Electronic Supplementary Material (ESI) for Metallomics.

Supporting Information For:

Remodeling of Zn^{2+} Homeostasis Upon Differentiation of Mammary Epithelial Cells

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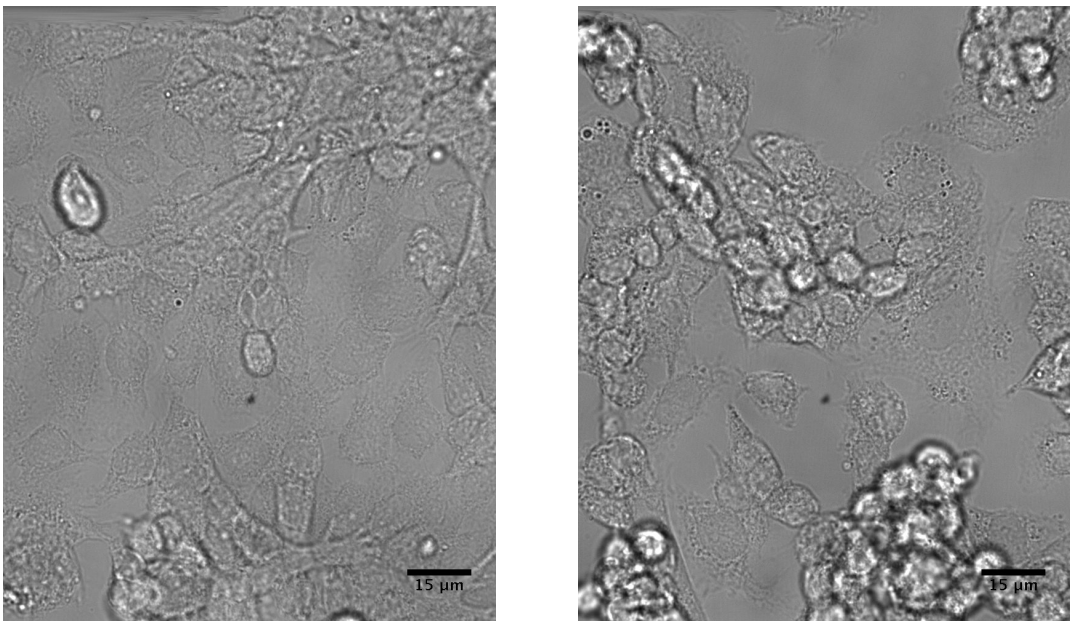


Figure S1: Brightfield DIC images of HC11 cells demonstrating cell morphology in Rday6 media (left) versus Dday6 media (right). As reported previously, differentiation causes clumping and clustering of the cells. Scale bar = 15 μm.

1. Detailed protocol and information for quantification of gene expression using qPCR

Quantitative analysis of the relative expression ratio (R) of target genes normalized to reference genes was performed with the Pfaffl method as previously described[1]. Briefly, the ratio (R) was calculated using this equation:

$$R = \frac{E\ (target)^{\Delta CT, target\ (control-sample)}}{E\ (ref)^{\Delta CT, ref\ (control-sample)}}\ .$$

The explanation of each term in this equation refers to the following **Table S1**.

Table S1: Explanation of terms for Equation for calculating R	
E (target)	The amplification efficiency of a target gene
E (ref)	The amplification efficiency of a reference gene
CT value	Cycle threshold, which is the number of cycles for signals to reach a certain threshold.
ΔCT, target (control - sample)	The CT value of a target gene under the control condition minus the CT value of the target gene under the experimental condition (sample)
ΔCT, ref (control - sample)	The CT value of a reference gene under the control condition minus the CT value of the reference gene under the experimental condition (sample)

In this study, target genes were CSN2, WAP, ZIP14, MT1 and MT2 and reference genes were ACTB and RPS9. Primer sequences and product size for each gene are presented in **Table S2**.

