Activation of the calcium sensing receptor attenuates TRPV6-dependent intestinal calcium absorption

by

Justin Joo Hyung Lee

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Department of Physiology
University of Alberta

© Justin Joo Hyung Lee, 2019

ABSTRACT

Plasma calcium (Ca^{2+}) is maintained by amending the release of parathyroid hormone and through direct effects of the Ca^{2+} sensing receptor (CaSR) in the renal tubule. Combined, these mechanisms alter intestinal Ca^{2+} absorption by modulating 1,25-dihydroxy vitamin D_3 production, bone resorption, and renal Ca^{2+} excretion. The CaSR is a therapeutic target in the treatment of secondary hyperparathyroidism and hypocalcaemia is a common complication seen with calcimimetic therapy. The CaSR is also expressed in intestinal epithelium, however, its role in regulating Ca^{2+} absorption is unknown. Chronic CaSR activation decreased expression of genes involved in Ca^{2+} absorption. In Ussing chambers, increasing extracellular Ca^{2+} or basolateral application of the calcimimetic cinacalcet decreased net Ca^{2+} absorption across intestinal preparations acutely. Conversely, Ca^{2+} absorption increased with decreasing extracellular Ca^{2+} concentration. These responses were absent in mice expressing a non-functional TRPV6, $TRPV6^{D541A}$. Cinacalcet also attenuated Ca^{2+} fluxes through TRPV6 in X*enopus* oocytes when co-expressed with the CaSR. Moreover, the phospholipase C inhibitor, U73122, prevented cinacalcet-mediated inhibition of Ca^{2+} flux. These results reveal a regulatory pathway whereby activation of the CaSR in the basolateral membrane of the bowel attenuates Ca^{2+} absorption via TRPV6 to prevent hypercalcaemia and help to explain how hypocalcaemia is induced by calcimimetics.

PREFACE

This thesis is modified from our published work "Lee, J. J., Liu, X., O'Neil, D., et al. (2019). Activation of the calcium sensing receptor attenuates TRPV6-dependent intestinal calcium absorption. *JCI Insight, 5*. doi:10.1172/jci.insight.128013".

This work could not have been completed without contribution from various individuals. The author, Dr. Henrik Dimke, and Dr. Todd Alexander conceived of and designed the research study. The author, Xiong Liu, Debbie O'Neill, Dr. Henrik Dimke, and Dr. Todd Alexander performed experiments (please, see Chapter 3 for details). The author, Xiong Liu, and Dr. Todd Alexander analyzed data. The author, Xiong Liu, Megan Beggs, Dr. Henrik Dimke, and Dr. Todd Alexander interpreted results of experiments. The author and Dr. Todd Alexander prepared figures and drafted the manuscript. All listed individuals edited, revised, and approved the final version of the manuscript. The author prepared and modified this thesis from the published manuscript.

ACKNOWLEDGEMENTS

I first and foremost thank all the present and past Alexander laboratory family: Dr. Todd Alexander, Megan Beggs, Wanling Pan, Debbie O'Neill, Señor Plain, Matthew Saurette, Saba Rehman, Kathy Tang, Shane Wiebe, and Juraj Rievaj. Special thank you to my supervisor Dr. Alexander for taking a chance on a naïve and eager undergraduate student. Your passion for science and your ability to bring out the best in your students is truly an inspiration. Also, I owe my sincerest gratitude to my mentor Megan Beggs, who paved the way and suffered through months of troubleshooting and optimization so that I could begin my project with ease. Any success I have had would not have been achieved without you and the rest of the Alexander lab family.

I would like extend my gratitude to the Faculty of Medicine & Dentistry, Natural Sciences and Engineering Research Council, Women's & Children's Health Research Institute and the Stollery Children's Hospital Foundation for providing financial support. I would also like to acknowledge Dr. Emmanuelle Cordat and laboratory members, as well as my committee members Dr. Justine Turner and Dr. Xing Zhen Chen for your guidance and feedback. Special thank you to the Department of Physiology staff and Dr. Jamie Mitchell for your support throughout my degree. In addition, I thank the anonymous reviewers and editors who have made me a more resilient person.

Lastly, I thank my friends and family for your love and support throughout my academics and endeavours. I would not be where I am without you all. Thank you.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION ... **1**
1.1 Calcium Homeostasis ... **2**
1.2 Regulation of Ca^{2+} Homeostasis: Endocrine Hormones ... **3**
1.2.1 Parathyroid Hormone (PTH) ... 4
1.2.2 Calcitonin ... 5
1.2.3 1, 25-dihydroxyvitamin D_3 (1,25[OH]$_2D_3$) ... 6
1.2.4 Fibroblast growth factor 23 (FGF23) ... 7
1.3 Regulation of Ca^{2+} Homeostasis: CaSR ... **8**
1.3.1 CaSR in the parathyroid gland ... 8
1.3.2 CaSR in the kidneys ... 10
1.3.3 CaSR in the bone ... 11
1.3.4 CaSR in the intestine ... 13
1.4 Intestinal Ca^{2+} Transport ... **13**
1.4.1 Paracellular Ca^{2+} Transport pathway ... 15
1.4.2 Transcellular Ca^{2+} transport pathway ... 16
1.5 Overall Rationale ... **19**
1.6 Hypothesis ... **19**

CHAPTER 2: MATERIALS AND METHODS ... **21**
2.1 Ethical approval and animals ... **22**
2.1.1 Intestinal segment isolation ... 22
2.1.2 Pig colon samples ... 23
2.2 Real-time quantitative PCR ... **23**
2.3 Immunoblotting ... **23**
2.4 Ussing chamber experiments ... **24**
2.5 Xenopus oocyte expression and two-electrode voltage clamp ... **26**
2.6 Statistics ... **27**

CHAPTER 3: RESULTS ... **28**
3.1 Changes in mRNA expression of transcellular Ca^{2+} absorption transporters in response to altered Ca^{2+} homeostasis ... **29**
3.1.1 Altered Ca^{2+} diets ... 29
3.1.2 Changes in the low Ca^{2+} diet is mediated by changes in the 1,25[OH]$_2D_3$ levels ... 29
3.1.3 Chronic cinacalcet treatment revealed an additional mechanism by which high Ca^{2+} might directly regulate Ca^{2+} absorption independent of 1,25[OH]$_2D_3$ ... 31
3.2 Extracellular Ca^{2+} inhibits transcellular Ca^{2+} absorption in the proximal colon ... **32**
3.3 Delineating the mechanism by which intestinal CaSR inhibits Ca^{2+} absorption ... **35**
3.3.1 Increased basolateral extracellular Ca^{2+} attenuates transcellular Ca^{2+} absorption ... 35
3.3.2 TRPV6 is necessary for altered transcellular Ca^{2+} absorption across the proximal large bowel ... 38
3.3.3 CaSR expression is sufficient for TRPV6 to respond to extracellular Ca^{2+} *in Xenopus oocytes* ... 40
3.3.4 CaSR activation inhibits transcellular Ca^{2+} *absorption via phospholipase C activation* ... 42

