HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2016 November 06.

Published in final edited form as:

Biochem Biophys Res Commun. 2015 November 6; 467(1): 152–156. doi:10.1016/j.bbrc.2015.09.087.

Deletion of the Intestinal Plasma Membrane Calcium Pump, Isoform 1, *Atp2b1*, in Mice is Associated with Decreased Bone Mineral Density and Impaired Responsiveness to 1, 25-Dihydroxyvitamin D_3

Zachary C. Ryan[1], **Theodore A. Craig**[1], **Adelaida G. Filoteo**[2], **Jennifer J. Westendorf**[2,3], **Elizabeth J. Cartwright**[4], **Ludwig Neyses**[4,5], **Emanuel E. Strehler**[2], and **Rajiv Kumar**[1,2]

[1]Nephrology Research, Department of Internal Medicine

[2]Department of Biochemistry and Molecular Biology

[3]Department of Orthopedic Surgery, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

[4]Institute of Cardiovascular Sciences, University of Manchester, Manchester M13 9PT, UK

[5]University of Luxembourg, L-4365 Esch-sur-Alzette, Luxembourg

Abstract

The physiological importance of the intestinal plasma membrane calcium pump, isoform 1, (*Pmca1, Atp2b1*), in calcium absorption and homeostasis has not been previously demonstrated *in vivo*. Since global germ-line deletion of the *Pmca1* in mice is associated with embryonic lethality, we selectively deleted the *Pmca1* in intestinal absorptive cells. Mice with *loxP* sites flanking exon 2 of the *Pmca1* gene ($Pmca1^{fl/fl}$) were crossed with mice expressing Cre recombinase in the intestine under control of the *villin* promoter to give mice in which the *Pmca1* had been deleted in the intestine ($Pmca1^{EKO}$ mice). $Pmca1^{EKO}$ mice were born at a reduced frequency and were small at the time of birth when compared to wild-type (*Wt*) litter mates. At two months of age, $Pmca1^{EKO}$ mice fed a 0.81% calcium, 0.34% phosphorus, normal vitamin D diet had reduced whole body bone mineral density ($P < 0.037$), and reduced femoral bone mineral density ($P < 0.015$). There was a trend towards lower serum calcium and higher serum parathyroid hormone (PTH) and 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) concentrations in $Pmca1^{EKO}$ mice compared to *Wt* mice but the changes were not statistically significant. The urinary phosphorus/creatinine ratio was increased in $Pmca1^{EKO}$ mice ($P < 0.004$). Following the administration of 200 ng of 1α,25$(OH)_2D_3$ intraperitoneally to *Wt* mice, active intestinal calcium transport increased ~2-fold, whereas $Pmca1^{EKO}$ mice administered an equal amount of 1α,25$(OH)_2D_3$ failed to show

---

Address inquiries to: Rajiv Kumar or Emanuel Strehler, Mayo Clinic, 200 1st Street SW, Rochester, MN 55905, USA, Telephone: 507-284-0020, Fax: 507-538-9536, rkumar@mayo.edu or strehler.emanuel@mayo.edu.

Conflict of Interest: The authors have no conflicts of interest to declare.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

an increase in active calcium transport. Deletion of the *Pmca1* in the intestine is associated with reduced growth and bone mineralization, and a failure to up-regulate calcium absorption in response to 1α,25$(OH)_2D_3$.

Keywords

Plasma membrane calcium pump; *Pmca1*; *Atp2b1*; intestine; bone density; 1α,25-dihydroxyvitamin D_3; calcium transport

Introduction

Absorption of dietary calcium (Ca) by the intestine is essential for the maintenance of normal Ca homeostasis [1]. The efficiency of Ca absorption increases or decreases inversely with the amount of dietary Ca, and adaptations to changes in Ca intake are dependent upon vitamin D and its active metabolite, 1α,25$(OH)_2D_3$ [1,2]. Ca transport in the intestine occurs by transcellular and para-cellular routes [1,3,4]. Passive, para-cellular Ca transport occurs at higher Ca concentrations and increases with increasing concentrations of luminal Ca [3,4]. In a Ca-transporting cell such as the enterocyte of the duodenum, apically situated, TRPV 5/6 cation channels mediate the increase in Ca uptake from the lumen into the cell [5]; intracellular Ca binding proteins such as calbindin D_{9K} and D_{28K} facilitate the movement of Ca across the cell [4,6]; and the basal-lateral plasma membrane Ca pump (PMCA) [7,8,9] and the Na-Ca exchanger (NCX) [10] assist in the extrusion of Ca from within the cell into the ECF. The Na gradient for the activity of the NCX is maintained by the Na-K ATPase. Intestinal transcellular Ca transport is regulated by vitamin D through its active metabolite, 1α,25$(OH)_2D$, which increases the expression of TRPV 6 channels [11], the intra-cellular concentrations of calbindin D_{9K} and D_{28K} [6,12,13,14], and the expression of the PMCA pump isoform 1 [15,16].

