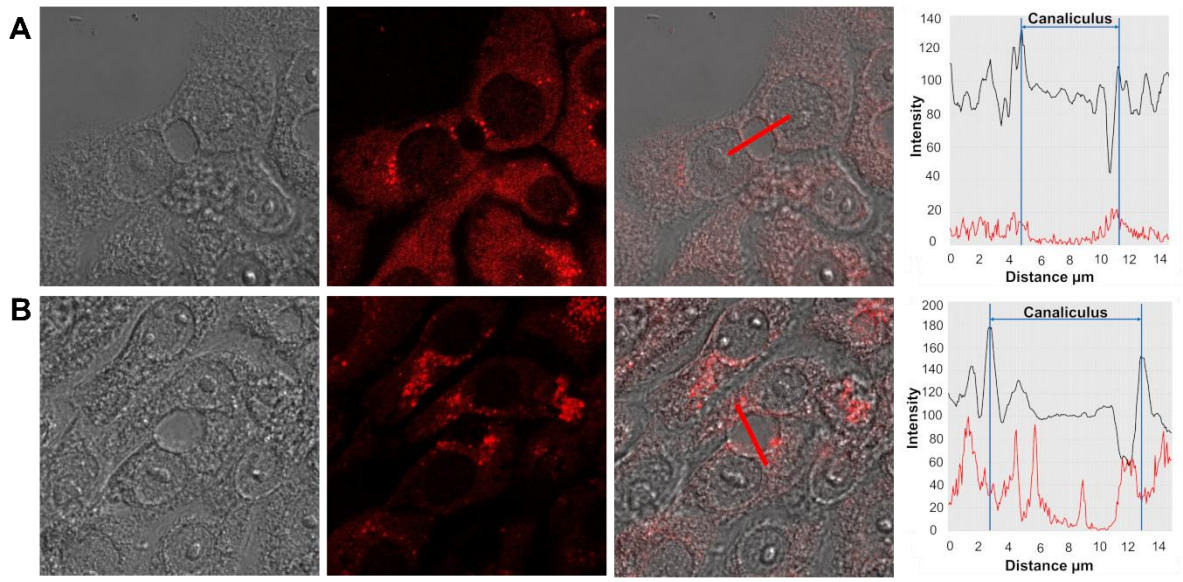

Fig. S2. Details of the analysis of Chel2* position in the region of the canaliculi shown in Fig. 3 in the main text. The analysis was performed on one 2D-section from Z-stack images. From left to right: phase contrast, Chel2* fluorescence, merge images of phase contrast and red fluorescence, intensity profiles along the 15 µm line crossing the canaliculi.

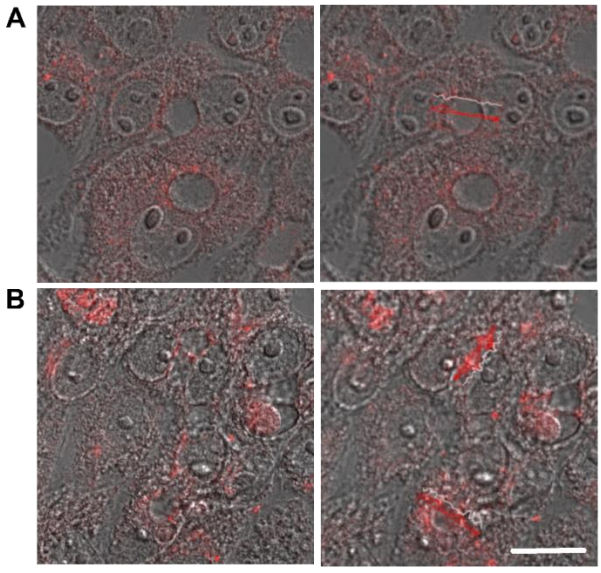

Fig. S3. Other images of canaliculi obtained as in Fig. 3 and Fig. S2. The right panels show the intensity profiles on the canaliculi.

Supplementary nano XRF images.