CHAPTER 4: DISCUSSION ... **45**

4.1 Proposed model for the CaSR-mediated reduced intestinal Ca^{2+} absorption by TRPV6 47
4.2 Significance in the Intestinal Ca^{2+} absorption and Regulation model 50
4.3 Therapeutic considerations 53
4.4 Conclusion 53
4.5 Future Directions 54
BIBLIOGRAPHY 57

TABLE OF FIGURES

Figure 1.1. Ca^{2+} homeostasis is mediated by the interaction between the bone, kidneys and intestine. 3

Figure 1.2. Regulation of Ca^{2+} and phosphate by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), and fibroblast growth factor 23 (FGF23). 5

Figure 1.3. Currently proposed intestinal Ca^{2+} absorption pathways. 14

Figure 2.1. Protocol used to measure unidirectional Ca^{2+} fluxes across intestinal preparations. 26

Figure 3.1. Relative intestinal mRNA expression of transcellular Ca^{2+} transport mediators under altered extracellular Ca^{2+} conditions. 30

Figure 3.2. Relative mRNA expression of transcellular calcium absorption mediators: TRPV6 (Trpv6), CalbindinD9K (Calb9k), Pmca1b (Pmca1b) normalized to GAPDH (A) and protein expression of CaSR in mouse and pig colon (B). 32

Figure 3.3. The effect of altering extracellular Ca^{2+} on Ca^{2+} fluxes across mouse proximal colon. .... **Error! Bookmark not defined.**

Figure 3.4. Expression of CaSR in the mouse intestine. 36

Figure 3.5. Effect of (A) apical or (B) basolateral CaSR activation on Ca^{2+} fluxes across mouse proximal colon. 37

Figure 3.6. The effect of basolateral CaSR activation on Ca^{2+} fluxes across mouse duodenum (A) and cecum (B). 37

Figure 3.7. Effect of extracellular Ca^{2+} on Ca^{2+} fluxes across proximal colon from $TRPV6^{WT/WT}$ or $TRPV6^{D541A/D541}$ mice. 39

Figure 3.8. Characterization of CaSR activation on TRPV6 function in Xenopus oocytes expressing TRPV6 and CaSR. 41

Figure 3.9. Effect of phospholipase C (PLC) inhibition on CaSR-mediated inhibition of Ca^{2+} absorption from mouse proximal colon. 43

Figure 4.1. Proposed Model of CaSR-mediated Inhibition of Ca^{2+} Absorption. 46

Figure 4.2. Distal Convoluted Tubule Comprise an Analogous Transcellular Ca^{2+} Transport Pathway Observed in the Intestine. 52

LIST OF TABLES

Table 1. *Real-time PCR primers and probes for mouse (Mus musculus)*

Table 2. *Real-time PCR primers and probes for pig (Sus scrofa)*

Table 3. Transepithelial Resistance (Ω*cm^2) of Ussing Chamber Experiments (Figures. 3.3, 3.5 - 3.7, 3.9)

TABLE OF ABBREVIATIONS

Ca^{2+} – calcium ion

PTH – parathyroid hormone

$1,25[OH]_2D_3$ – 1, 25-dihydroxyvitamin D_3 (active Vitamin D_3)

$25[OH]D_3$ – 25-hydroxyvitamin D_3

FGF23 – fibroblast growth factor 23

CaSR – calcium sensing receptor

FHH – familiar hypocalciuric hypercalcaemia

ADH – autosomal dominant hypocalcaemia

cAMP – cyclic adenosine monophosphate

GPCR – G-protein coupled receptor

TAL – thick ascending limb

TRPV6 – transient receptor potential cation channel subfamily V member 6

CABP9K – calbindin-D_{9k}

NCX1 – sodium-calcium exchanger 1

PMCA1b – plasma membrane calcium ATPase 1b

TRPV5– transient receptor potential cation channel subfamily V member 5

$^{45}Ca^{2+}$ – calcium-45

NHE3 – sodium-chloride exchanger 3

GLUT2 – glucose transporter 2

BBV – brush border vesicle

BLV – basolateral vesicle

Isc – short-circuit current

Chapter 1: Introduction

1.1 Calcium Homeostasis

Calcium (Ca^{2+}) homeostasis is vital to many physiological functions, including muscle contraction, neuronal function, bone development, and cellular signaling. Not surprisingly, as little as 5% change in plasma Ca^{2+} can produce detrimental clinical outcomes. A significant increase in plasma Ca^{2+} can lead to soft tissue calcification, potentially leading to kidney failure, atherosclerosis, and immobility, while a decrease can produce muscle tetany, including of the diaphragm, resulting in asphyxia. Therefore, the body tightly regulates plasma Ca^{2+} levels by altering epithelial Ca^{2+} transport across the intestine, kidneys, and bone (**Figure 1**).

The greatest and most readily available source of Ca^{2+} is the bone, storing >99% of the body's Ca^{2+} in hydroxyapatite, while the kidneys and intestine concomitantly regulate minute-to-minute Ca^{2+} (re)absorption/secretion. During bone development, the body undergoes a net positive Ca^{2+} balance (*i.e.* greater Ca^{2+} being absorbed from diet and deposited, than secreted and excreted) (Bronner & Pansu, 1999). However, as individuals transition from adolescence to adulthood, the growth plates close and the need for a positive Ca^{2+} balance is abolished. Under neutral Ca^{2+} balance, the Ca^{2+} transport processes at the intestine, kidneys and bones are constantly regulated by a combination of endocrine and receptor-mediated cellular regulation to maintain plasma Ca^{2+} level at equilibrium. When the body experiences negative Ca^{2+} balance (*e.g.* greater Ca^{2+} is being excreted), it begins to leach Ca^{2+} stores from the bone, weakening them ("Office of the Surgeon General (US). 2004. Bone Health and Osteoporosis: A Report of the Surgeon General. Rockville (MD): Office of the Surgeon General (US)."). Thus, it is the goal of this thesis to investigate other homeostatic mechanisms involved in maintaining the body's Ca^{2+} integrity, in particular the intestine, so as to permit maximal bone mineral density.