There is a paucity of information regarding the requirement of various intestinal Ca transporter proteins in transcellular Ca transport *in vivo*. Deletions of *TrpV6* and *calbindin* D_{9K} genes are not associated with alterations in intestinal Ca transport *in vivo* in the basal state and following the administration of 1α,25$(OH)_2D_3$ [17,18]. One report suggests that basal Ca transport on an adequate Ca diet is normal in *TrpV6* knockout mice but adaptations to a low Ca diet are impaired [19]. The role of PMCA 1, the major Ca pump in the intestine, has been difficult to establish *in vivo* because appropriate animal models with tissue-specific deletions have not been available. Germ line deletion of the predominant *Pmca1* gene that is expressed in the intestine and kidney, is associated with early embryonic death, thus making it impossible to investigate the role of this protein *in vivo* [20]. *Liu et al. previously demonstrated a possible role for the enterocyte Pmca1b in Ca homeostasis [21]. These authors showed that murine knockout of enterocyte protein 4.1R which associates with PMCA1b and reduces its expression, results in significantly impaired small intestinal calcium absorption and secondary hyperparathyroidism. However, experiments in which the Pmca1 has been deleted in the enterocyte have not been performed.*

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

We now report the intestine-specific deletion of the *Pmca1* gene in mice. We show that these mice are viable and have a phenotype suggesting altered bone and mineral homeostasis.

Methods and Materials

Animal care

All protocols were approved by the University of Manchester and Mayo Clinic institutional animal care committees. Mice were fed a 0.81% Ca, 0.34 percent phosphorus (non-phytate) diet containing 2.3 I.U. of vitamin D_3 per gram (PicoLab Rodent Diet 20).

Generation of floxed Pmca1 mice

The targeting strategy to create mice with a floxed *Pmca1* gene is shown in Fig. 1. The *Pmca1* gene on mouse chromosome 10 contains 21 exons spread over more than 100 kb of DNA. Exon 2 contains the ATG initiation codon and was therefore targeted for insertion of the flanking *LoxP* sites: deletion of this exon prevents the production of a functional Pmca1 protein because of the absence of an appropriate downstream initiation codon in the proper reading frame.

Generation of mice with small intestine-specific knockout of Pmca1

We used *villin-Cre* transgenic mice [22] from Jackson Labs (B6.SJL-Tg(Vil-cre)997Gum/J) to obtain mice with intestinal enterocyte-specific deletion of the *Pmca1* gene (abbreviated as $Pmca1^{EKO}$ mice). The *villin* gene promoter drives *Cre* recombinase expression in the small intestine starting in late embryogenesis and throughout adulthood [22] and has been successfully used to knock out specific genes in the absorptive epithelium of the duodenum, jejunum and ileum [23,24]. Of note, the 12.4-kilobase region of the mouse villin gene promoter used in these mice has been shown to drive high level expression of two different reporter genes (LacZ and Cre recombinase) within the entire intestinal epithelium, whereas no expression is noted in the embryonic kidney [22]. We crossed *villin-Cre* transgenic male mice with female $Pmca1^{fl/fl}$ mice to generate *villin*-Cre-$Pmca1^{+/fl}$*(+/-)* offspring, which were then back-crossed to $Pmca1^{fl/fl}$ mice to create the desired *villin*-Cre-$Pmca1^{\mathrm{fl/fl}}$ (-/-) genotype (henceforth abbreviated as $Pmca1^{EKO}$ mice) (Fig. 2A, boxed). The following primers were used for genotyping: Primers 1f and 1r (Fig. 1) for WT *Pmca1* (754 bp) and $Pmca1^{fl/fl}$ (898 bp): 1f – 5′-CTG GCC TCA CCT AGT TTG CTA AAC C-3′; 1r – 5′-CTG TGG AGT ACA TGC TTC GTT CTG C-3′. Primers for vil-cre (1100 bp): Forward - 5′-GTG TGG GAC AGA GAA CAA ACC-3′; Reverse - 5′-ACA TCT TCA GGT TCT GCG GG-3′.

Primers 2f and 2r (Fig. 1) for *Pmca1* in DNA extracts of the intestinal scrapings (WT allele 3.4 kb, KO allele 1.2 kb): 2f – 5′-AAT GCT CTC TGA GCG TAT GGT CTG G-3′; 2r – 5′-CCA GAG ACC ATT CAT GGC TTC TAC C-3′.

Confirmation of deletion of Pmca1 from intestinal mucosal cells

Enterocytes from the duodenum of wild-type or knockout mice were isolated by treatment of 8 cm duodenal segments with Type 1A collagenase (100 active U/mL) in oxygenated

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

enterocyte isolation buffer (125 mM NaCl, 10 mM D-Fructose, 0.15 mM $CaCl_2$, 30 mM Tris-HCl (pH 7.4)) at 37 °C for 15 minutes with shaking. At 15-minute intervals, duodenal segments were placed in fresh enterocyte isolation buffer, for a total of four cycles. Detached cells were centrifuged, washed in buffer, and RNA was isolated using RNA spin columns (Clontech). *Pmca1* and *Pmca4* mRNA was identified by RT-PCR using the following primers: *Pmca1*: 5′ primer: 5′- CGGAAAATACAGGAGAGCTATGG-3; 3′ primer: 5′-CTTTCCAAACACTGCTTCTCTTC-3′ (123 bp PCR product); *Pmca4*: 5′ primer: 5′-TGTGGGCAGTGAGTGAGAAT-3′; 3′ primer: 5′-TCAGGTTGGGTCATGAAGGT-3′ (110 bp PCR product). Primers for *Rpl13a* reference gene: 5′ primer: 5′-CCCTCCACCCTATGACAAGA-3′; 3′ primer: 5′-GCCCCAGGTAAGCAAACTT-3′.

