**ZnT4 (SLC30A4)-null** (“lethal milk”) mice have defects in mammary gland secretion and hallmarks of precocious involution during lactation.

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**RUNNING HEAD**

ZnT4-null mice have secretion defects during lactation

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Abstract

During lactation, highly-specialized secretory mammary epithelial cells (MECs) produce and secrete huge quantities of nutrients and non-nutritive factors into breast milk. The zinc (Zn) transporter ZnT4 (SLC30A4) transports Zn into the trans-Golgi apparatus for lactose synthesis, and across the apical cell membrane for efflux from MECs into milk. This is consistent with observations in “lethal milk” (lm/lm) mice, which have a truncation mutation in SLC30A4, and present with not only low milk Zn concentration, but also smaller mammary glands, decreased milk volume and lactation failure by lactation day 2. However, the molecular underpinnings of these defects are not understood. Here, we used lactating C57bl/6Jlm/lm (ZnT4-null) mice to explore the consequences of a ZnT4-null phenotype on mammary gland function during early lactation. Lactating C57bl/6Jlm/lm mice had significantly fewer, smaller and collapsed alveoli comprised of swollen, lipid-filled MECs during early lactation. These defects were associated with decreased Akt expression and STAT5 activation, indicative of defects in MEC secretion. In addition, increased expression of ZnT2, TNFα and cleaved e-cadherin concomitant with increased activation of STAT3 implicated the loss of ZnT4 in precocious activation of involution. Collectively, our study indicates that loss of ZnT4 has profound consequences on MEC secretion and may promote tissue remodeling in the mammary gland during early lactation.

Keywords: zinc, lactation, mammary gland, SLC30A4, ZnT4
Introduction

ZnT4 (SLC30A4) is a zinc (Zn) transporter that is expressed in numerous tissues including the liver (13), lung (13), small intestine (28), and mammary gland (13). ZnT4 is particularly important for mammary gland function as ZnT4 expression increases dramatically during lactation (23). Previous studies in vitro established that ZnT4 transports Zn into the trans-Golgi apparatus of the mammary epithelial cell (MEC) and delivers Zn to critical Zn-dependent proteins including galactosyltransferase (a resident Golgi protein that is vital for lactose production) and carbonic anhydrase VI (a secreted milk enzyme that is vital for infant alimentary tract pH balance) (15). The “lethal milk” mouse (C57bl/6J^lm/lm) carries a spontaneous truncation mutation in SLC30A4, resulting in non-sense mediated decay of ZnT4 mRNA (29). During lactation, ZnT4-null mice have ~35% lower milk Zn concentration than their wild-type littermates (37). While this implicates ZnT4 in Zn secretion into milk, studies in breastfeeding women have yet to identify defects in ZnT4 in women with low milk Zn levels or transient neonatal Zn deficiency in exclusively breastfed infants (4, 14, 20). Moreover, ZnT4-null mice have smaller mammary glands, lower milk volume, and undergo overt lactation failure during early lactation (37), implicating ZnT4 in processes that are critical for mammary gland development and function beyond Zn secretion into milk.

The majority of mammary gland development occurs postnatally. Once the ductal tree is established during puberty, mammary gland development is stalled until pregnancy during which time extensive epithelial cell proliferation and ductal branching occurs, followed by development of the alveolar buds into complex lobular-alveolar structures that will eventually produce and secrete milk during lactation (2, 12, 45). There are two requisite stages of early lactation, secretory differentiation (lactogenesis I; development of the capacity to produce and secrete...
milk), and secretory activation (lactogenesis II; initiation of copious milk production). Hormones such as estrogen, progesterone, prolactin, growth hormone, glucocorticoids and insulin are required for secretory differentiation, while the trigger for secretory activation appears to be the withdrawal of estrogen and progesterone along with the stimulation of prolactin secretion (reviewed in Pang and Hartmann, 2007, (34)). Increased prolactin levels stimulate Jak2/STAT5 and PI3K-Akt signaling for the transcription of milk proteins and the regulation of the process of lactation, including carbohydrate metabolism, lipid biosynthesis and the transport of milk constituents across the apical membrane into the alveolar lumen (31, 32, 40). Lactation failure can result from impaired differentiation or defects in secretory activation, which is characterized by reduced milk volume, low milk lactose levels (44) and accumulation of milk protein and lipid droplets in the alveolar epithelium (26, 40). In addition, precocious involution may underlie lactation failure (7, 49). Because the ZnT4-null phenotype is associated with smaller mammary glands, low milk volume and lactation failure, we used C57bl/6J<sup>lm/lm</sup> mice to explore effects of the lack of ZnT4 on MEC secretion during early lactation.