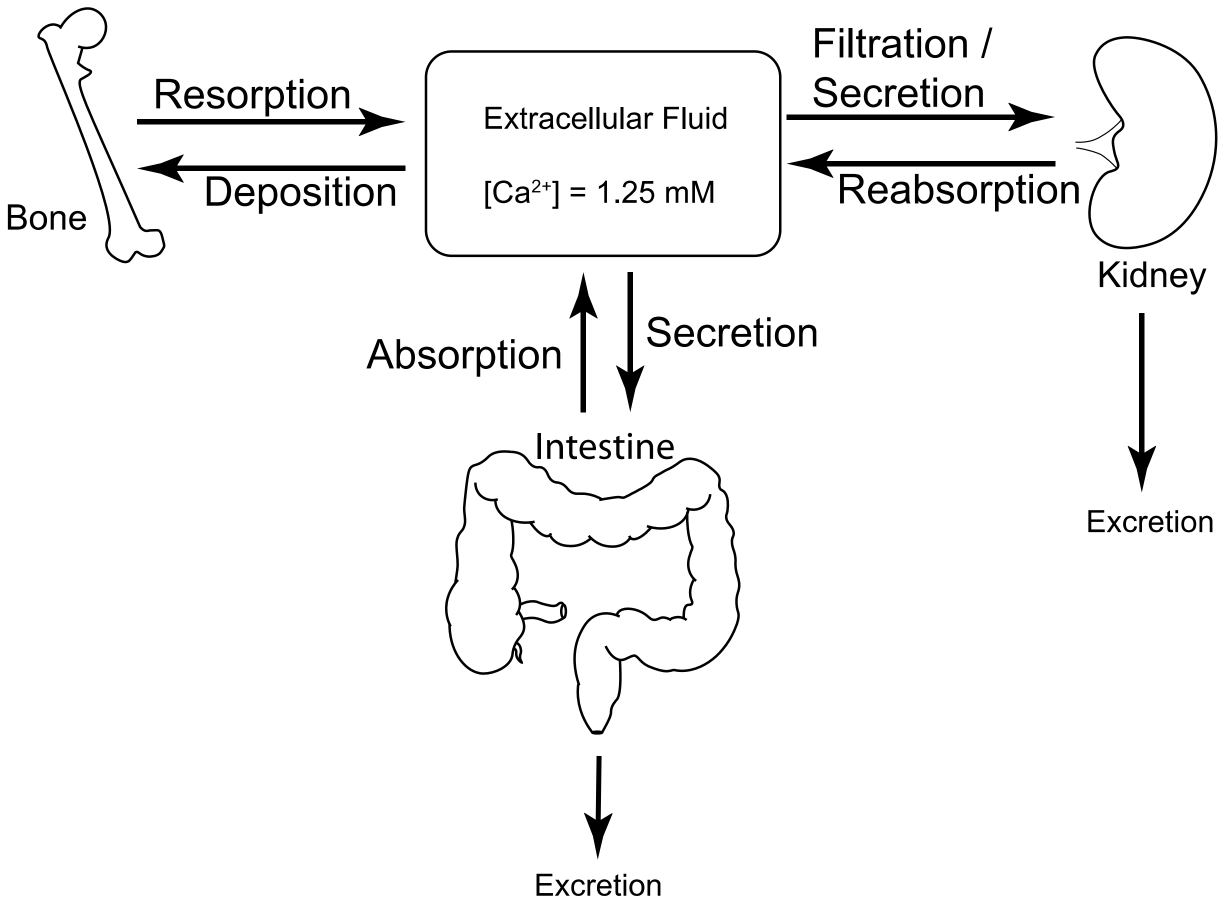

Figure 1.1. **Ca^{2+} homeostasis is mediated by the interaction between the bone, kidneys and intestine.**

Extracellular Ca^{2+} and serum Ca^{2+} levels are maintained by the regulated interplay between bone Ca^{2+} deposition / resorption, Ca^{2+} secretion/reabsorption by the kidneys, and Ca^{2+} absorption / secretion in the intestine.

1.2 Regulation of Ca^{2+} Homeostasis: Endocrine Hormones

It has been appreciated for some time that particular group of endocrine hormones, known as the calciotropic hormones, primarily regulate plasma Ca^{2+} levels. These hormones include parathyroid hormone produced from parathyroid glands; calcitonin from parafollicular cells of thyroid gland; active vitamin D_3 (1, 25-dihydroxyvitamin D_3), which is activated in the renal proximal tubule; and fibroblast growth factor 23 from bone. It is important to note that Ca^{2+} and phosphate homeostasis are inextricably linked (Blau & Collins, 2015; James C. Fleet, 2017;

Khundmiri et al., 2016; J. J. Lee et al., 2017; Martin et al., 2012). Thus, the abovementioned calciotropic hormones also have a significant role in regulation of phosphate homeostasis. Herein, this thesis will focus on their calciotropic functions.

1.2.1 Parathyroid Hormone (PTH)

Since the early 1900s, the relationship between the parathyroid gland and Ca^{2+} metabolism have been well established. Prior to its discovery, it was recognized that individuals with damaged or removed parathyroid gland were more prone to tetany: a hypocalcemic symptom (Bergeim et al., 1914; Collip, 1925; Maccallum & Voegtlin, 1909). This has led to decades of research, where the extracts of parathyroid gland and its effect on the body were delineated. PTH, produced and secreted from the parathyroid gland under hypocalcemic conditions, is an 84 amino acid polypeptide hormone that ultimately increases plasma Ca^{2+} levels (Potts et al., 1971). Its calciotropic function primarily occurs at the kidneys and bone, where PTH-PTH receptor (PTHR) binding results in increased Ca^{2+} reabsorption and resorption, respectively (**Figure 2**) (J. J. Lee et al., 2017; Poole & Reeve, 2005). PTH also indirectly increases Ca^{2+} absorption in the intestine by activating vitamin D_3, which will be further explained in Section 1.2.3. PTHR is a 7-transmembrane G-protein coupled receptor, which initiates cellular signaling cascade, ultimately affecting either membrane expression and/or gene regulation of transporters associated with Ca^{2+} reabsorption or altering osteoblast activity with respect to bone resorption (Datta & Abou-Samra, 2009; J. J. Lee et al., 2017; Mannstadt et al., 1999).