Measurement of serum and urinary Ca and P, 1α,25(OH)$_2$D, PTH, and FGF-23

These were measured using methods described previously [25].

Measurement of bone mineral density

DEXA was performed as described previously [25,26].

Assessment of calcium transport in response to the administration of 1α,25(OH)$_2$D$_3$

Intestinal Ca transport was assessed by the everted gut sac method of Martin and DeLuca following the administration of 50 ng 1α,25(OH)$_2$D$_3$ in 50 μL ethanol intra-peritoneally 16 h prior to measurement [27].

Results

Pmca1^{EKO} mice are viable and breed normally. We confirmed deletion of exon 2 in intestinal epithelium using two independent strategies shown in Fig. 2B and C. **Panel 2B** shows results obtained using primers 1f and 1r (Fig. 1). Since the binding site for primer 1r lies within the *LoxP* sites, and is predicted to be deleted as a result of Cre-recombinase activity in the intestine, no DNA product should be generated. This is what is observed in homozygous *Pmca1*$^{\mathrm{EKO}}$ mice; heterozygotes display the appropriate sized 754 bp band. A second approach was used to confirm these results. Primer pair 2f/2r (Fig. 1) was used for PCR on genomic DNA isolated from the small intestine of a *Pmca1*$^{\mathrm{EKO}}$ and a wild type *Pmca1*$^{\mathrm{fl/fl}}$ mouse. Removal of the floxed exon 2 region results in a smaller 1.3 kb fragment in the *Pmca1*$^{\mathrm{EKO}}$ tissue as a result of deletion of exon 2, whereas the expected fragment in the wild type *Pmca1*$^{\mathrm{fl/fl}}$ is 3.4 kb as indicated. We confirmed the lack of expression of the *Pmca1* mRNA in intestinal scrapings by performing RT-PCR with appropriate primers. In *Pmca1^{EKO}* mice, *Pmca1* mRNA expression is absent in enterocytes, whereas it is readily detected in *Pmca1*$^{\mathrm{fl/fl}}$ mice (Fig. 2D). These data show that we have generated mice in which the *Pmca1* gene has been disabled in enterocytes.

Pmca1^{EKO} mice are born at a frequency lower than the predicted 25% Mendelian frequency. The mice are smaller than wild-type or *Pmca1$^{fl/fl}$* mice at birth and into adulthood (Fig. 3). The results of measurements of serum and urinary minerals, serum calciotropic hormones and FGF-23, and bone mineral density are shown in Table 1. Whole body bone mineral

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

density (BMD) and femoral BMD were decreased in $Pmca1^{EKO}$ mice compared to control mice. Spinal BMD tended to be lower but the results were not statistically significant. Serum calcium, phosphorus, parathyroid hormone, FGF-23, and $1\alpha,25(OH)_2D$ was similar in knockout and wild-type mice. Urinary calcium and phosphorus tended to be lower in $Pmca1^{EKO}$ mice compared to wild-type mice (P = 0.15 calcium and P = 0.08 for phosphorus). Following the administration of $1\alpha,25(OH)_2D_3$ intestinal calcium transport increased in wild-type mice but failed to do so in $Pmca1^{EKO}$ knockout mice.

Discussion

The plasma membrane calcium pump is a widely distributed protein that plays an important role in cellular calcium homeostasis [7,28]. Four genes (*Pmca1-4/Atp2b1-4* in mice) encode mammalian PMCA isoforms [29,30]. The mouse *Pmca1* is ubiquitously expressed starting early in embryo development (day 9.5 *post coitum*), although its levels vary in different tissues and cell types [29,31]. By contrast, the other PMCA isoforms appear later in development and show a more restricted expression pattern [31,32]. PMCA2 and PMCA3 are most abundant in muscle and brain; PMCA2 is also highly expressed in secretory epithelial tissues such as lactating mammary glands [33]. Expression of PMCA4 is widespread, and recent evidence suggests that its major role may be in localized Ca signaling rather than in bulk Ca extrusion [34,35].

There is limited information concerning the physiological role of the various plasma membrane calcium pumps *in vivo*. Germ line deletion of *Pmca1* in mice results in embryonic lethality [20,36]. Mice that are null for *Pmca2* appear normal at birth [36,37,38,39,40,41]. By 10 days of age they have difficulty in maintaining their balance. Deafness becomes apparent, and it has been suggested that this isoforms of the PMCA maintains extracellular calcium concentrations around the hair bundles of the outer hair cells. Milk calcium excretion is also reduced in these mice. No data are available concerning the deletion of the *Pmca3* in mice. Deletion of the *Pmca4* gene results in male infertility possibly as a result of calcium overload and apoptotic cell death [20,36,42].

Given embryonic lethality [20,36] observed in mice with germ line deletion of *Pmca1*, we attempted to delete the *Pmca1* in the intestine, a tissue in which we have previously shown PMCA1 to be present along the baso-lateral membranes of enterocytes of the proximal intestine [9]. In addition, *Pmca1* is upregulated in the intestine in response to treatment with $1\alpha,25(OH)_2D_3$, suggesting an important role of this pump in active calcium transport [15].

