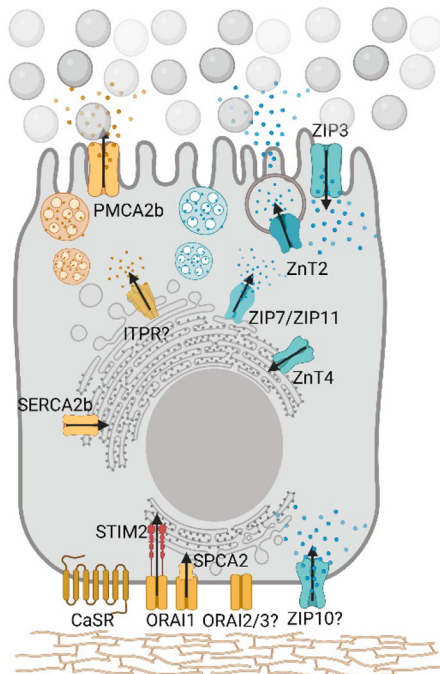


# The ins and outs of mammary gland calcium and zinc transport: A brief review\*

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## Graphical Abstract

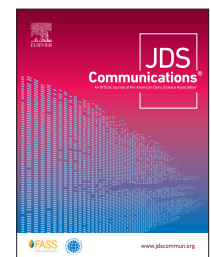


## Summary

The transport of calcium and zinc in the mammary gland is tightly regulated through mineral-specific uptake, sequestration, and export mechanisms. Calcium transport involves calcium sensing via CaSR, uptake across the basolateral membrane via Orai1 through interactions with STIM2 and SPCA2 for sequestration into the endoplasmic reticulum and Golgi apparatus, respectively, and efflux across the apical membrane via PMCA2b or by secretion in casein micelles. On the other hand, zinc transport is tightly regulated by 2 families of zinc transporters and involves uptake across the basolateral membrane possibly by ZIP10, sequestration/efflux by the Golgi apparatus via ZnT4 and ZIP7/ZIP11, and import into secretory vesicles via ZnT2 or casein micelles for secretion across the apical membrane.

## Highlights

- Calcium is regulated by a diverse array of calcium channels and transporters.
- Zinc is regulated by members of 2 different gene families of zinc transporters.
- Both calcium and zinc accumulate in the Golgi apparatus during lactation.
- Further research is needed to understand molecular and genetic regulation.



\*Presented as part of the Lactation Biology Symposium: Nutrient Transport in the Mammary Gland held at the ADSA Annual Meeting, June 2022. Department of Biomedical and Nutritional Sciences, University of Massachusetts, Lowell 01854. †Corresponding author: [shannon\\_kelleher@uml.edu](mailto:shannon_kelleher@uml.edu). © 2023, The Authors. Published by Elsevier Inc. and FASS Inc. on behalf of the American Dairy Science Association®. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>). Received June 26, 2022. Accepted August 31, 2022.

# The ins and outs of mammary gland calcium and zinc transport: A brief review\*

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**Abstract:** Milk is an excellent source of all macrominerals and trace elements, which are essential for proper function of a wide variety of vital processes. The concentrations of minerals in milk are influenced by numerous factors, including stage of lactation, time of day, nutritional and health status of the mother, as well as maternal genotype and environmental exposures. Additionally, tight regulation of mineral transport within the secretory mammary epithelial cell itself is critical for the production and secretion of milk. In this brief review, we focus on the current understanding of how the essential divalent cations calcium (Ca) and zinc (Zn) are transported in the mammary gland (MG) with a focus on molecular regulation and the consequence of genotype. A deeper grasp of mechanisms and factors affecting Ca and Zn transport in the MG is important to understanding milk production, mineral output, and MG health to inform intervention design and novel diagnostic and therapeutic strategies in production animals and humans.