Table S2: Primer sequences for RT-(q)PCR

Gene name	Application	Forward Primer Sequence (5' to 3') [Final Concentration]	Reverse Primer Sequence (5' to 3') [Final Concentration]	Product Size (bp)
ACTB	RT-PCR RT-qPCR	AGCCATGTACGTAGCCATCC [50 nM]	CTCTCAGCTGTGGTGGTGAA [50 nM]	228
RPS9	RT-PCR RT-qPCR	ATTTACCCTGGCCAAGATCC [50 nM]	AGCTTCATCTTGCCCTCATC [50 nM]	141
CSN2	RT-PCR RT-qPCR	TCACTCCAGCATCCAGTCACA [50 nM]	GGCCCAAGAGATGGCACCA [50 nM]	126
WAP	RT-PCR RT-qPCR	CCGTGCCCAATGAAGATAGA [50 nM]	GCTGCTCACTGAAGGGTTAT [50 nM]	125
ZIP14	RT-qPCR	CGGTCCCAGACAACAAGATT [250 nM]	TGGCTTGTGGTGTGAAATGT [250 nM]	116
MT1	RT-qPCR	CTCCGTAGCTCCAGCTTCAC [250 nM]	AGGAGCAGCAGCTCTTCTTG [250 nM]	137
MT2	RT-qPCR	CAAACCGATCTCTCGTCGAT [200 nM]	CATTTGCATTGTTTGCATTTG [200 nM]	121

To determine the amplification efficiency (E), a standard curve was generated by plotting the CT value of each qPCR reaction with a 10-fold serial dilution of template against the $\log_{10}$ (dilution of template). A best-fit linear trend line was created and the slope was acquired from this curve. E

was calculated with this equation $E = 10^{[-1/slope]}$. For example, in the Figure A.A.1, the slope of the standard curve of RPS9 reaction with 10^{-1}, 10^{-2}, 10^{-3} template DNA dilution was plugged in the reaction: $E = 10^{(-1/3.4285)} = 1.95$. The E for each gene amplification was calculated in the same way and is shown in **Table S3**.

Figure S2: Standard curve of RPS9 qPCR reaction

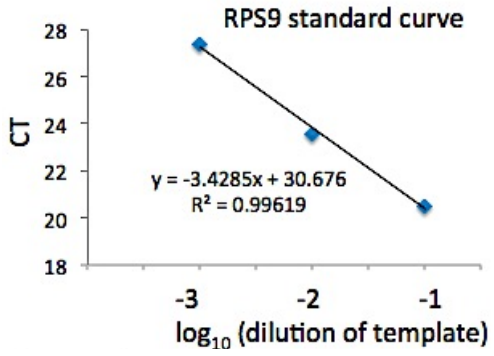


Table S3: Amplification efficiency for each gene	
Gene	E
ACTB	1.87
RPS9	1.95
CSN2	1.98
WAP	2.01
ZIP14	1.93
MT1	1.85
MT2	2.09

2. Detailed ICP-MS protocol and sample preparation

A standard curve was created for each of the two internal standards (Y and Ga) that were spiked into experimental samples and for the metal of interest (Zn). Using these standard curves, we normalized the amount of Zn to the internal standard that was spiked into the experimental samples. A complete view of ICP-MS samples set up is shown in the Table S4:

Table S4. ICP-MS Samples Set-up

Sample	Description	Y	Ga	Zn	Other
1	Y and Ga Standard Curve	5 ppb	5 ppb	-	
2		10 ppb	10 ppb		
3		25 ppb	25 ppb		
4	Zn Standard Curve	5 ppb	5 ppb	25ppb	
5		5 ppb	5 ppb	100 ppb	
6		5 ppb	5 ppb	200 ppb	
7	Blank	-	-	-	water + nitric acid
8	P day2 Biological Replicates #1-4	5 ppb	5 ppb	-	
9		5 ppb	5 ppb		
10		5 ppb	5 ppb		
11		5 ppb	5 ppb		
12	R day1 Biological Replicates #1-4	5 ppb	5 ppb	-	
13		5 ppb	5 ppb		
14		5 ppb	5 ppb		
15		5 ppb	5 ppb		
16	D 12hr Biological Replicates #1-4	5 ppb	5 ppb	-	
17		5 ppb	5 ppb		
18		5 ppb	5 ppb		
19		5 ppb	5 ppb		
20	D day1 Biological Replicates #1-4	5 ppb	5 ppb	-	
21		5 ppb	5 ppb		
22		5 ppb	5 ppb		
23		5 ppb	5 ppb		
24	D day3 Biological Replicates #1-4	5 ppb	5 ppb	-	
25		5 ppb	5 ppb		
26		5 ppb	5 ppb		
27		5 ppb	5 ppb		
28	D day6 Biological Replicates #1-4	5 ppb	5 ppb	-	
29		5 ppb	5 ppb		
30		5 ppb	5 ppb		
31		5 ppb	5 ppb		