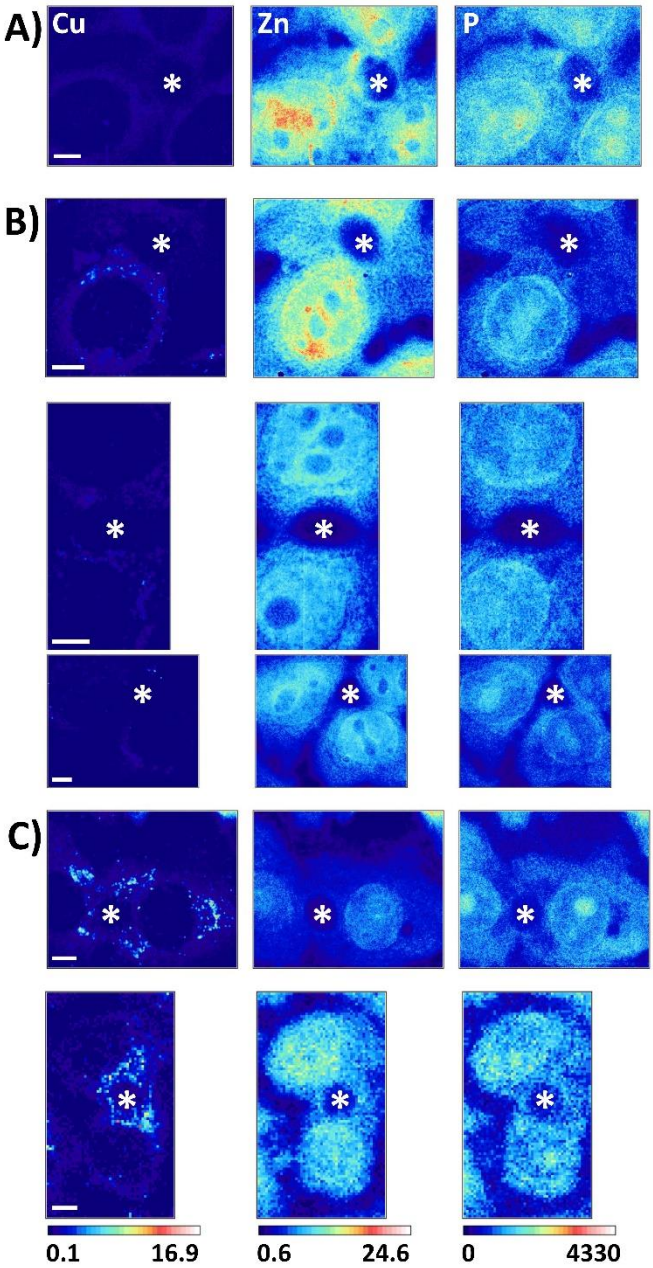

Fig. S4. Cell content analysed by nano XRF in WIF-B9 epithelia forming canaliculi under 3 different conditions: (A) basal Cu, (B) after 2 h with 1 µM Cu and (C) after 2 h with 1 µM Cu followed by addition of 50 µM Chel2 for 3 h. Under each condition, the Cu, Zn and P maps are shown. Scale bar 5 µm. The pixel size is 150x150 nm^2, except the last series at the bottom, which was acquired with a pixel size of 500x500 nm^2. Intensity scales are given in ng/cm^2.

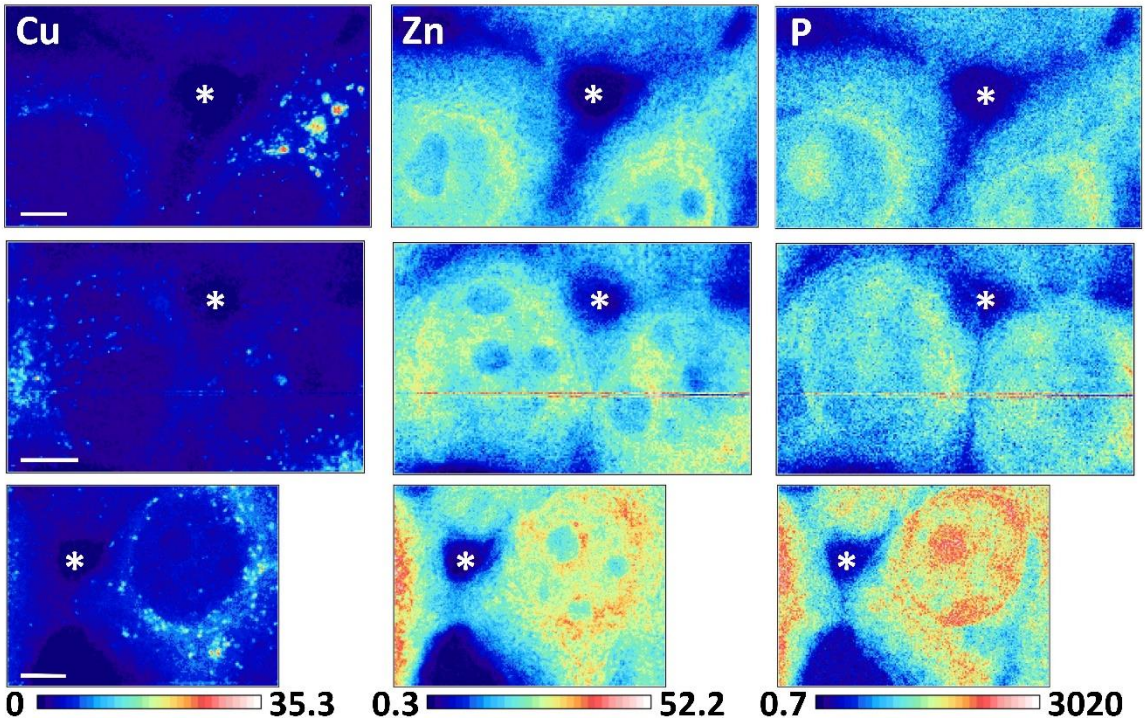

Fig. S5. Cell content analysed by nano XRF in WIF-B9 epithelia forming canaliculi after 2 h with 15 µM Cu Under each condition, the Cu, Zn and P maps are shown. Scale bar 5 µm. The pixel size is 150x150 nm^2. Intensity scales are given in ng/cm^2.