Milk is a complex biological fluid (Andreas et al., 2015) that includes all essential macrominerals and trace elements found in concentrations reflecting the physiological needs of the neonate. Regardless of species, milk mineral concentrations change over the course of lactation, time of day, and milking interval, and are dependent upon complex interactions between maternal diet, genetics, health status, and environmental factors (Roy et al., 2020). Moreover, essential minerals are also important for MG function. In this brief review, we focus on Ca and Zn transport as they represent the macromineral and trace element, respectively, found in highest concentration in milk, and both are divalent cations that regulate numerous intracellular functions. Because of this, they both require tightly regulated uptake from maternal circulation into the secretory mammary epithelial cell (MEC), followed by sequestration into and efflux from intracellular storage pools, and finally efflux or secretion into the alveolar lumen, all of which are facilitated by a diverse array of often mineral-specific membrane transport proteins and channels. Furthermore, we provide a brief discussion of the consequences of genotype on mammary gland (MG) Ca and Zn transport and milk concentrations.

The divalent cation Ca is the macromineral found in highest concentration in milk. Dairy milks contain ~1.2 g of Ca/L (human milk contains ~350 mg/L), partitioned into ionic and protein-associated pools. Milk Ca concentration depends upon breed/genetics, stage of lactation, parity, and diet (Gaignon et al., 2018; Qin et al., 2021). In addition, the ionized Ca pool within MEC plays a critical role in a diverse array of intracellular signaling cascades fundamental for MG function, such as cell proliferation, differentiation, apoptosis, secretion, gene expression, and ion transport (Dolman and Tepikin, 2006). To meet the enormous Ca demand during lactation, the MG communicates with Ca storage pools in bone. The extracellular Ca-sensing receptor (CaSR) is a G-protein-coupled receptor expressed in numerous tissues that responds to changes in extracellular free Ca ions ( $\text{Ca}^{2+}$ ) within the physiologic range (Kim and Wysolmerski, 2016). In the MG, the CaSR is localized to the basal membrane

(Cheng et al., 1998) and activation stimulates PKC and attenuates cAMP pathways (Mamillapalli et al., 2008). The expression of CaSR increases during lactation (VanHouten et al., 2004) and serves at least 2 principal roles: (1) it senses systemic  $\text{Ca}^{2+}$  levels and, in response, reduces secretion of parathyroid hormone-related protein into maternal circulation to reduce  $\text{Ca}^{2+}$  reabsorption from bone, and (2) stimulation increases activity of plasma membrane Ca transport protein 2 (PMCA2) to directly increase transepithelial  $\text{Ca}^{2+}$  efflux into milk (VanHouten et al., 2007).

Numerous transporters and channels play a role in Ca transport in the MG, and the reader is referred to Montalbetti et al. (2014) for an excellent review. Calcium must first be taken up by the MEC across the basal membrane; however, the precise mechanisms responsible for Ca uptake from maternal circulation are not well understood. Recent evidence suggests Ca taken up by the MEC may be directly sequestered into the endoplasmic reticulum (ER) and Golgi apparatus. The ORAI Ca release-activated Ca modulator (ORAI1–3) proteins are pore-forming subunits of the store-operated Ca entry (SOCE) channels. Stromal interaction molecules (STIM1–2) are ER-embedded  $\text{Ca}^{2+}$  sensing proteins essential to the activation of  $\text{Ca}^{2+}$  influx that respond to reduced ER  $\text{Ca}^{2+}$  stores by aggregating and interacting with apposed cell membrane ORAI proteins, causing ORAI to open and directly replenish ER  $\text{Ca}^{2+}$  stores from the extracellular space. Our current understanding of the molecular interactions between ORAI and STIM proteins was recently reviewed (Johnson et al., 2022). ORAI1 is expressed on the basal membrane and is of primary importance during lactation as it is profoundly upregulated in the lactating MG (McAndrew et al., 2011), and appears responsible for ~50% of  $\text{Ca}^{2+}$  uptake (Cross et al., 2013; Davis et al., 2015). Interestingly, ORAI1-mediated intracellular  $\text{Ca}^{2+}$  signaling is also critical for myoepithelial function, thus playing a role in milk ejection as well (Davis et al., 2015). Importance of ORAI1 is reflected in its role as a component of the ER stress response that occurs during the transient negative energy balance that occurs after calving (Zhang et al.,

\*Presented as part of the Lactation Biology Symposium: Nutrient Transport in the Mammary Gland held at the ADSA Annual Meeting, June 2022.