We now demonstrate that we have successfully deleted the *Pmca1* in the intestine using Cre-lox methods. $Pmca1^{EKO}$ mice are viable but are born at a reduced frequency. The precise reason for this is uncertain at the present time but suggests the presence of embryonic lethality, perhaps due to "leaky" villin-cre expression in very early embryogenesis. Additionally, we observe that $Pmca1^{EKO}$ mice are smaller than their wild-type littermates, once again suggesting an important role for the *Pmca1* in fetal development. Further experiments will be necessary to understand the role of this important pump during embryonic development.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Pmca1^{EKO} mice have a reduced bone mineral density compared to wild-type littermates suggesting a failure of absorption of Ca from the intestine resulting in reduced bone mineral deposition. Importantly, we found a lack of response of the intestine in *Pmca1*^{EKO} mice to exogenously administered 1α,25$(OH)_2D_3$. These findings are supported by a tendency towards lower urinary calcium concentrations in *Pmca1*^{EKO} mice. *The mean serum Ca concentration of Pmca1*^{EKO} *mice was lower than in Pmca1*$^{fl/fl}$ *(2.07 vs. 2.14 mM) although differences were not statistically significant. In accordance with the lower serum calcium, PTH, and 1α,25*$(OH)_2D$ *concentrations* tended to be higher in *Pmca1*^{EKO} mice compared to wild-type mice. *The urinary phosphorus concentration normalized for creatinine was higher in Pmca1*^{EKO} *mice compared with Pmca1*$^{fl/fl}$ *mice (P<0.0004). Collectively, these findings are consistent with mild secondary hyperparathyroidism which may have contributed to the reduced BMD in addition to the intestinal calcium malabsorption manifest as a failure to respond to exogenous 1α,25*$(OH)_2D_3$*. The data thus underline the importance of Pmca1 as the major Ca extrusion system in 1α,25*$(OH)_2D_3$*-responsive transcellular Ca absorption*. Similar concentrations of serum minerals and regulatory hormones in *Pmca1*^{EKO} mice are not surprising since the animals were fed a normal calcium diet. When dietary calcium is normal or high, a significant proportion of calcium absorption in the intestine occurs by passive para-cellular mechanisms, and hence would not be expected to change as a result of deletion of the *Pmca1*.

Our findings are consistent with those of Liu et al, who found that ablation of enterocyte 4.1R protein, a basolateral enterocyte protein which binds to and reduces *Pmca1b* expression, reduced intestinal calcium transport and BMD and caused secondary hyperparathyroidism [21]. Of note, in 4.1R$^{-/-}$ mice, 4.1R protein expression was additionally absent in the brain, kidney proximal tubules and erythrocytes resulting in neurobehavioral deficits and hemolytic anemia [43,44]. Our findings are consistent with a role of the *Pmca1* in intestinal calcium transport and overall calcium homeostasis. Future studies will investigate the importance of the intestinal Pmca1 in calcium homeostasis in conditions of dietary calcium restriction and during aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported in part by NIH grant DK066013.

References

1. Tebben, PJ.; Kumar, R. The Hormonal Regulation of Calcium Metabolism. In: Alpern, RJ.; Moe, OW.; Caplan, M., editors. Seldin and Giebisch's The Kidney, Physiology and Pathophysiology. Academic Press; New York: 2013. p. 2273-2330.
2. DeLuca HF. Overview of general physiologic features and functions of vitamin D. Am J Clin Nutr. 2004; 80:1689S–1696S. [PubMed: 15585789]
3. Wasserman RH, Corradino RA, Fullmer CS, Taylor AN. Some aspects of vitamin D action; calcium absorption and the vitamin D-dependent calcium-binding protein. Vitamins & Hormones. 1974; 32:299–324. [PubMed: 4376296]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