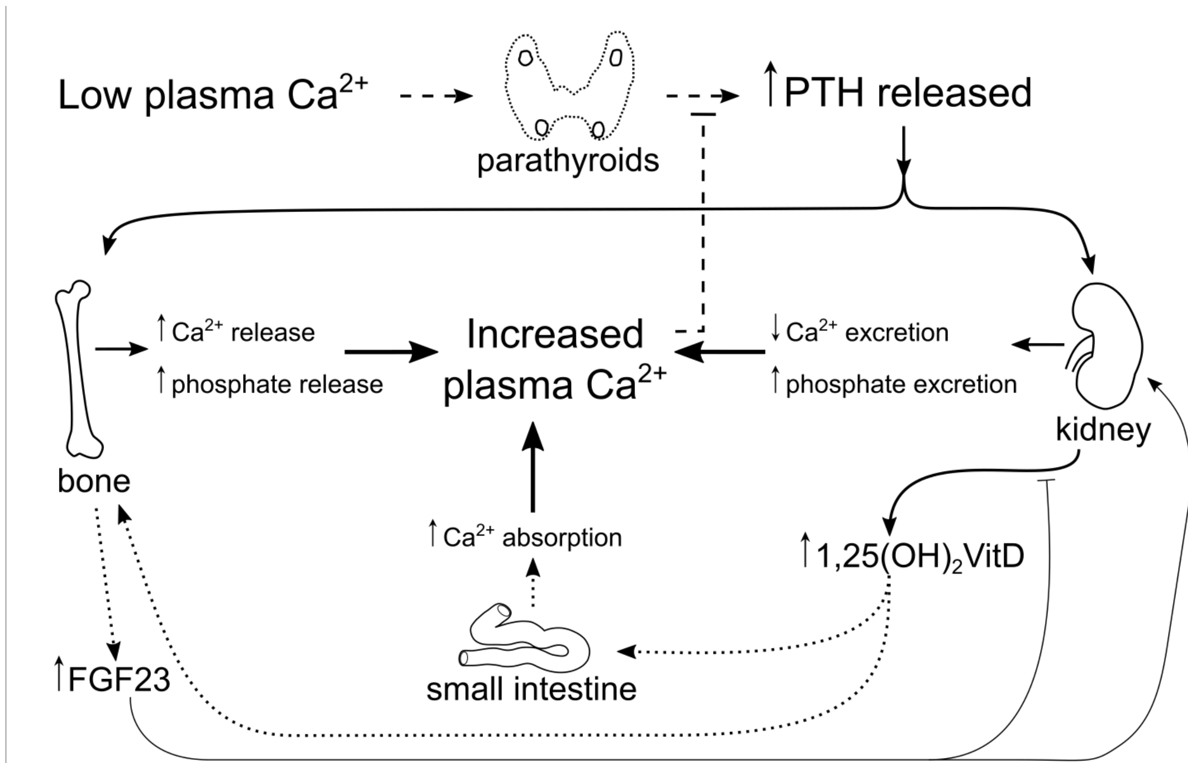

Figure 1.2. **Regulation of Ca^{2+} and phosphate by parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$), and fibroblast growth factor 23 (FGF23).**

Low plasma Ca^{2+} stimulates the release of PTH from the parathyroid glands. PTH stimulates resorption of bone, releasing Ca^{2+} and phosphates into the plasma. In the kidney, PTH increases urinary Ca^{2+} reabsorption and phosphate excretion. PTH indirectly increases Ca^{2+} absorption in the intestine via increasing production of 1,25(OH)$_2$D$_3$ (modified from (J. J. Lee et al., 2017) with permission).

1.2.2 Calcitonin

Following the discovery of PTH and its function, researchers started to recognize an opposing phenomenon, where the plasma Ca^{2+} of para- and thyroidectomised animals under hypercalcemic conditions transiently decreased when subjected to parathyroid extracts (Copp & Cameron, 1961; Copp et al., 1962). It was realized that this transient hypocalcemic effect was mediated by another peptidergic hormone now known as calcitonin, produced and secreted from the parafollicular cells of the thyroid gland. Not unlike the PTH, calcitonin secretion also occurs

upon changes in plasma Ca^{2+} levels) (Talmage et al., 1980). However, in contrast to PTH, its secretion occurs when plasma Ca^{2+} increases, as it functions to reduce plasma Ca^{2+} levels (Roth et al., 1974). Its hypocalcemic actions primarily target the bone, where it reduces the osteoclast activity, inhibiting bone resorption and maintaining bone Ca^{2+} integrity (Chambers & Magnus, 1982; Dacquin et al., 2004; Davey et al., 2008). The potential impact of calcitonin on renal or intestinal handling of Ca^{2+} is not fully understood.

1.2.3 1, 25-dihydroxyvitamin D_3 (1,25[OH]$_2D_3$)

Early calciotropic scientists recognized a seasonal change in plasma Ca^{2+} levels among rabbits, where the animals showed higher plasma Ca^{2+} levels in the summer months with greater sun light exposure (W. H. Brown, 1929; Havard & Hoyle, 1928; Hess et al., 1922). This led to the association between ultra-violet (UV) light exposure and change in plasma Ca^{2+} levels. This discovery was nonetheless the photochemical synthesis of inactive vitamin D_3 (also known as cholecalciferol) from 7-dehydrocholestrerol in the epidermis (Holick et al., 1977; Okano et al., 1978). Now, it is understood that the inactive vitamin D_3 is then hydroxylated in the hepatocytes by vitamin D 25-hydroxylase (*CYP2R1*) to form 25-hydroxyvitamin D_3 (25[OH]D_3; also known as calcifediol) (Holick & DeLuca, 1971; Horsting & DeLuca, 1969; Tucker et al., 1973). Thereafter, 25[OH]D_3 undergoes its final activation step, where another hydroxylase, 25-hydroxyvitamin D_3 1-α-hydroxylase (*CYP27B1*) in the proximal tubular epithelial cells, catalyzes the production of 1,25[OH]$_2D_3$ (Garabedian et al., 1974; Kawashima et al., 1981). 1,25[OH]$_2D_3$ primarily acts at the intestinal epithelial cells to increase the absorption of Ca^{2+} from diet, ultimately increasing plasma Ca^{2+} [described in more detail in Section 1.4] (Christakos, 2012; Christakos et al., 2011; Wasserman & Fullmer, 1995).