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2020). While ORAI2 and ORAI3 expression in the MG are both slightly increased during lactation, their localization, regulation, and contribution to Ca uptake are not yet understood. Moreover, while STIM1 plays a minimal role, STIM2 expression in the MG is modestly increased during lactation (McAndrew et al., 2011) implicating it in  $\text{Ca}^{2+}$  influx and ER  $\text{Ca}^{2+}$  sequestration, which may be necessary for milk protein production, control of concomitant ER stress, or may serve as a storage pool until released from the ER by an unknown mechanism.

In addition to the ER, the Golgi apparatus is also a key regulator of the spatiotemporal regulation of cytosolic  $\text{Ca}^{2+}$  levels. Changes in Ca concentration either within the Golgi lumen or juxtaposed in the cytoplasm, regulate Golgi functions such as the sorting and processing of secretory and membrane proteins. Two Ca pumps control  $\text{Ca}^{2+}$  uptake into the Golgi apparatus. The sarco/endoplasmic reticulum  $\text{Ca}^{2+}$  transport ATPase (SERCA1–3) proteins are mainly found in the ER and *cis*-golgi and belong to the P-type pump family (Christodoulou et al., 2021). To our knowledge, only SERCA2b is expressed in MEC; however, its role is not understood. The more abundant secretory pathway  $\text{Ca}^{2+}/\text{Mn}^{2+}$  transport ATPase (SPCA1 and SPCA2) proteins are found throughout the Golgi apparatus and in secretory vesicles, and SPCA1 and SPCA2 have discrete functions. Ubiquitous expression of SPCA1 suggests it provides  $\text{Ca}^{2+}$  for general intraluminal functions, such as structure, protein sorting, and vesicle trafficking (Lissandron et al., 2010). In contrast, much more is known regarding the role of SPCA2 in the MG as SPCA2 expression is restricted to highly specialized secretory cells suggesting a unique role for SPCA2 and  $\text{Ca}^{2+}$  uptake into the Golgi apparatus in secretory function. Recent studies show SPCA2 interacts with ORAI1 influxing  $\text{Ca}^{2+}$  directly into the Golgi apparatus from the extracellular environment, revealing store-independent Ca influx pathways (SICE) are also vital during lactation (Cross et al., 2013). While Golgi  $\text{Ca}^{2+}$  sequestration is likely responsible for the abundant Ca secreted with casein micelles comprising ~30% of milk Ca, additional studies are required to understand the role of Ca sequestration in the Golgi apparatus as it pertains to MG function.

Emerging evidence suggests ER and Golgi  $\text{Ca}^{2+}$  pools may be directly mobilized in response to cellular cues in the MG. Inositol 1,4,5-trisphosphate receptor (ITPR) mediates  $\text{Ca}^{2+}$  release from ER and Golgi stores in response to binding of  $\text{IP}_3$  to propagate complex spatial and temporal  $\text{Ca}^{2+}$  signaling cascades regulating diverse physiological responses (Foskett et al., 2007) and is expressed in almost all animal cells. Myoepithelial cells, the contractile cells that surround the luminal MEC, are  $\text{IP}_3$ -sensitive suggesting a role of ITPR in stimulating milk ejection (Nakano et al., 2001). In addition,  $\text{Ca}^{2+}$  is released from the ER in MEC in response to  $\text{IP}_3$  suggesting a role for ITPRs in luminal cells as well. However,  $\text{IP}_3$ -sensitive  $\text{Ca}^{2+}$  release from MEC is greater during pregnancy compared with lactation (Yoshimoto et al., 1990); thus, the function of  $\text{IP}_3$ -stimulated  $\text{Ca}^{2+}$  stores requires further investigation.