4. Wasserman RH, Fullmer CS. Vitamin D and intestinal calcium transport: facts, speculations and hypotheses. J Nutr. 1995; 125:1971S–1979S. [PubMed: 7602379]
5. Dimke H, Hoenderop JG, Bindels RJ. Molecular basis of epithelial Ca2+ and Mg2+ transport: insights from the TRP channel family. The Journal of physiology. 2011; 589:1535–1542. [PubMed: 21041532]
6. Wasserman RH, Smith CA, Brindak ME, De Talamoni N, Fullmer CS, Penniston JT, Kumar R. Vitamin D and mineral deficiencies increase the plasma membrane calcium pump of chicken intestine. Gastroenterology. 1992; 102:886–894. [PubMed: 1311274]
7. Brini M, Cali T, Ottolini D, Carafoli E. The plasma membrane calcium pump in health and disease. The FEBS journal. 2013
8. Brini M, Carafoli E. Calcium pumps in health and disease. Physiological reviews. 2009; 89:1341–1378. [PubMed: 19789383]
9. Borke JL, Caride A, Verma AK, Penniston JT, Kumar R. Cellular and segmental distribution of Ca2(+)-pump epitopes in rat intestine. Pflugers Archiv : European journal of physiology. 1990; 417:120–122. [PubMed: 1705698]
10. Philipson KD, Nicoll DA. Sodium-calcium exchange: a molecular perspective. Annual review of physiology. 2000; 62:111–133.
11. Meyer MB, Zella LA, Nerenz RD, Pike JW. Characterizing early events associated with the activation of target genes by 1,25-dihydroxyvitamin D3 in mouse kidney and intestine in vivo. J Biol Chem. 2007; 282:22344–22352. [PubMed: 17556365]
12. Taylor AN, Wasserman RH. Vitamin D-induced calcium-binding protein: comparative aspects in kidney and intestine. The American journal of physiology. 1972; 223:110–114. [PubMed: 5039053]
13. Wasserman RH, Brindak ME, Meyer SA, Fullmer CS. Evidence for multiple effects of vitamin D3 on calcium absorption: response of rachitic chicks, with or without partial vitamin D3 repletion, to 1,25-dihydroxyvitamin D3. Proceedings of the National Academy of Sciences of the United States of America. 1982; 79:7939–7943. [PubMed: 6961461]
14. Wasserman RH, Taylor AN. Vitamin d3-induced calcium-binding protein in chick intestinal mucosa. Science. 1966; 152:791–793. [PubMed: 17797460]
15. Cai Q, Chandler JS, Wasserman RH, Kumar R, Penniston JT. Vitamin D and adaptation to dietary calcium and phosphate deficiencies increase intestinal plasma membrane calcium pump gene expression. Proceedings of the National Academy of Sciences of the United States of America. 1993; 90:1345–1349. [PubMed: 7679502]
16. Lee SM, Riley EM, Meyer MB, Benkusky NA, Plum LA, DeLuca HF, Pike JW. 1,25-Dihydroxyvitamin D3 Controls a Cohort of Vitamin D Receptor Target Genes in the Proximal Intestine That Is Enriched for Calcium-regulating Components. The Journal of biological chemistry. 2015; 290:18199–18215. [PubMed: 26041780]
17. Benn BS, Ajibade D, Porta A, Dhawan P, Hediger M, Peng JB, Jiang Y, Oh GT, Jeung EB, Lieben L, Bouillon R, Carmeliet G, Christakos S. Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. Endocrinology. 2008; 149:3196–3205. [PubMed: 18325990]
18. Kutuzova GD, Sundersingh F, Vaughan J, Tadi BP, Ansay SE, Christakos S, Deluca HF. TRPV6 is not required for 1alpha,25-dihydroxyvitamin D3-induced intestinal calcium absorption in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105:19655–19659. [PubMed: 19073913]
19. Lieben L, Benn BS, Ajibade D, Stockmans I, Moermans K, Hediger MA, Peng JB, Christakos S, Bouillon R, Carmeliet G. Trpv6 mediates intestinal calcium absorption during calcium restriction and contributes to bone homeostasis. Bone. 2010; 47:301–308. [PubMed: 20399919]
20. Okunade GW, Miller ML, Pyne GJ, Sutliff RL, O'Connor KT, Neumann JC, Andringa A, Miller DA, Prasad V, Doetschman T, Paul RJ, Shull GE. Targeted ablation of plasma membrane Ca2+-ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a critical role in hyperactivated sperm motility and male fertility for PMCA4. The Journal of biological chemistry. 2004; 279:33742–33750. [PubMed: 15178683]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

21. Liu C, Weng H, Chen L, Yang S, Wang H, Debnath G, Guo X, Wu L, Mohandas N, An X. Impaired intestinal calcium absorption in protein 4.1R-deficient mice due to altered expression of plasma membrane calcium ATPase 1b (PMCA1b). The Journal of biological chemistry. 2013; 288:11407–11415. [PubMed: 23460639]
22. Madison BB, Dunbar L, Qiao XT, Braunstein K, Braunstein E, Gumucio DL. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. The Journal of biological chemistry. 2002; 277:33275–33283. [PubMed: 12065599]
23. Jones RG, Li X, Gray PD, Kuang J, Clayton F, Samowitz WS, Madison BB, Gumucio DL, Kuwada SK. Conditional deletion of beta1 integrins in the intestinal epithelium causes a loss of Hedgehog expression, intestinal hyperplasia, and early postnatal lethality. The Journal of cell biology. 2006; 175:505–514. [PubMed: 17088430]
24. Battle MA, Bondow BJ, Iverson MA, Adams SJ, Jandacek RJ, Tso P, Duncan SA. GATA4 is essential for jejunal function in mice. Gastroenterology. 2008; 135:1676–1686 e1671. [PubMed: 18812176]
25. Ryan ZC, Ketha H, McNulty MS, McGee-Lawrence M, Craig TA, Grande JP, Westendorf JJ, Singh RJ, Kumar R. Sclerostin alters serum vitamin D metabolite and fibroblast growth factor 23 concentrations and the urinary excretion of calcium. Proc Natl Acad Sci U S A. 2013; 110:6199–6204. [PubMed: 23530237]
26. McGee-Lawrence ME, Bradley EW, Dudakovic A, Carlson SW, Ryan ZC, Kumar R, Dadsetan M, Yaszemski MJ, Chen Q, An KN, Westendorf JJ. Histone deacetylase 3 is required for maintenance of bone mass during aging. Bone. 2013; 52:296–307. [PubMed: 23085085]
27. Martin DL, DeLuca HF. Influence of sodium on calcium transport by the rat small intestine. The American journal of physiology. 1969; 216:1351–1359. [PubMed: 5786721]
28. Lopreiato R, Giacomello M, Carafoli E. The plasma membrane calcium pump: new ways to look at an old enzyme. The Journal of biological chemistry. 2014; 289:10261–10268. [PubMed: 24570005]
29. Strehler EE, Zacharias DA. Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. Physiological reviews. 2001; 81:21–50. [PubMed: 11152753]
30. Strehler EE, Caride AJ, Filoteo AG, Xiong Y, Penniston JT, Enyedi A. Plasma membrane Ca2+ ATPases as dynamic regulators of cellular calcium handling. Annals of the New York Academy of Sciences. 2007; 1099:226–236. [PubMed: 17446463]
31. Zacharias DA, Kappen C. Developmental expression of the four plasma membrane calcium ATPase (Pmca) genes in the mouse. Biochimica et biophysica acta. 1999; 1428:397–405. [PubMed: 10434059]
32. Strehler EE, Treiman M. Calcium pumps of plasma membrane and cell interior. Current molecular medicine. 2004; 4:323–335. [PubMed: 15101689]
33. Reinhardt TA, Filoteo AG, Penniston JT, Horst RL. Ca(2+)-ATPase protein expression in mammary tissue. American journal of physiology Cell physiology. 2000; 279:C1595–1602. [PubMed: 11029307]
34. Strehler EE, Filoteo AG, Penniston JT, Caride AJ. Plasma-membrane Ca(2+) pumps: structural diversity as the basis for functional versatility. Biochemical Society transactions. 2007; 35:919–922. [PubMed: 17956246]
35. Oceandy D, Mohamed TM, Cartwright EJ, Neyses L. Local signals with global impacts and clinical implications: lessons from the plasma membrane calcium pump (PMCA4). Biochimica et biophysica acta. 2011; 1813:974–978. [PubMed: 21167220]
36. Prasad V, Okunade GW, Miller ML, Shull GE. Phenotypes of SERCA and PMCA knockout mice. Biochemical and biophysical research communications. 2004; 322:1192–1203. [PubMed: 15336967]
37. Kozel PJ, Friedman RA, Erway LC, Yamoah EN, Liu LH, Riddle T, Duffy JJ, Doetschman T, Miller ML, Cardell EL, Shull GE. Balance and hearing deficits in mice with a null mutation in the gene encoding plasma membrane Ca2+-ATPase isoform 2. The Journal of biological chemistry. 1998; 273:18693–18696. [PubMed: 9668038]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