One of the indirect mechanisms whereby PTH increases plasma Ca^{2+} is via increasing the production of $1,25[OH]_2D_3$ in the proximal tubules of kidneys, where PTH-PTHR binding results in upregulation of *CYP27B1* gene (Kremer & Goltzman, 1982; Siegel et al., 1986; Welsh et al., 1991). Clinically, the PTH-vitamin D_3 axis is perhaps most evident in individuals with severe vitamin D_3 deficiency either due to diet or chronic kidney disease (therefore unable to convert inactive $25[OH]D_3$ to $1,25[OH]_2D_3$). This can result in secondary hyperparathyroidism – a disease that abnormally produces PTH as a result of another condition (e.g. lack of vitamin D_3) producing hypocalcaemia. (Cipriani et al., 2018). Not surprisingly, vitamin D_3 is one of the most recommended supplementary products to aid Ca^{2+} absorption.

1.2.4 Fibroblast growth factor 23 (FGF23)

The most recent calciotropic factor to be discovered is FGF23, a 32 kDa peptide hormone produced from the osteocytes and osteoblasts of bone (Rodriguez-Ortiz & Rodriguez, 2015). The discovery of FGF23 was made with efforts to identify the genes responsible for hereditary disorders responsible for phosphate imbalance (E. White et al., 2000). It was recognized that 4 patients with autosomal dominant hypophosphatemic rickets, which is clinically manifested as low plasma phosphorus and poor bone deposition, carried a missense mutation in the *FGF23* gene (E. White et al., 2000). FGF23 was also found to have direct implications on Ca^{2+} homeostasis by impairing *CYP27B1* expression in the proximal tubules and consequently reducing $1,25[OH]_2D_3$ activation (Perwad et al., 2007; Takeshita et al., 2018). The receptor by which FGF23 carries its inhibitory effect in the proximal tubules are also expressed in the parathyroid gland (Silver & Naveh-Many, 2010). Thus, it is reasonable to speculate that FGF23 may inhibit PTH secretion via a similar inhibitory mechanism. However, it is possible that PTH secretion may subside with

compensatory normalization of plasma Ca^{2+} levels, as well as direct inhibitory actions of FGF23 at the sites of PTH action. Since its discovery, the focus of FGF23 research has been primarily on phosphate homeostasis. Recognizing its elusive involvement in Ca^{2+} homeostasis, it will certainly be of interest to researchers in this field.

1.3 Regulation of Ca^{2+} Homeostasis: CaSR

More recently, the homeostatic mechanisms permitting direct sensing of extracellular Ca^{2+} by the nephron or bone and subsequently altering tubular Ca^{2+} reabsorption or bone remodeling were delineated (Goltzman & Hendy, 2015; Toka et al., 2015). This direct sensing of extracellular Ca^{2+} occurs, at least in part, by the 7-transmembrane G-protein coupled Ca^{2+} sensing receptor (CaSR) (E. M. Brown, 2013). Not surprisingly, mutation in the CaSR gene causes diseases that display abnormal Ca^{2+} balance. For example, inactivating mutations of the *CaSR* cause familiar hypocalciuric hypercalcaemia (FHH), while activating mutations of the *CaSR* cause autosomal dominant hypocalcaemia (ADH) (Mancilla et al., 1998; Pidasheva et al., 2005).

1.3.1 CaSR in the parathyroid gland

Following the discovery of PTH, it became apparent that there is a Ca^{2+} sensing mechanism in the parathyroid gland whereby an increase in plasma Ca^{2+} decreases PTH release and vice versa. Using collagenase digestion of parathyroid gland tissue, researchers isolated parathyroid cells and examined the secretion of PTH upon changes in extracellular Ca^{2+} levels (E. M. Brown et al., 1978a; E. M. Brown et al., 1976; Conigrave et al., 2004; Morrissey & Cohn, 1978). In these *in vitro* conditions, researchers were able to directly alter extracellular Ca^{2+} levels to high (1.5 mM) and low (0.5 mM) concentrations. High extracellular Ca^{2+} suppressed PTH secretion, while the opposite was observed with low extracellular Ca^{2+}.

In a series of manuscripts, Brown et al. described the mechanism of endogenous PTH secretion, which is stimulated by cAMP-mediated G-protein coupled receptor (GPCR) activation (coupled to $G\alpha_s$-protein) (E. M. Brown et al., 1978b; E. M. Brown et al., 1979; E. M. Brown et al., 1977; E. M. Brown et al., 1985). This led to the hypothesis and examination of whether the parathyroid Ca^{2+} sensor may be a GPCR that inhibits cAMP generation, also known as $G_{i/o}$PCR. Although experiments employing pharmacological blockade of the $G_{i/o}$PCR with pertussis toxin support this hypothesis (E. M. Brown et al., 1990), other work suggested an alternative signalling pathway by which the parathyroid Ca^{2+} sensor signals. This is based on the observation that bovine parathyroid cells exposed to high extracellular Ca^{2+} levels had 1) increased cytosolic Ca^{2+} levels and 2) greater production of radiolabelled inositol phosphates – both cellular consequences of $G_{q/11}$-PCR activation (E. Brown et al., 1987; Nemeth & Scarpa, 1986). This seems highly plausible as, similar to that of CaSR mutations, patients with inactivating/activating mutations in the *GNA11* gene, which codes for the $G\alpha_{11}$-protein, cause familial hypocalciuric hypercalcaemia type 2 and autosomal dominant hypocalcaemia type 2, respectively (Nesbit et al., 2013). Although these studies implicate specific Gα-protein pathways, it is likely that there may be crosstalk between the cellular signaling pathways and further research is certainly required to delineate the molecular mechanism of CaSR-mediated decreased secretion of PTH.

Understanding that the Ca^{2+} sensor in the parathyroid cell is likely a GPCR, the next goal was to examine the cDNA library of parathyroid cell extract and isolate the gene of interest (E. M. Brown et al., 1993). This was done by expressing the size-fractionated bovine parathyroid cDNA extracts in Xenopus oocytes and identifying the fractions with the characteristics of the Ca^{2+} sensing GPCR described above. Isolation of this extract led to the discovery of a 5.3 kb clone containing a GPCR, now known as the CaSR (E. M. Brown et al., 1993).

1.3.2 CaSR in the kidneys

It has been previously hypothesized that the kidney itself contains a Ca^{2+} sensing mechanism. This hypothesis was based on microperfusion studies in dogs, where hypercalcaemia appeared to increase urinary excretion of Ca^{2+}, independent of PTH (Edwards et al., 1974; Glassman et al., 1974; Mohammad et al., 1974). Following the discovery of the bovine CaSR, its orthologues were identified and cloned from other species (E. M. Brown et al., 1993; Garrett et al., 1995; Riccardi et al., 1995) and CaSR expression was identified throughout the renal epithelial cells with the greatest abundance in the thick ascending limb (TAL) (Loretz et al., 2004; Riccardi et al., 1998). The precise location of the CaSR in the kidney (i.e. basolateral vs apical membrane and which nephron segments) has been debated. In brief, there have been reports of CaSR localization in the apical membrane of proximal tubular cells (Riccardi et al., 1998), basolateral membranes of TAL (Loupy et al., 2012; Riccardi et al., 1998; Toka et al., 2012), and basolateral membranes of distal convoluted tubules (Riccardi et al., 1998). Although expression of the CaSR in the proximal tubule and distal convoluted tubule has been contested (Loupy et al., 2012).