**Materials and Methods**

**Animals**

This study was approved by the IACUC Committee at the Pennsylvania State University, which is accredited by the American Association for the Accreditation of Laboratory Animal Care (IACUC #36027). Female heterozygous mice (strain name: C57BL/6Slc30a4<sup>Im</sup>/J) were obtained commercially (Stock #: 000219, Jackson Labs, Bar Harbor, ME) and individually housed in polycarbonate cages. Mice were fed a commercially available diet based on AIN93G and maintained on a 12 h light/dark cycle under controlled temperature and humidity. Male and
female C57bl/6J\textsuperscript{wt/lm} mice were bred and allowed to deliver naturally. Offspring were genotyped using a method adapted from Jackson Labs (http://jaxmice.jax.org). Briefly, DNA was isolated from ear snips of weanling mice using the Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO) followed by PCR amplification of a 979 bp \textit{SLC30A4} sequence located on chromosome 2 (primers: 5'-TTCCCACAGCATGGTATCTAGT-3'; 5'-ATACGTACAGATGGATC-3'). PCR products were restriction digested with \textit{NdeI} for 3 h at 37°C. Subsequent products were run on a 1% agarose gel containing 0.1% ethidium bromide and visualized using UV light. To study effects of the loss of ZnT4 on mammary gland function during lactation, 8-10 week old C57bl/6J\textsuperscript{wt/wt} and C57bl/6J\textsuperscript{lm/lm} female mice were bred with male C57bl/6J\textsuperscript{wt/wt} mice, allowed to deliver normally and nurse their litters up to lactation day 2 (LD2). Litter size was normalized to 5 pups at birth and offspring were removed from dams 2 h prior to euthanization by CO\textsubscript{2} inhalation. Milk production was confirmed by gently squeezing the nipple and observing milk release and noting the presence of milk spots in the stomachs of the offspring. Blood was collected into heparinized tubes, plasma was separated and mammary glands were collected as previously described (5).

\textbf{Zinc Measurement}

Plasma Zn concentration was determined by atomic absorption spectrophotometry (n=5/genotype) as previously described (5). Zn analysis was validated using Bovine Liver Standard 1577 (National Bureau of Standards).
**Immunoblotting**