Plasma membrane  $\text{Ca}^{2+}$  ATPase (PMCA1–4) proteins are a family of P-type  $\text{Ca}^{2+}$  pumps that efflux  $\text{Ca}^{2+}$  out of the cytoplasm (Reinhardt and Horst, 1999). PMCA1 and PMCA4 are ubiquitously expressed; however, PMCA2 expression is primarily restricted to the MG and moreover, expression of a specific splice variant (PMCA2b) is localized to the apical membrane, thus much is known about PMCA function during lactation. Expression is dramatically upregulated during pregnancy and lactation (Reinhardt

et al., 2004) and further stimulated by CaSR, permitting persistent efflux of  $\text{Ca}^{2+}$  across the apical membrane reflecting its major role in ~60–70% of  $\text{Ca}^{2+}$  transported into milk (Reinhardt et al., 2004).

Milk Ca concentration depends upon stage of lactation, parity, and diet (Gaignon et al., 2018; Qin et al., 2021). While human mutations in *ORAI* and *STIM* (Vaeth et al., 2020), *SERCA* (Hovnanian, 2007), *SPCA2* (Shull et al., 2011), and *PMCA2* (Giacomello et al., 2011) are associated with different disease states, genetic variants in Ca transporters or channels that affect MG function or milk Ca concentration have not yet been described. Targeted studies are required to understand potential roles for Ca transporters and ion channels in MG function and Ca homeostasis.

The divalent cation Zn is the trace element found in greatest concentration in milk. Dairy milks contain ~5 mg Zn/L (human milk contains ~1.2 mg/L). Like Ca, Zn is required for a plethora of cellular processes. Zinc is a co-factor for >10% of the proteome and regulates a diverse array of cellular functions critical for MG function including DNA synthesis and repair, transcription, translation, proliferation, apoptosis, autophagy, management of oxidative stress, and the cell cycle (McCormick et al., 2014). Zinc is transported into the cell and between subcellular compartments by members of 2 different gene families of Zn transporters (ZIP and ZnT proteins) and has been elegantly reviewed by Kambe et al. (2021). In general, the ZIP family of Zn transporters (*SLC39A*; ZIP1–14) import Zn into the cytoplasm, either from the extracellular space or from within an intracellular compartment. In contrast, the ZnT family of Zn transporters (*SLC30A*; ZnT1–10) export Zn from the cytoplasm, into subcellular organelles or out of the cell entirely.

Mammary gland Zn transport is only partially understood and has been extensively reviewed by McCormick et al. (2014). Zinc uptake from maternal circulation likely results from ZIP5, ZIP8, and ZIP10, as they are localized to the basal membrane of MEC (McCormick et al., 2014). While ZIP5 expression remains relatively constant, ZIP8 expression profoundly increases during lactation (Kelleher et al., 2012). However, in addition to Zn, ZIP8 transports selenium, cadmium, iron, and manganese, and thus its contribution to MEC Zn uptake is not understood. In contrast, ZIP10 expression is profoundly increased during lactation and it appears to be Zn-specific, suggesting ZIP10 may be primarily responsible for the large amount of Zn taken up by the lactating MEC (McCormick et al., 2010). ZIP3 expression also increases during lactation (Kelleher et al., 2009); however, ZIP3 is localized to the apical membrane and responsible for Zn reuptake from the secreted milk pool; thus, additional studies are required to understand its physiological relevance.

Once taken up by the MEC, like Ca, Zn can also be sequestered in the ER and Golgi apparatus. ZnT5 and ZnT6 may transport Zn into the early secretory (ER/*cis*-Golgi) pathway of MEC (Suzuki et al., 2005) and supply Zn for resident Zn-requiring enzymes (Fukunaka et al., 2009). In contrast, ZnT4 transports Zn into the *trans*-Golgi apparatus (McCormick and Kelleher, 2012), and ZnT4 expression in the MG is profoundly increased during lactation (Kelleher et al., 2012). A spontaneous mutation in the ZnT4 gene in mice leads to nonsense-mediated mRNA decay and is associated with a ~35% reduction in milk Zn level and reduced milk production (McCormick et al., 2016). Juxtaposed to ZnT4, ZIP7 and ZIP11 efflux Zn from the Golgi apparatus and expression is also profoundly increased in the lactating MG. Although specific

functions of ZIP7 and ZIP11 have not been revealed, increased abundance of ZnT4, ZIP7, and ZIP11 suggests the Golgi apparatus provides an important mobilizable Zn pool in MEC, and further studies are required to understand the role and regulation of Golgi apparatus Zn pools during lactation.