38. Ficarella R, Di Leva F, Bortolozzi M, Ortolano S, Donaudy F, Petrillo M, Melchionda S, Lelli A, Domi T, Fedrizzi L, Lim D, Shull GE, Gasparini P, Brini M, Mammano F, Carafoli E. A functional study of plasma-membrane calcium-pump isoform 2 mutants causing digenic deafness. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:1516–1521. [PubMed: 17234811]
39. Takahashi K, Kitamura K. A point mutation in a plasma membrane Ca(2+)-ATPase gene causes deafness in Wriggle Mouse Sagami. Biochemical and biophysical research communications. 1999; 261:773–778. [PubMed: 10441500]
40. Street VA, McKee-Johnson JW, Fonseca RC, Tempel BL, Noben-Trauth K. Mutations in a plasma membrane Ca2+-ATPase gene cause deafness in deafwaddler mice. Nature genetics. 1998; 19:390–394. [PubMed: 9697703]
41. Reinhardt TA, Lippolis JD, Shull GE, Horst RL. Null mutation in the gene encoding plasma membrane Ca2+-ATPase isoform 2 impairs calcium transport into milk. The Journal of biological chemistry. 2004; 279:42369–42373. [PubMed: 15302868]
42. Schuh K, Cartwright EJ, Jankevics E, Bundschu K, Liebermann J, Williams JC, Armesilla AL, Emerson M, Oceandy D, Knobeloch KP, Neyses L. Plasma membrane Ca2+ ATPase 4 is required for sperm motility and male fertility. The Journal of biological chemistry. 2004; 279:28220–28226. [PubMed: 15078889]
43. Shi ZT, Afzal V, Coller B, Patel D, Chasis JA, Parra M, Lee G, Paszty C, Stevens M, Walensky L, Peters LL, Mohandas N, Rubin E, Conboy JG. Protein 4.1R-deficient mice are viable but have erythroid membrane skeleton abnormalities. The Journal of clinical investigation. 1999; 103:331–340. [PubMed: 9927493]
44. Walensky LD, Shi ZT, Blackshaw S, DeVries AC, Demas GE, Gascard P, Nelson RJ, Conboy JG, Rubin EM, Snyder SH, Mohandas N. Neurobehavioral deficits in mice lacking the erythrocyte membrane cytoskeletal protein 4.1. Current biology : CB. 1998; 8:1269–1272. [PubMed: 9822582]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

- The *Pmca1* was deleted in the intestine to examine its role in Ca homeostasis *in vivo*
- Mice with intestinal *Pmca1* deletion are born with reduced frequency and are stunted at birth
- At two months of age mice with intestinal *Pmca1* deletion have reduced bone mineral density
- $1\alpha,25(OH)_2D_3$-induced Ca transport is impaired in mice with intestinal *Pmca1* deletion