HC11 samples were prepared as follows:

(1) Collect ~10 x 10^6 cells from cell culture dish: trypsinize cells, spin down cells at 1000 rpm for 5 min.

(2) Re-suspend cells in 2 mL of phosphate-free HHBSS buffer, count cells and record cell numbers, transfer cells to metal-free conical tubes, spin down cells again at 1000 rpm for 5 min.

(3) Discard supernatant. Cells can be frozen here. To continue the procedure, dry cells in the heat block (50 degree C) overnight in a fume hood.

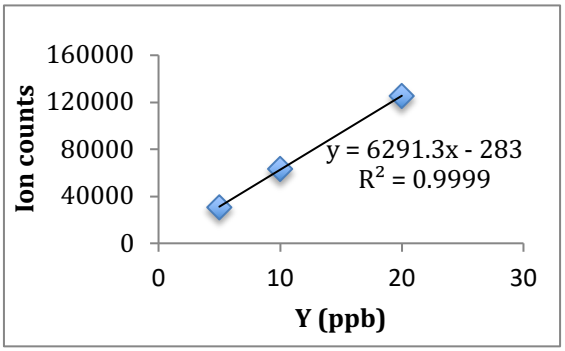
(4) Next day, add 200 μL nitric acid (trace-metal grade) to the cell pellet to digest the cells. 200 μL nitric acid is enough to digest 10 million cells. The volume was adjusted as necessary for larger or smaller number of cells. To lyse cells, heat sample in hot water bath (80-100 degree C) for 30 min.

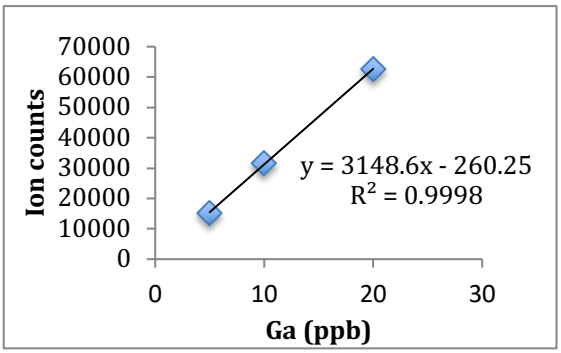
(5) Add desired amount of internal control element to the sample (5 ppb Y and Ga in each sample).

(6) After samples cool down, add 6400 μL of Chelex-100 treated H_2O to each sample to make the final the HNO_3 concentration ~2%. Samples are subjected to ICP-MS analysis.

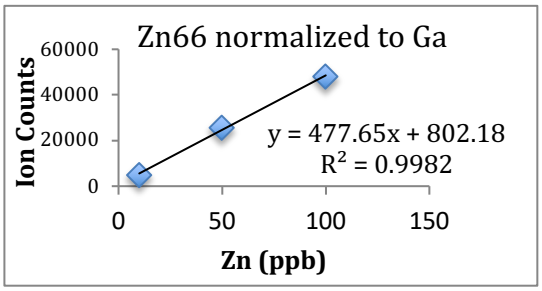
Data Analysis using the example in the above table:

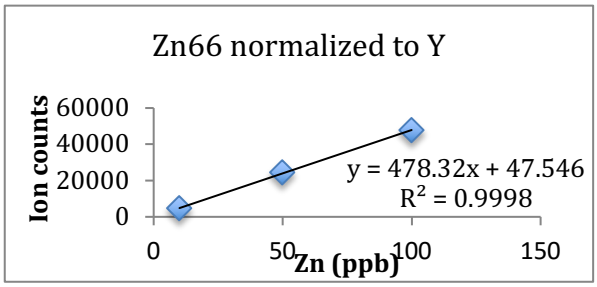
1. Generate standard curves for Y and Ga. Subtract Y/Ga ion counts of the blank sample #7 from the Y/Ga ion counts of all other individual standard samples #1-3. The standard curve is generated by plotting ion counts against ppb.





2. Generate zinc standard curve. For each Y, Ga or Zn element, subtract ion counts of the blank sample #7 from the ion counts of all other individual standard samples #1-3. Convert ion counts of Y and Ga to ppb units for samples #4-6 using the Y and Ga Standard curve. Then normalize Zn ion counts of each sample to 5 ppb Y and 5 pbb Ga, respectively. Zn standard curve is generated by plotting Zn ion counts with added Zn ppb.





3. Calculate Zn(ppb) in experimental samples.

For each Y, Ga or Zn element, subtract ion counts of the blank sample #7 from the ion counts of all other individual experimental samples. Convert ion counts of Y and Ga to ppb units for samples #4-6 using the Y and Ga Standard curve. Then normalize Zn ion counts of each sample to 5 ppb Y and 5 pbb Ga, respectively. Zn (ppb) amount was converted from Zn ion counts using the Zn standard curve.

4. To convert Zn (ppb) to [Zn] per cell, first, ppb was converted to molar concentration (M): 1 ppb Zn = 1 gram (g) of Zn / 109 grams (g) of water = 15 nM (Zn atomic mass: 65.38 g/mol, 1 g of water = 1 mL of water); next, with the known volume of the sample, 15 nM was converted to total mols of Zn (Zn mols). Zn (mols) per cell was acquired by dividing total Zn (mols) by the total cell number. To calculate [Zn] per cell, divide the Zn (mols) per cell by single cell volume (L). Single cell dimension was measured using light microscopy: length: 20 μm; width: 15 μm; height: 10 μm. Single cell volume = 20 μm x 15 μm x 10 μm = 3000 μm^3 = 3 x 10^{-9} cm^3 = 3 x 10^{-9} mL = 3 x 10^{-12} L. Normalized Zn concentration for all samples are shown in the Table S5.

Table S5. Normalized Zn Concentration for All Samples

Sample	Description	Zn Normalized to Y (μM Per Cell)	Avg / Standard Deviation	Zn Normalized to Ga (μM Per Cell)	Avg / Standard Deviation
1	P day2 Biological Replicates #1-4	205.7	200.0/9.6	196.3	195.1/11.3
2		185.8		181.8	
3		202.9		193.1	
4		205.8		209.2	
5	R day1 Biological Replicates #1-4	172.0	178.6/15.5	180.8	176.1/4.1
6		163.1		171.8	
7		179.7		173.6	
8		199.4		178.2	
9	D 12hr Biological Replicates #1-4	184.7	185.2/17.8	200.9	179.9/15.6
10		165.2		166.7	
11		182.5		169.4	
12		208.6		182.5	
13	D day1 Biological Replicates #1-4	218.7	172.1/23.6	226.9	173.1/6.7
14		185.4		182.3	
15		142.3		146.4	
16		142.1		136.9	
17	D day3 Biological Replicates #1-4	226.2	187.7/10.1	238.7	189.2/13.1
18		186.4		203.6	
19		167.3		166.6	
20		171.0		148.0	
21	D day6 Biological replicates #1-4	205.4	169.5/20.6	203.6	164.4/24.9
22		176.2		214.3	
23		151.6		148.0	
24		144.8		131.7	

Materials for this protocol:
(1) Trace-metal grade Nitric Acid: *02650 FLUKA*, Nitric acid. CAS number: 7696-372.