Mammary tissue (n=3-5/genotype) was homogenized in HEPES-based lysis buffer containing protease inhibitor and processed as previously described (17). Cellular debris and nuclei were pelleted by centrifugation at 500 g for 5 min. Supernatant was centrifuged at 100,000 g for 20 min, and the total membrane pellet was resuspended in lysis buffer. Protein concentration was determined by Bradford assay. Total membrane protein (50–100 μg) was diluted in Laemmli sample buffer containing 100 mM dithiothrietol and incubated at 95°C for 5 min. Samples were electrophoresed using SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked in 1% BSA for 1 h at room temperature and incubated with the following primary antibodies for 1 h: ZnT4, (1:1000, (19)); ZnT2 (1 μg/mL, (17)); cytokeratin 8 (CK8, 1:1000; #ab9023, Abcam, Cambridge, MA) phospho-STAT3 (1:1000; #9131, Cell Signaling Technology, Inc, Danvers, MA), STAT3 (1:1000; #8768, Cell Signaling), TNFα (0.1µg/mL; #ab9739, Abcam), phospho-STAT5 (1:1000; #9359, Cell Signaling), STAT5 (1:1000; #9363, Cell Signaling), Akt (1:1000; #4691, Cell Signaling), e-cadherin (1:200; #sc-21791, Santa Cruz Biotechnology, Inc, Dallas, TX) and β-actin (1:5000; #A5316, Sigma-Aldrich). The Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln, NE) was used to quantify changes in ZnT4 abundance. ZnT4 and β–actin antibodies were diluted in LI-COR buffer and incubated overnight at 4 ºC. Membranes were washed three times for 5 min in PBS with 0.1% Tween (PBS-T) and incubated for 1 h at room temperature with infrared secondary antibodies (IRDye® 800CW goat anti-rabbit IgG and IRDye® 680RD goat anti-mouse IgG diluted 1:20,000 in LI-COR buffer). The membranes were washed three times for 5 min in PBS-T and rinsed in PBS before scanning on the Odyssey® CLX imaging system. Protein quantification was performed on Odyssey Image Studio Ver 2.0. Chemiluminescence was used to quantify all other immunooblots. Membranes were
washed three times for 5 min in PBS-T or TBS with 0.1% Tween (TBS-T) and incubated for 1 h
at room temperature with donkey anti-rabbit IgG or goat anti-mouse IgG conjugated to
horseradish peroxidase (GE Healthcare Life Sciences, Pittsburgh, PA, 1:20,000). The
membranes were washed three times for 5 min in PBS-T or TBS-T, detected with SuperSignal
West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA) and
visualized using the Fluorchem™ M System (Protein Simple, Wallingford, CT). Membranes
were stripped and reprobed for either β-actin (ZnT4 and CK8) or CK8 (Akt, p-STAT5, STAT5,
p-STAT3, STAT3 and TNF) for normalization as noted. STAT (de)activation was calculated by
taking the ratio of p-STAT to normalized total STAT.

Histology

Mammary glands were fixed in 4% phosphate-buffered paraformaldehyde overnight at 4°C
followed by dehydration in 70% ethanol diluted in PBS. Tissue was sectioned and mounted onto
positively-charged slides. Hematoxylin & Eosin (H&E) staining was performed as previously
described (16). Alveolar diameter was measured using Adobe Photoshop CS3 (v10.0; San Jose,
CA). Alveolar diameter was determined using the “ruler” tool to measure two perpendicular
diameters, which were averaged to calculate the alveolar diameter. The mean alveoli diameter
from 16 random alveoli/mouse ± SD was calculated (n=4/genotype). Sections were examined by
light microscopy (Leica DM IL LED; Leica Microsystems GmbH, Wetzlar, Germany) and
images were captured using Leica Application Suite (version 3.6.0) at magnifications as
indicated. Immunohistochemistry –Sections were incubated with adipophilin (ADRP) antibody
(1:50; sc-32888, Santa Cruz) diluted into 10% serum/1% BSA/0.3% Triton X-100/PBS
overnight at 4°C and detected by immunofluorescence using anti-mouse IgG AlexaFluor® 488
(1µg/mL, Life Technologies, Carlsbad, CA). Sections were viewed at 63X magnification using the Leica Inverted Confocal Microscope SP8 (Leica Micro Systems). Three different images/mammary gland were captured (n= 3/genotype).

Whole Mount Staining and Analysis
The fourth inguinal mammary glands were collected and placed on a microscope slide at room temperature for 5 min, followed by overnight incubation in Carnoy’s fixative (60% ethanol, 30% chloroform, 10% acetic acid). Mammary glands were rehydrated in 70% ethanol (2 x 15 min), followed by 50%, 30%, and 10% ethanol (2 x 10 min each), and finally water (1 x 5 min). Rehydrated glands were stained overnight in carmine alum solution (2% carmine dye, 5% aluminum potassium sulfate), destained for 1 h in destain solution (70% ethanol, 2% HCl), and dehydrated in 70%, 95%, and 100% ethanol (2 x 15 min each) followed by xylenes for 30 min. All incubations were performed at room temperature. Mammary glands were mounted with Permount mounting medium on glass slides. Images of whole mount mammary glands were captured with a Nikon SMZ1500 stereoscope and analyzed with Adobe Photoshop CS3.