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

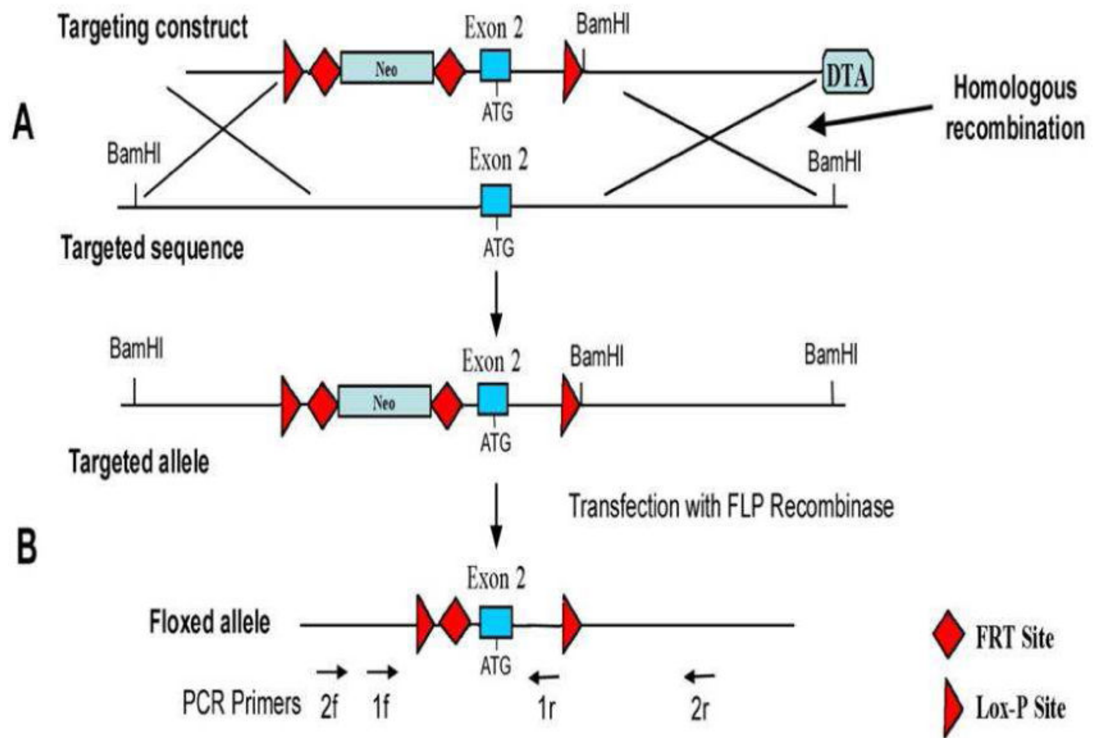

Figure 1.

Generation of Pmca1$^{fl/fl}$ mice. The targeting construct consists of two *LoxP* sites flanking exon 2 of the *Pmca1 (Atp2b1)* gene, a short 5′ arm homology of 1.7 kb, a neomycin resistance gene (*Neo*) flanked by FRT sites, a long (5.7 kb) 3′ homology arm, a diphtheria toxin A (DTA) expression cassette and an exogenous *Bam*HI restriction site. The construct underwent homologous recombination in mouse embryonic stem (ES) cells to produce the targeted allele (A). Targeted ES cells were transfected with FLP recombinase to remove the neo cassette and generate the floxed Pmca1 allele (B). Targeted ES cells were injected into C57Bl/6 blastocysts and resultant chimeric mice were mated to test for germ line transmission of the floxed allele. Heterozygotes were crossed to give homozygous Pmca1$^{fl/fl}$ mice. The location of PCR primers for genotyping (1f, 1r) and analysis of deletion of the floxed exon 2 (2f, 2r) are shown on the bottom.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