Milk Zn levels are high during early lactation and decline as lactation continues (Kelleher and Lönnerdal, 2003). During early lactation, ZnT1 is localized to the apical membrane and may play a role in transiently enhancing Zn efflux into milk (Kelleher and Lönnerdal, 2003). However, Zn is primarily secreted into milk via exocytotic vesicles by ZnT2 (Lopez and Kelleher, 2009). The lactogenic hormone prolactin increases transcription of ZnT2 through activation of JAK2/STAT5 signaling (Qian et al., 2009), and posttranslationally regulates ZnT2 by stimulating ubiquitination, trafficking, and finally proteasomal degradation (Seo et al., 2014). Numerous mutations in human ZnT2 (*SLC30A2*) have been identified in breastfeeding women and gene variants are associated with both low and high Zn levels in milk. Loss-of-function (LoF) variants result in pathologically low levels of Zn in milk and lead to severe Zn deficiency in exclusively breastfed infants, a disorder referred to as “transient neonatal Zn deficiency” (TNZD) (Chowanadisai et al., 2006; Itsumura et al., 2013; Lova Navarro et al., 2014; Alam et al., 2015; Golan et al., 2016; Li et al., 2020). While TNZD was once thought of as uncommon, Golan and colleagues estimated ~1:2,334 exclusively breastfed newborns may be at risk for TNZD due to the prevalence of LoF variants in ZnT2 in the human genome (Golan et al., 2019). Genetic variants in *SLC30A2* (rs1091709 and rs1373623) are also associated with low milk Zn levels in Danish Holstein cows (Buitenhuis et al., 2014). Moreover, ZnT2 function extends well beyond Zn secretion into milk. Recently, a role for ZnT2 in vesicular acidification and the development of the general secretory pathway was identified (Lee et al., 2017). As a result, ZnT2-null mice have impaired MEC polarity and defects in alveolar expansion, leading to insufficient trafficking of prolactin receptor to the cell surface and compromised milk production. In addition, TNF $\alpha$  stimulates AP3 binding and re-localization of ZnT2 to lysosomes following weaning, illustrating a key role for ZnT2 in lysosomal-mediated cell death during MG remodeling as well (Hennigar and Kelleher, 2015). *SLC30A2* was also 1 of 11 candidate genes associated with milk protein and fat traits in Holstein bulls (Jiang et al., 2016), reflecting the important role of ZnT2 in MG biology across species.

In conclusion, the MG must transfer a large quantity of Ca and Zn into milk while tightly regulating intracellular transport to maintain MG function, and numerous mechanisms remain to be understood. Although progress has been made toward understanding basic transport mechanisms, similar gaps in knowledge remain. For example, a lack of understanding regarding molecular mechanisms through which Ca and Zn are taken up by the MG still exists. Moreover, a key role for the ER/Golgi apparatus in mineral transport is consistent, suggesting a critical role in buffering intracellular stores for optimal MG function, and further studies are required to understand its role and relevance to lactation. Moreover, as divalent cations, perturbations in the transport of one may interfere with intracellular functions of the other (Rossowska and Nakamoto, 1993; Gore et al., 2004), and further studies are required to understand the molecular integration of these minerals. Finally, it is critical that a deeper understanding of role of genetics on MG function, mineral transport, and milk production is required

to inform intervention design and novel diagnostic and therapeutic strategies in production animals and humans.

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## Notes

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This study received no external funding.

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

The authors have not stated any conflicts of interest.