Supplementary data for the ^{65}Cu experiments on mice.

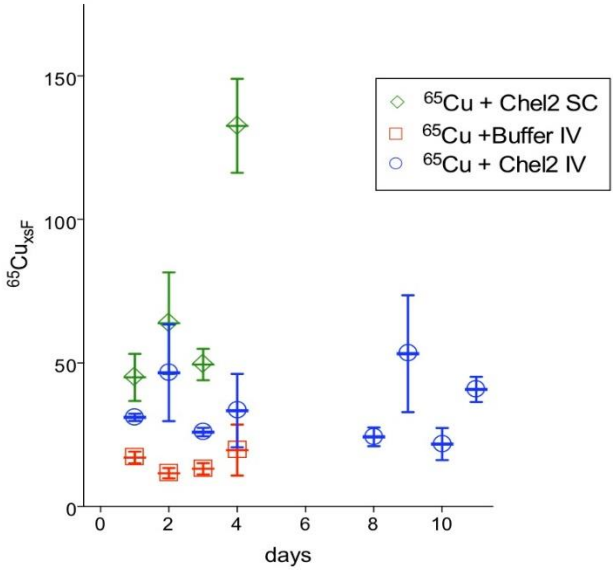

Fig. S6. Effect of Chel2 injections (starting at day 0) on the fraction of hepatic ^{65}Cu excreted in the faeces of mice ($^{65}Cu_{xsF}$ in permil) following an IV injection of ^{65}Cu at day -4. (□) mock treatment: 4 mice received 4 IV injections of buffer from day 0 to day 4; (○) Chel2 IV-treatment: 7 mice received 8 IV injections of Chel2 (30 mg/kg) from day 0 to day 10; (◇) Chel2 SC-treatment: 8 mice received 4 SC injections of Chel2 (50 mg/kg) from day 0 to day 4. For each day and each treatment, the data is represented by its mean and standard error of the mean (SEM).

Table S1. $^{65}Cu_{xsF,}$ the fraction of hepatic ^{65}Cu excreted in the faeces in permil, measured by MC-ICPMS during the experiments on mice.

	$^{65}Cu_{xsF}$ (‰)		
	Chel2	**Chel2**	**Buffer**
day	**IV-treatment**	**SC-treatment**	**Mock**
J-3	61.75	104.95	181.80
J-3	143.71	106.56	58.60
J-3	34.80	189.87	
J-3		30.96	78.29
J-3	55.90	31.63	
J-3	31.18		
J-3		110.48	
J-3	79.39	74.89	
mean	67.79	92.76	106.23
SEM	16.84	20.68	38.21
N	6	7	3
J+1	28.49	55.83	15.05
J+1	31.26	24.19	19.12
J+1	34.38	60.15	
J+1	30.15	39.82	
mean	31.07	45.00	17.08

SEM	1.24	8.20	2.04
N	4	4	2
J+2	36.66	115.77	9.80
J+2	23.72	41.74	13.40
J+2		57.29	
J+2	79.41	40.67	
mean	46.60	63.87	11.60
SEM	16.83	17.71	1.80
N	3	4	2
J+3	25.07	65.82	15.10
J+3	23.28	44.11	11.16
J+3	25.15	44.45	
J+3	30.23	43.44	
mean	25.93	49.45	13.13
SEM	1.50	5.46	1.97
N	4	4	2
J+4	11.88	153.80	10.75
J+4	32.25	94.97	28.54
J+4		116.13	
J+4	56.09	165.43	
mean	33.41	132.58	19.64
SEM	12.78	16.37	8.89
N	3	4	2
J+8	19.15		
J+8	30.38		
J+8	23.33		
J+8			
mean	24.29		
SEM	3.28		
N	3		
J+9	31.7546		
J+9	34.0677		
J+9			
J+9	93.9369		
mean	53.2531		
SEM	20.3529		
N	3		
J+10	20.6505		
J+10	37.9594		
J+10	14.4674		
J+10	14.0151		
mean	21.7731		
SEM	5.6037		
N	4		

J+11	35.9911
J+11	36.7724
J+11	
J+11	49.6083
mean	40.7906
SEM	4.4146
N	3

Table S2. Statistical analysis of the 3 datasets of Table S1 and Fig. 6, $^{65}Cu_{xsF,}$ the fraction of hepatic ^{65}Cu excreted in the faeces.

Group	N	Median	25%	75%
Mock	8	14	11	18
IV-treatment	27	30	23	37
SC-treatment	16	57	42	111
H = 28 with 2 degrees of freedom		P < 0.001		

According to Kruskal-Wallis One Way Analysis of Variance on Ranks, the differences in the median values are greater than would be expected by chance. Therefore there is a statistically significant difference, which leads to pairwise comparisons hereafter.

Comparison	Difference of Ranks	P
SC vs Mock	33	<0.001
SC vs IV	17	<0.001
IV vs Mock	16	0.024

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