In the kidneys, approximately 99% of plasma Ca^{2+} are filtered and reabsorbed (Bonny & Edwards, 2013). There are two major pathways mediating Ca^{2+} reabsorption along the nephron. Filtered Ca^{2+} is reabsorbed via the paracellular pathway (i.e. between the cells) via a sodium-dependent osmotic and electrochemical gradient in the proximal tubule and the thick ascending limb; while a transcellular Ca^{2+} transport pathway has been identified in the distal convoluted tubule [readers are directed to (Bonny & Edwards, 2013) for a detailed review of Ca^{2+} reabsorption throughout the nephron]. There is significant evidence that a renal CaSR regulates Ca^{2+} reabsorption in the TAL. The Alexander laboratory previously demonstrated that activation of the

renal CaSR *in vivo* and *in vitro* results in increased claudin-14 expression (a tight junction protein which decreases permeability of Ca^{2+} across TAL) thereby increasing urinary Ca^{2+} excretion (Dimke et al., 2013). Current work aims to delineate the cellular signalling pathway downstream of CaSR activation that increases claudin-14 expression. These studies are physiologically relevant and highly plausible as high plasma Ca^{2+} (i.e. hypercalcaemia) can be detected by the basolateral CaSR in the TAL, which inhibits Ca^{2+} reabsorption across this segment. In contrast to the TAL, the functional role(s) of the CaSR in the proximal tubule and distal tubule remain controversial. Under *in vitro* conditions, activation of proximal tubular CaSR appears to have an inhibitory effect of the PTH-mediated cellular consequences, including natriuresis and phosphaturesis via inactivation of sodium-phosphate cotransporters and sodium-hydrogen exchanger (respectively) (J. J. Lee et al., 2017), while the distal tubular CaSR appears to increase transcellular Ca^{2+} transport across this segment (Topala et al., 2009). Though plausible, further studies including *in vivo* or *ex vivo* activation of CaSR in these segments perhaps using micropuncture/microperfusion techniques should be conducted to definitively demonstrate the functional effects of renal CaSR activation.

1.3.3 CaSR in bone

It is reasonable to assume that the body's largest Ca^{2+} deposit, the bone, should have a Ca^{2+} sensing mechanism and be able to locally regulate bone resorption/deposition independent of calciotropic hormones. However, unlike the epithelial transport of Ca^{2+} in the kidneys or the intestine, the molecular mechanism of bone resorption/deposition involves a multitude of complex interconnected mechanisms mediated by osteoclasts and osteoblasts, influenced by combinations of calciotropic hormones, that ultimately alter the amount of hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) dissolved and /or synthesized from the extracellular matrix of bone [for more detailed reviews that

highlight the mechanistic perspectives of osteoblast and osteoclast mediated regulation of hydroxyapatite, readers are directed to (Marie, 2010; Raggatt & Partridge, 2010)]. Due to this complexity, the role of a local Ca^{2+} sensing mechanism has been difficult to elucidate.

Though debated, there have been reports of CaSR expression in osteoblasts, osteoclasts, and skeleton (Dvorak et al., 2004; Pi et al., 1999; Yamaguchi et al., 1998). There is functional evidence suggesting osteoblasts, osteoclasts, and their progenitor cells to have Ca^{2+} sensing mechanisms. Exposure to high extracellular Ca^{2+} conditions in *in vitro* enhance pre-osteoblastic differentiation to mature osteoblasts (Sugimoto et al., 1994). In contrast, high extracellular $Ca^{2+,}$ as well as pharmacological activation of the CaSR, inhibit the differentiation of pre-osteoclastic progenitor cells (Kanatani et al., 1999; Sugimoto et al., 1993). If these actions are indeed mediated by the CaSR, this has physiological consequences in maintaining Ca^{2+} homeostasis. High plasma Ca^{2+} increases osteoblast activity/formation while decreasing osteoclast activity/formation, enhancing bone deposition while inhibiting resorption. Unfortunately, it has been difficult to replicate these observations under *in vivo* conditions, as many hormonal or molecular compensatory mechanisms are activated to maintain bone integrity (Rodriguez et al., 2007). The generation of osteoblast-specific CaSR knockout mice has provided insight into the *in vivo* relevance of skeletal CaSR. The knockout mice have profound bone defects similar to that of patients with inactivating CaSR mutations. They display lower bone mass, poor skeletal development, and reduced bone turnover (i.e. reduced osteoblast/clast activity) (Chang et al., 2008; Kantham et al., 2009; Yamaguchi & Sugimoto, 2007). Together, these findings suggest that an apparent Ca^{2+} sensing mechanism in the bone plays a physiological role in regulating local Ca^{2+} levels. However, further studies confirming the expression of the CaSR in osteoblasts and osteoclasts, delineating the cellular mechanisms involved in CaSR-mediated osteoblast and

osteoclast differentiation and activity regulation, and bone phenotype of bone cell-specific CaSR knockout animal models are needed to more clearly define the functional role of the CaSR in the bone.

1.3.4 CaSR in the intestine

The intestine is responsible for regulating absorption of Ca^{2+} available in the diet. Following the discovery of the CaSR, its expression and localization throughout the intestinal tract was also examined. Various researchers found expression of the CaSR on both the apical and basolateral membranes of intestinal epithelial cells in rodents (Chattopadhyay et al., 1998; Gama et al., 1997). It was then hypothesized that the intestinal CaSR may have a functional role in maintaining Ca^{2+} homeostasis through regulating Ca^{2+} absorption. However, research following defining the intestinal localization focused on modulation of various general digestive functions including gastric enzyme secretion, proliferation/differentiation of epithelial cells, and intestinal barrier integrity/fluid secretion (S. Cheng et al., 2002; S. X. Cheng et al., 2014; Geibel et al., 2006) [readers are directed to (Tang et al., 2016) for detailed review]. It is therefore surprising that thus far no one has examined the role of the CaSR in regulating intestinal Ca^{2+} absorption, given its known role in maintaining Ca^{2+} homeostasis throughout the rest of the body. It is therefore the goal of this thesis to examine whether the intestinal CaSR does indeed regulate Ca^{2+} absorption/secretion across the intestine, in an attempt to maintain Ca^{2+} homeostasis.