Statistical Analyses
Data are expressed as mean ± SD and were analyzed by Student’s t-test. The sample size is described for each experiment. Statistical analysis was conducted using GraphPad Prism 5.0 (San Diego, CA) and significance was demonstrated at $P < 0.05$. 

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Results

Confirmation of ZnT4-null phenotype.

Mouse genotype was confirmed using a modified PCR genotyping protocol adapted from Jackson Laboratories (Bar Harbor, ME). SLC30A4-amplified PCR products were digested with the endonuclease NdeI and visualized on a 1% agarose gel. Product size indicated SLC30A4 genotype (data not shown). To confirm that ZnT4 protein expression was abolished in C57bl/6J<sup>lm</sup>/<sup>lm</sup> mice, immunoblotting for ZnT4 in the mammary gland was performed (Figure 1A). Additionally, we noted that plasma Zn concentration did not differ between C57bl/6J<sup>wt</sup>/<sup>wt</sup> (11.1 ± 1.2 μM) and C57bl/6J<sup>lm</sup>/<sup>lm</sup> (10.7 ± 0.6 μM) mice, consistent with previous reports (37). This indicates that effects of a ZnT4-null phenotype on mammary gland function are not a consequence of systemic Zn deficiency, as previously suggested (36).

ZnT-4 null mice have profound defects in mammary gland morphology.

Previous studies noted that C57bl/6J<sup>lm</sup>/<sup>lm</sup> mice produce ~50% less milk that contains ~50% less lactose, have ~25% less mammary gland mass and failed to lactate beyond lactation day 2 compared with their wild-type littermates (36). To better understand the physiological defects during lactation, we studied the mammary glands of wild-type and C57bl/6J<sup>lm</sup>/<sup>lm</sup> mice on lactation day 2. Here, we identified distinct defects in the morphology of the mammary glands from lactating C57bl/6J<sup>lm</sup>/<sup>lm</sup> mice (Figure 1). Whole mount imaging (Figure 1B) and H&E-stained cross-sections (Figure 1C) of mammary glands from lactating C57bl/6J<sup>lm</sup>/<sup>lm</sup> mice visualized a substantial reduction in the density of the secreting epithelium and the retention of adipocytes in the stroma. In addition, the mean alveoli diameter in the mammary glands of C57bl/6J<sup>lm</sup>/<sup>lm</sup> mice was significantly smaller (Figure 1D; <i>P</i> < 0.05), the alveoli were not well-
expanded and the MECs were distended, all of which is consistent with less mammary gland
mass and reduced secretory epithelium. To verify this, we measured the abundance of CK8,
which is exclusively expressed in luminal, secretory MECs (43), and found that the lactating
mammary glands of C57bl/6J^{lm/lm} mice had a ~52% reduction in CK8 (Figure 1 E-F; $P < 0.05$).
In addition, the relative abundance of the differentiation-promoting transcription factor phospho-
STAT5 was substantially reduced (Figure 1G-H; $P < 0.01$), consistent with the loss of
differentiated epithelium. This indicates that ZnT4-null mice have altered mammary gland
morphology and a substantial reduction in the secretory epithelium.