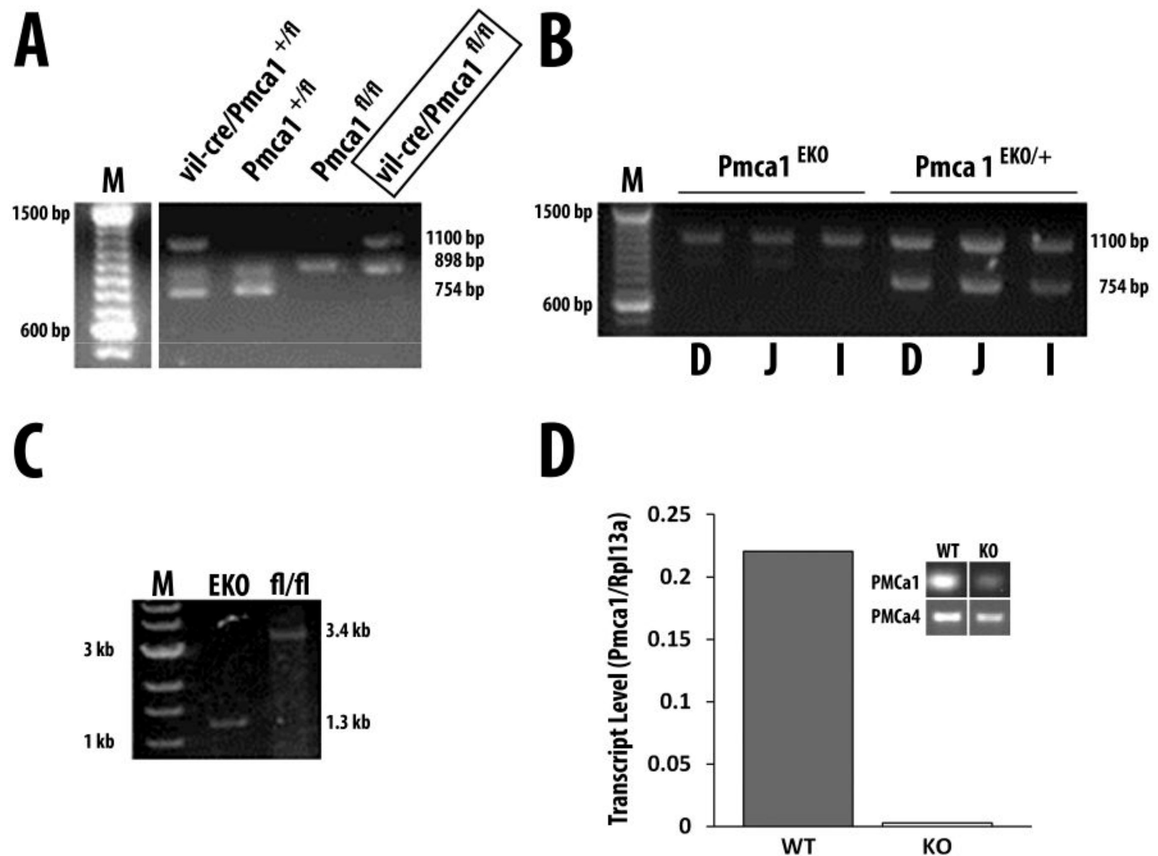

Figure 2.

Genotyping of *Pmca1*^{EKO} mice and confirmation of *Atp2b1* exon 2 deletion in the small intestine. **A.** Genotyping of offspring of a *vil-cre/Pmca1*$^{+/fl}$ × *Pmca1*$^{fl/fl}$ pairing reveals *vil-cre/Pmca1*$^{+/fl}$, *Pmca1*$^{+/fl}$, *Pmca1*$^{fl/fl}$, and *vil-cre/Pmca1*$^{fl/fl}$ individuals as indicated by the presence of PCR fragments of 754 bp for the wild-type *Atp2b1* allele, 898 bp for the floxed *Atp2b1* allele, and 1100 bp in *villin-Cre* hemizygous mice. The single *vil-cre/Pmca1*$^{fl/fl}$ (= *Pmca1*^{EKO}) genotype is boxed. M, 100 bp DNA ladder. **B** and **C.** PCR confirming enterocyte-specific deletion of the *Pmca1* exon 2 in *Pmca1*^{EKO} mice. In **B**, primer pair 1f/1r (Fig. 1) was used to amplify genomic DNA from mucosal cell scrapings of the duodenum (D), jejunum (J) and ileum (I) of a *Pmca1*^{EKO} and a heterozygote *Pmca1*^{EKO}/+ mouse as indicated. The presence of the villin-cre gene was confirmed by separate primers resulting in the expected 1100 bp band. Note the absence of a *Pmca1*-specific band in the *Pmca1*^{EKO} tissues, while the expected 754 bp *Atp2b1* band is observed in the heterozygote. M, DNA size marker ladder. In **C**, primer pair 2f/2r (Fig. 1) was used for PCR on genomic DNA isolated from the small intestine of a *Pmca1*^{EKO} and a control *Pmca1*$^{fl/fl}$ mouse. Removal of the floxed exon 2 region results in a 1.3 kb fragment in the *Pmca1*^{EKO} tissue, whereas the expected fragment in the *Pmca1*$^{fl/fl}$ control is 3.4 kb as indicated. M, DNA size marker lane. **D**, *Pmca1* mRNA expression was assessed by quantitative RT-PCR in the intestinal epithelium isolated by collagenase treatment of duodenal loops of *Pmca1*^{EKO} mice. Inset shows agarose gel of *Pmca1* and *Pmca4* from *Pmca1*^{EKO} (KO) and *Pmca1*$^{fl/fl}$ (WT)

epithelium isolated by sequential collagenase treatment of everted gut sacs and assessed by semi-quantitative RT-PCR (residual amount of *Pmca1* is derived from non-epithelial cells).

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Figure 3.
Pmca1^{EKO} mice are stunted. Five week-old littermates of three *Pmca1*^{EKO} animals and a *Pmca1*$^{fl/lf}$ control. Note the reduced size of the *Pmca1*^{EKO} animals.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1

Bone mineral density, biochemical parameters and intestinal calcium transport in everted gut sacs of wild-type (*Pmca1*$^{fl/fl}$) and knockout (*Pmca1*^{EKO}) mice.

	WT (*Pmca1*$^{fl/fl}$)			KO (*Pmca1*^{EKO})			
	Mean	**SE**	**n**	**Mean**	**SE**	**n**	**P**
BMD Body (g/cm^2)	0.051	0.001	13	0.046	0.002	10	0.037
BMD Spine (g/cm^2)	0.049	0.001	13	0.046	0.002	10	0.21
BMD Femur (g/cm^2)	0.059	0.002	13	0.051	0.002	10	0.015
PTH (pg/mL)	120.4	26.3	7	135.2	12.1	7	0.62
FGF-23 (pg/mL)	103.6	17.6	8	131.8	15.4	8	0.25
$1\alpha,25(OH)_2D$ (pg/mL)	30.96	5.5	8	38.4	5.9	9	0.38
Serum Ca (mM)	2.14	0.14	7	2.07	0.1	9	0.69
Serum phosphorus (P) (mM)	2.8	0.1	8	2.9	0.1	9	0.46
Urine volume (μL/24 h)	788	129	8	1267	196	9	0.07
Urine Ca (mg/24 h)	0.026	0.003	8	0.040	0.01	8	0.15
Urine P (mg/24 h)	1.498	0.326	8	2.211	0.217	9	0.08
Urine Ca/creatinine	0.605	0.091	8	0.720	0.147	8	0.52
Urine P/creatinine	37.256	2.818	8	53.282	2.213	9	0.0004
Intestinal ^{45}Ca Transport (Inside/Outside)	1.97	0.06	2	1.08	0.01	2	0.005