1.4 Intestinal Ca^{2+} Transport

Although the CaSR has been identified in the intestine, its role in the regulation of intestinal Ca^{2+} absorption has not been described. Intestinal Ca^{2+} absorption/secretion occurs via a paracellular or transcellular pathway. The paracellular pathway is a passive process, where Ca^{2+}

flux is facilitated by a tight junction protein complex, driven by the transepithelial electrochemical gradient (**Figure 3**). Although plasma Ca^{2+} is relatively stable, various luminal conditions, including sojourn time, pH, the osmolality of luminal contents, and transepithelial potential differences alter luminal Ca^{2+} concentration, allowing for conditional bidirectional Ca^{2+} transport (Bronner & Pansu, 1999). The transcellular pathway is an active process, where Ca^{2+} is transported through the cell, driven by a unidirectional active transport; thus, it only contributes to Ca^{2+} absorption (i.e. lumen to blood).

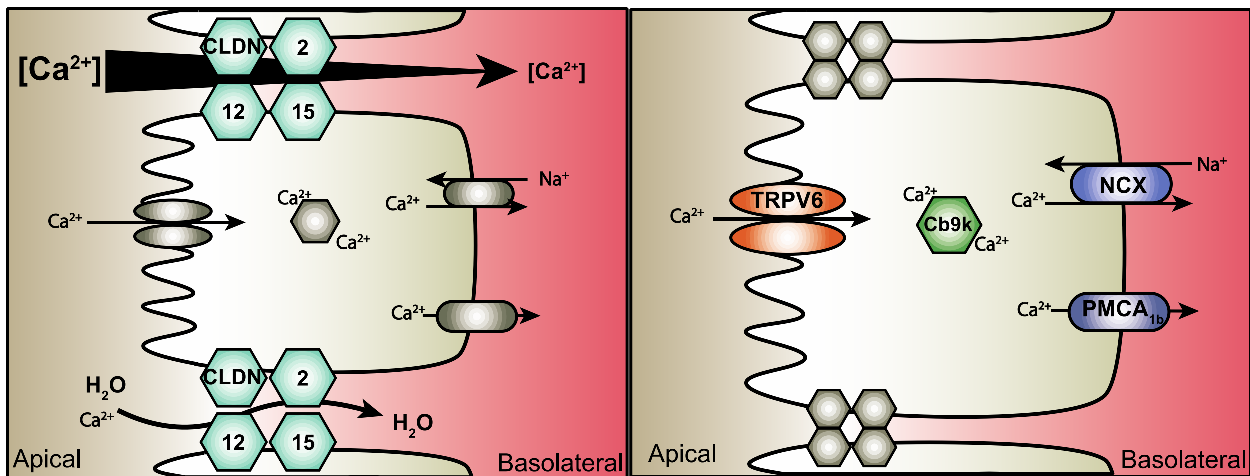

Figure 1.3. **Currently proposed intestinal Ca^{2+} absorption pathways.**

Left: Passive paracellular Ca^{2+} transport is mediated by the claudin family of tight junction proteins: claudin (CLDN), where Claudin-2, -12, and -15 are currently proposed to facilitate Ca^{2+} transport. Of note, this pathway can result in Ca^{2+} secretion depending on the electrochemical gradient. Right: Active transcellular Ca^{2+} transport occurs in 3 steps: (i) Ca^{2+} entry by transient receptor potential channel, subfamily V, member 6 (TRPV6); (ii) intracellular diffusion by Calbindin-D_{9k} (CABP9K; Cb9k); and (iii) basolateral Ca^{2+} extrusion by plasma membrane Ca^{2+} ATPase (PMCA1b) and Na^{+}-Ca^{2+} exchanger 1 (NCX1).

1.4.1 Paracellular Ca^{2+} Transport pathway

A major mechanism by which the body transports ions across epithelia is via paracellular pathways. Evidence for paracellular Ca^{2+} transport in the intestine is largely derived from radioactive Ca^{2+} flux studies performed in Ussing chambers. Throughout the whole length of the intestine, a voltage-dependent Ca^{2+} transport process has been observed, suggesting a Ca^{2+} transport pathway that allows free diffusion between cells (Karbach, 1992). Having identified the presence of transepithelial paracellular transport pathways, researchers began searching for the paracellular transport mediators. This led to the discovery of a family of tight junction proteins called claudins (Krause et al., 2009; Rosenthal et al., 2017). Of the claudin family, isoforms claudin-2, -12, and -15 have been localized and implicated in forming cation-permeable pores between intestinal epithelial cells (Amasheh et al., 2002; Fujita et al., 2006; Fujita et al., 2008; Yu et al., 2009; Yu et al., 2010). Though genetic deletion of claudin-2, -12, and -15 have shown some alteration in total sodium and water transport, no direct examination of Ca^{2+} permeability has been performed (Fujita et al., 2008; Wada et al., 2013). The Alexander laboratory has generated claudin-2, -12 knockout and claudin-2 and -12 double-knockout mice and is currently investigating the Ca^{2+} phenotype and paracellular Ca^{2+} permeability across the intestinal segments of these mice. There is evidence of regulation of these paracellular transport mediators via $1,25[OH]_2D_3$ (Fujita et al., 2008; Wasserman, 2004). However, much work needs to be done to establish the mechanism of claudin protein function, including how the claudins form a pore; whether they form pores as multimers or via complexing with other isoforms; and how these complexes may affect the ion selectivity of paracellular pores.

1.4.2 Transcellular Ca^{2+} transport pathway

The duodenum, cecum and proximal colon are sites of significant intestinal transcellular Ca^{2+} absorption (Christakos et al., 2011; Hoenderop et al., 2005). These segments display a voltage-independent mucosal-to-serosal (i.e. lumen-to-blood) Ca^{2+} flux, indicating the presence of an active transcellular Ca^{2+} transport pathway (Karbach, 1989; Karbach & Feldmeier, 1993; Rievaj et al., 2013). This is in part mediated by the expression of the apically expressed Ca^{2+} channel TRPV6, the intracellular Ca^{2+} buffering protein calbindin-D_{9k} (CABP9K), and the basolateral Ca^{2+} extruding proteins the plasma membrane Ca^{2+} ATPase 1b (PMCA1b) and the Na^{+}/Ca^{2+}-exchanger (NCX1) (Hoenderop et al., 2005). Not surprisingly, these mediators are found in the duodenum, cecum, and proximal colon.