**Secretion is impaired in lactating C57bl/6J^{lm/lm} mice.**

Because ZnT4-null mice had reduced secretory epithelium, we next assessed the secretory
capacity of these MECs. Magnified H&E images of lactating mammary glands of wild-type mice
illustrated a fully-functional mammary gland, where the lipid droplets were distributed toward
the apical membrane and actively fusing into the alveolar lumen (Figure 2A). In contrast, the
mammary glands of C57bl/6J^{lm/lm} mice showed an accumulation of lipid droplets within the
MECs lining the alveoli, suggesting impairments in MEC secretion. To verify this, mammary
glands were stained for adipophilin (Figure 2B), a lipid-droplet specific marker that is present in
differentiated mammary glands during lactation (39). Adipophilin was observed in both the
mammary glands of wild-type and C57bl/6J^{lm/lm} mice, indicating that both genotypes had
successfully undergone mammary gland differentiation. However, while adipophilin-lined lipid
droplets were abundantly found in the alveolar lumen in the mammary gland of wild-type mice,
adipophilin-lined lipid droplets accumulated in the epithelium of C57bl/6J^{lm/lm} mice. Moreover,
Akt expression, which is normally higher in secreting MECs, was significantly reduced in the
mammary glands of C57bl/6J<sup>lm/lm</sup> mice compared to wild-type littermates (Figure 2C, D; \( P < 0.01 \)), further confirming a defect in MEC secretion.

ZnT2 expression is enhanced in C57bl/6J<sup>lm/lm</sup> mice.

Previous studies also found that lactating C57bl/6J<sup>lm/lm</sup> mice still produce milk containing ~50-65% of the Zn that is found in the milk of their wild-type littermates (1, 37). This suggests that other Zn transporters participate in, or may compensate for, the lack of ZnT4. Given the vital role previously established for ZnT2 in Zn secretion into milk (4, 14, 20), we measured ZnT2 abundance by immunoblotting and found that indeed, ZnT2 abundance was ~9-fold greater in the mammary glands of C57bl/6J<sup>lm/lm</sup> mice compared to their wild-type littermates (Figure 3A-B; \( P < 0.05 \)). This suggests that increased ZnT2 abundance may compensate for the loss of ZnT4 in C57bl/6J<sup>lm/lm</sup> mice. Alternatively, previous studies showed that ZnT2-mediated Zn accumulation into lysosomes activates mammary gland involution, a process that is stimulated by TNFα (9). Consistent with this hypothesis, we found that the abundance of the involution-promoting transcription factor phospho-STAT3 was significantly higher in C57bl/6J<sup>lm/lm</sup> mice (Figure 3C-D; \( P < 0.01 \)). Moreover, we noted that TNFα abundance was 3-fold higher in C57bl/6J<sup>lm/lm</sup> mice (Figure 3E-F; \( P < 0.05 \)), consistent with the greater adipocyte mass found in these mammary glands and its role in mammary gland involution (11, 51). Moreover, during involution membrane adhesion is disrupted and is indicated by e-cadherin truncation (48). Likewise, mammary glands from C57bl/6J<sup>lm/lm</sup> mice had significantly greater truncated e-cadherin compared to their wild-type littermates (Figure 3G-H; \( P < 0.05 \)). Collectively, this suggests that in addition to defects in secretion, the mammary glands of C57bl/6J<sup>lm/lm</sup> mice underwent precocious involution.
Discussion

Since its initial characterization by Piletz and Ganschow in 1978, it has long been presumed that the primary defect in “lethal milk” mice is decreased Zn secretion into milk (1, 36, 37). However, lesser known phenotypes found in lactating C57bl/6J^{lm/lm} mice are reduced lactose production, low milk volume (36), smaller mammary gland mass and lactation failure (37). Here, we established that the “lethal milk” phenotype in ZnT4-null mice extends well-beyond Zn secretion into milk. Using lactating C57bl/6J^{lm/lm} mice, we demonstrated that decreased milk volume, smaller mammary gland mass and lactation failure resulted from distinct morphological and physiological defects that suggest both impaired epithelial secretion and precocious involution.