(2) Chelex-100 treated H_2O: Add Chelex-100 resin to MiliQ H_2O and stir overnight. The next day, filter H_2O. Chelex resin can be re-used.
(3) Y standard solution (10ppm): 125 mL - IV-STOCK-53-125 mL, Inorganic Ventures.
(4) Ga standard solution (1000pm): Gallium for ICP - CGGA1-125 mL, Inorganic Ventures.
(5) Zn standard solution (1000pm): VWR. VWR catalog #: RCMSZN1KN-100

Table S6. Top 10 zinc-dependent genes differentially expressed at D day6 compared to R day1 with highest fold change or lowest p-value.

	Downregulated Genes			Upregulated Genes		
	Gene Full Name (Symbol)	**Log2FC**	**(-) Log10 p-value**	**Gene Full Name (Symbol)**	**Log2FC**	**(-) Log10 p-value**
TOP 10 Genes with Highest FC	Histocompatibility (minor) HA-1 (Hmha1)	-4.7	10.1	Kallikrein 1-related peptidase b3 (Klk1b3)	7.9	10.0
	Amidohydrolase domain containing 1 (Amdhd1)	-4.6	46.3	Tripartite motif-containing 55 (Trim55)	7.0	7.6
	Snail family transcription repressor 1 (Snai1)	-4.5	305.8	Kruppel-like factor 15 (Klf15)	5.6	101.4
	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1 (Acap1)	-4.5	25.8	Kallikrein 1-related pepidase b4 (Klk1b4)	5.5	3.9
	Sp7 transcription factor 7 (Sp7)	-4.4	4.8	Melanophilin (Mlph)	5.4	119.8
	Nitric oxide synthase 2, inducible (Nos2)	-4.2	29.9	Zinc finger protein 385B (Zfp385b)	5.4	27.1
	Dystrophin related protein 2 (Drp2)	-4.0	15.4	Zinc finger imprinted 1(Zim1)	5.2	3.8
	Neuralized E3 ubiquitin protein ligase 1A (Neurl1a)	-4.0	64.0	Dipeptidase 1 (Dpep1)	4.6	25.2
	Activation-induced cytidine deaminase (Aicda)	-4.0	15.6	Angiotensin I converting enzyme 2 (Ace2)	4.2	75.9
	Tripartite motif-containing 9 (Trim9)	-3.9	33.1	Solute carrier family 39 member 8 (Slc39a8)	4.2	10.7
TOP 10 Genes with Lowest p-value	Snail family transcription repressor 1 (Snai1)	-4.5	305.8	Amyloid beta precursor protein (App)	1.7	277.0
	Cysteine and glycine-rich protein 2 (Csrp2)	-3.7	293.0	Carboxypeptidase Q (Cpq)	2.0	256.3
	PDZ and LIM domain 7 (Pdlim7)	-2.1	282.5	Collagen type IX alpha 1 (Col9a1)	2.5	231.7
	PR domain containing 1 with ZNF domain (Prdm1)	-3.2	266.5	pleckstrin homology domain containing family F member 1 (Plekhf1)	2.2	184.0
	Zinc finger protein 703 (Zfp703)	-1.5	203.4	Jade family PHD finger 2 (Jade2)	1.7	182.1
	A disintegrin and metallopeptidase domain 12 (Adam12)	-3.0	203.0	RAS protein activator like 1 (Rasal1)	2.3	162.0
	Superoxide dismutase 3 (Sod3)	-2.3	193.1	Ring finger protein 149 (Rnf149)	1.3	152.9
	Zinc finger protein 385A (Zfp385a)	-2.1	193.1	Solute carrier family 39 member 14 (Slc39a14)	1.8	151.5
	Matrix metallopeptidase 11 (Mmp11)	-2.4	165.3	Zinc finger protein 36 (Zfp36)	1.6	142.7
	Kruppel-like factor 10 (Klf10)	-1.4	165.0	Human immunodeficiency virus type I enhancer binding protein 3 (Hivep3)	4.2	136.4

Reference:

1. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT–PCR.

Nucleic Acids Res **29**, e45–e45 (2001).