A key finding from our study was the observation that the mammary glands of lactating C57bl/6J^{lm/lm} mice had substantial reductions in the secreting epithelium with fewer and poorly formed alveoli. Defects in lactation outcomes in ZnT4-null mice were associated with decreased activation of STAT5, which is a critical regulator of mammary gland differentiation (27). It is possible that these defects may have partially resulted from impaired expansion during pregnancy. Rigorous analysis utilizing timed-pregnancy is required to better understand if and how the loss of ZnT4 impairs mammary expansion during pregnancy. Recent studies have shown that cell signaling can be affected by alterations in subcellular Zn pools (reviewed in (24)), which has critical implications for mammary gland function (5, 10, 21, 42). For example, LMO4, a member of the LIM-only class of Zn finger transcription factors, associates with gp130 (30), co-activates Janus kinase 1 (JAK1), the tyrosine phosphatase SHP2, and suppressor of cytokine signaling 3 (SOCS3) (30), is a positive regulator of MEC proliferation and is critical for mammary expansion during pregnancy (46). In addition, accumulation of cytoplasmic lipid...
droplets was noted in the MECs, which is a classic hallmark of impaired secretion (39). Secretory activity is largely governed through the Akt signaling pathway (25). Consistent with this, we found that in the absence of ZnT4, Akt expression was much lower in the lactating mammary gland. Accumulation of intracellular Zn decreases Akt expression (53) and the inability to mobilize Zn pools from the Golgi apparatus may also reduce Akt signaling (47). Whether reduced Akt expression in C57bl/6j\textsuperscript{lim/lim} mice was a direct effect of dysregulated Zn homeostasis in the Golgi apparatus, global defects in Golgi organization and signaling scaffolds (35), ZnT2-mediated Zn accumulation into vesicles withholding Zn from activating Akt phosphorylation or further alterations in cellular Zn homeostasis, is not currently understood. Alternatively, loss of ZnT4 may impair secretory activation, which occurs at the transition between gestation and lactation. A series of transcriptional (2) and translational (38) molecular events occur during this time, and dysregulation in key molecules impact secretory activation. For example, adipocyte enhancer binding protein 1 (AEBP1) negatively regulates PTEN and NFkB, and while AEBP1-null mice do not have defects in mammary gland expansion during pregnancy, the appearance of large cytoplasmic lipid droplets combined with the accumulation of milk proteins in the MECs at late pregnancy and parturition, reflects failed secretory activation (52). Although we also noted the presence of large cytoplasmic lipid droplets in ZnT4-null mice, carefully timed studies at different developmental stages are required to determine if ZnT4-null mice have defects in secretory activation.

Several reports suggest mammary gland function may be particularly sensitive to changes in Zn metabolism. Lactating mice fed a marginal Zn diet (5) also have a substantial reduction in secretory epithelium and defects in function during lactation. However, changes in ZnT4 or ZnT2 expression are not the culprit, thus the molecular underpinnings remain unknown. While
ZnT2-null mice also have a substantial reduction in secretory epithelium, this results from cytoplasmic Zn accumulation, the loss of phospho-STAT5 signaling and profound defects in mammary gland differentiation and secretion as opposed to precise involution (21). In contrast, while ZnT4-null mice also have a substantial reduction in secretory epithelium, these defects are associated with increased ZnT2 and TNFα expression and precocious activation of phospho-STAT3, which is indicative of mammary gland remodeling (9, 10). Similarly, activation of precocious involution was associated with increased TNFα and ZnT2 expression and the activation of phospho-STAT3 in the mammary glands of lactating mice fed an obesogenic diet (11). Thus, the molecular drivers of the responses in these three murine models appear to overlap. For example, while the loss of ZnT2 likely leads to toxic Zn accumulation in the mammary gland in ZnT2-null mice (21), ZnT4-null mice had greater expression of ZnT2, which in turn is a Zn-responsive vesicular Zn importer (8) that protects against Zn toxicity (33). Thus, an attempt to protect against Zn toxicity may underlie the sparse mammary epithelium in ZnT4-null mice. Alternatively, increased ZnT2 expression might augment Zn import into mitochondria (41), reducing ATP production (22) and/or increasing apoptosis (41), either of which would have important implications for MEC function. In addition, these defects may also be a consequence of the adipocyte-rich stroma that is retained in ZnT4-null mammary glands, which may secrete estrogen (3), pro-inflammatory cytokines (50) such as TNFα, and other anti-lactogenic factors. Further studies are required to understand the molecular interaction between ZnT4 with ZnT2 in maintaining the secretory epithelium of the mammary gland during lactation.

**Perspectives and Significance**
While breastfeeding is the best way to nourish the developing neonate, many women have difficulty successfully activating or maintaining lactation, or produce breastmilk of sub-optimal composition. Such difficulties can lead to hypernatremia, nutritional deficiencies, increased morbidity and growth failure. Increasing evidence in production animals, rodent models, and more recently in humans, implicates genetic variation in key molecules as a modifier of lactation outcomes. Further studies are required to understand the physiological relevance of genotype and the molecular underpinnings of defects on lactation biology so that women suffering from or at risk for sub-optimal lactation can be identified and alternative neonatal feeding strategies can be explored. With this intention, we aimed to better understand the lactation defects in ZnT4-null mice. Previous studies noted that ZnT4-null mice produced milk that contained sub-optimal levels of Zn and suggested that the role of ZnT4 was to provide zinc for secretion into milk. While milk zinc levels are lower in ZnT4-null mice, findings from the present study demonstrate that loss of ZnT4 function leads to profound loss of secretory epithelium, reduced secretory capacity and precocious involution. This may have important implications for breastfeeding women as numerous genetic variants in ZnT4 have been identified in genome wide association studies. Collectively, these data contribute to a growing body of evidence suggesting that maternal genetics plays an important role in lactation success and neonatal health.

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Disclosures

The authors have no conflicts of interest to disclose.
Figure Legends

**Figure 1.** A ZnT4-null phenotype in C57bl/6J<sup>lm/lm</sup> mice leads to reduced secretory epithelium.

(A) Representative immunoblot of ZnT4 in total membrane fractions prepared from wild-type (WT) and C57bl/6J<sup>lm/lm</sup> (lm) mammary glands. β-actin was used as a loading control. (B) Representative whole mount images of mammary glands from WT and lm mice on lactation day 2. Magnification =0.5X, scale bar = 0.5 mm. (C) H&E stained images of mammary glands from WT and lm mice on lactation day 2 at 4X magnification (i, ii; scale bar = 250 μm) and 10X magnification (iii, iv; scale bar = 100 μm). (D) Data represent mean alveolar diameter (μm) ± SD, n = 4 mice/genotype. *indicates significant effect of genotype, P<0.05. (E) Representative immunoblots of the luminal epithelial cell marker, cytokeratin 8 (CK8) in cell lysates from WT and lm mammary glands. β-actin was used as a loading control. (F) Data represent mean CK8 protein abundance normalized to β-actin ± SD, n=3 mice/genotype. *indicates significant effect of genotype, P<0.05. (G) Representative immunoblots of phospho-STAT5 (p-STAT5) and total STAT5 in cell lysates from mammary glands of WT and lm mice. CK8 was used as a loading control. (H) Quantification of STAT5 activation. Data represent mean ratio of p-STAT5/ total STAT5 normalized to CK8 ± SD, n=3 mice/genotype. **indicates significant effect of genotype, P<0.01.

**Figure 2.** Secretory defects in C57bl/6J<sup>lm/lm</sup> mammary glands.

(A) H&E stained images of mammary glands from wild-type (WT) and C57bl/6J<sup>lm/lm</sup> (lm) mice on lactation day 2 at 100x magnification, scale bar= 100 μm. Black arrow marks active fusion of lipid droplets. (B) Representative images of adipophilin (ADRP, green) in mammary glands from
WT and *lm* mice on lactation day 2. Nuclei were counterstained with DAPI (blue).

Magnification= 63X. scale bar= 100 µm. (C) Representative immunoblots of Akt in cell lysates from WT and *lm* mammary glands. Cytokeratin 8 (CK8) was used for normalization. (D) Data represent mean Akt protein abundance relative to CK8 ± SD, n=3 mice/genotype. **indicates significant effect of genotype, *P*<0.01.

**Figure 3. ZnT2 abundance is increased in the C57bl/6J *lm/lm* mammary gland.**

Representative immunoblot of ZnT2 in total membrane fractions from mammary glands of C57bl/6J *lm/lm* (*lm*) mice and their wild-type (WT) littermates. Cytokeratin 8 (CK8) was used for normalization. (B) Data represent mean ZnT2 protein abundance relative to cytokeratin 8 (CK8) ± SD, n=3 mice/genotype from 2 different experiments. *indicates significant effect of genotype, *P*<0.05. (C) Representative immunoblots of phospho-STAT3 (p-STAT3) and total STAT3 in cell lysates from mammary glands of WT and *lm* mice. CK8 was used for normalization. (D) Quantification of STAT3 activation. Data represent mean ratio of p-STAT3/total STAT3 normalized to CK8 ± SD, n=3 mice/genotype. **indicates significant effect of genotype, *P*<0.01. (E) Representative immunoblots of TNFα in cell lysates from mammary glands of WT and *lm* mice. CK8 was used as a loading control. (F) Data represent mean TNFα protein abundance relative to CK8 ± SD, n=3 mice/genotype. *indicates significant effect of genotype, *P*<0.05. (G) Representative immunoblots of mature (120 kDa) and truncated (97 kDa) e-cadherin (e-cad) in cell lysates from mammary glands of WT and *lm* mice. CK8 was used for normalization. (H) E-cadherin truncation. Data represent mean ratio of truncated/mature e-cad normalized to CK8 ± SD, n=3 mice/genotype. *indicates significant effect of genotype, *P*<0.05.
References


FIGURE 1.

A

[Images of immunoblotting showing protein bands labeled ZnT4 (47 kDa) and β-actin (42 kDa) for WT and lm samples.]

B

[Images of histological sections labeled WT and lm showing ductal structures.]

C

[Images of histological sections labeled WT and lm showing tissue morphology.]

D

[Graph showing ductal diameter (μm) with WT and lm samples. * indicates statistical significance.]

E

[Images of immunoblotting showing protein bands labeled CK8 (50 kDa) and β-actin (42 kDa) for WT and lm samples.]

F

[Graph showing CK8 abundance (normalized to β-actin) with WT and lm samples. * indicates statistical significance.]

G

[Images of immunoblotting showing protein bands labeled p-STAT5 (90 kDa), STAT5 (90 kDa), CK8 (50 kDa) for WT and lm samples.]

H

[Graph showing STAT5 activation (normalized to total STAT5) with WT and lm samples. ** indicates statistical significance.]
FIGURE 2.

A. WT vs. Im

B. WT vs. Im

C. Western Blot

D. Akt abundance (relative to CK8)
FIGURE 3.

A

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B

ZnT2 abundance (relative to CK8)

WT | Im  
---|-----|
0   | **| 6 |

C

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D

STAT3 activation (normalized to total STAT3 levels)

WT | Im  
---|-----|
0.01 | **|| 0.04 |

E

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Im</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK8</td>
<td>17 kDa</td>
<td>50 kDa</td>
</tr>
</tbody>
</table>

F

TNF abundance (relative to CK8)

WT | Im  
---|-----|
0 | *| 3 |

G

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Im</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cad</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK8</td>
<td>120 kDa</td>
<td>97 kDa</td>
</tr>
</tbody>
</table>

H

E-cadherin truncation (normalized to mature e-cad)

WT | Im  
---|-----|
1.5 | *| 2.5 |