The discovery of TRPV6 in the intestine followed from development of sequencing techniques that permitted researchers to differentiate TRPV6 from TRPV5, a close family member that is highly expressed in the kidneys where it mediates significant Ca^{2+} reabsorption from the distal tubule (Barley et al., 2001; Peng et al., 2001; Peng et al., 1999; van Abel et al., 2003; Van Cromphaut et al., 2001). Under *in vitro* conditions, researchers identified a role for TRPV6 in cellular Ca^{2+} uptake, as expression of exogenous *TRPV6* in cells increased Ca^{2+} entry (Barley et al., 2001; Peng et al., 1999; Vassilev et al., 2001). With respect to the functional role of TRPV6 in overall Ca^{2+} homeostasis, some argue that TRPV6 is a vital factor in Ca^{2+} absorption and homeostasis, as there are significant alterations in intestinal Ca^{2+} absorption and bone mineral density in TRPV6 knockout mice or mice with activating mutations in TRPV6 (Bianco et al., 2007; Cui et al., 2012). Others suggest a less essential role of TRPV6 in Ca^{2+} homeostasis based on observations from another TRPV6 knockout mouse or the D541A knock-in mutation (pore mutation, unable to transport Ca^{2+}). These models do not demonstrate a significant difference in

intestinal Ca^{2+} absorption nor a dramatic bone phenotype (Lieben et al., 2010; Weissgerber et al., 2011; Woudenberg-Vrenken et al., 2012). Regardless, a major methodological limitation of these studies is technique used to measure Ca^{2+} absorption *in vivo, i.e.* oral gavage of radioactive Ca^{2+} and then measuring the amount taken up into blood. Unfortunately, this technique does not account for various confounding variables, including acute/chronic calciotropic hormone effect, local compensatory factors, as well as the potential secretion of absorbed Ca^{2+} by the paracellular pathway (Bianco et al., 2007; Lieben et al., 2010). Therefore, it is clear that more conclusive research is required to further implicate the role of TRPV6 in intestinal Ca^{2+} absorption.

Intracellular Ca^{2+} levels are maintained at nanomolar concentrations to prevent an increase which signals apoptosis (Choi et al., 2011). Understanding that $1,25[OH]_2D_3$ can increase Ca^{2+} absorption in the intestine, Wasserman et al. identified a protein in chick intestinal epithelium with a number of Ca^{2+} binding EF-hand domains that was sensitive to vitamin D_3 administration, now known as CABP9K (Wasserman et al., 1969; Wasserman & Taylor, 1966). Following this discovery, it was found that the CABP9K co-localizes with TRPV6, suggesting a shuttling role for the transcellular Ca^{2+} transport pathway (Shimura & Wasserman, 1984). Although a series of CABP9K genetic modification studies have been attempted, its functional role *in vivo* has been difficult to examine due to its vital role in maintaining cytosolic Ca^{2+} in the body and the likelihood of compensatory mechanisms that are activated in the absence of CABP9K. However, researchers have found that the expression profile of CABP9K follows that of TRPV6 under Ca^{2+} rich or deficient environments, where low Ca^{2+} diet both increased CABP9K and TRPV6 expression in wild type, but not CABP9K knock out mice (Ko et al., 2009a; Ko et al., 2009b; G. S. Lee et al., 2007). Together, there is molecular evidence to suggest a role in intestinal Ca^{2+} absorption, however, its functional involvement remains to be clearly elucidated.

The basolateral extrusion of cytosolic Ca^{2+} is thought to be mediated by PMCA1b and NCX1. Both proteins are expressed in epithelial cells (Kim et al., 2011; Liu et al., 2013; Reilly & Shugrue, 1992) and their Ca^{2+} transport mechanisms have been clearly demonstrated (Di Leva et al., 2008; Ottolia & Philipson, 2013). There is indirect evidence that these proteins are involved in transcellular Ca^{2+} absorption, as greater expression of PMCA1b has been detected in intestinal segments of transcellular Ca^{2+} absorption (Freeman et al., 1995) and both intestinal expression of PMCA1b and NCX1 were greater in mice on low Ca^{2+} chow (Centeno et al., 2004). Due to the importance of Ca^{2+} extruding mechanism and their ubiquitous expression in cells throughout the body, animal models lacking Ca^{2+} pumps have not been viable (Cho et al., 2003; Koushik et al., 2001; Prasad et al., 2004). In addition, limited availability of specific pharmacological agents targeting these proteins increases the difficulty of examining the functional involvement of these proteins. Regardless, unless there is new evidence to dispute otherwise, PMCA1b and NCX1 are the most logical and plausible basolateral extrusion proteins based on the evidence currently available.

Not surprisingly, the intestinal Ca^{2+} absorption/secretion process is subjected to regulation. Hypocalcaemia leads to increased PTH secretion, which stimulates the production of $1,25[OH]_2D_3$ and thus increases intestinal Ca^{2+} transport (Cui et al., 2009; J. C. Fleet et al., 2002; Song et al., 2003; Walters et al., 2006). $1,25[OH]_2D_3$ increases intestinal Ca^{2+} absorption by increasing the expression of TRPV6, a phenomenon that correlates with intestinal Ca^{2+} absorption (Alexander et al., 2009; Christakos et al., 2011; Pan et al., 2012). The resulting increased Ca^{2+} influx in turn enhances the expression of CABP9K (Benn et al., 2008; Wongdee & Charoenphandhu, 2015; Xue & Fleet, 2009). Conversely, hypercalcaemia inhibits PTH release and consequently reduces intestinal Ca^{2+} uptake, by limiting active $1,25[OH]_2D_3$ synthesis. However, this latter regulatory

mechanism would be rather slow with respect to attenuating hypercalcaemia. As minute-to-minute regulation of plasma Ca^{2+} level is crucial, it was the goal of this thesis to examine whether intestinal CaSR expression directly affects Ca^{2+} transport across the sensing segment of the intestine.

1.5 Overall Rationale

The CaSR is expressed throughout the intestine (Chattopadhyay et al., 1998; S. Cheng et al., 2002; Gama et al., 1997), where it regulates fluid, sodium and chloride secretion (S. Cheng et al., 2002; Geibel et al., 2006; Tang et al., 2016). However, a direct role in Ca^{2+} homeostasis has not been reported (E. M. Brown, 2013; Tang et al., 2016). We therefore set out to examine the effect of extracellular Ca^{2+} on intestinal Ca^{2+} absorption. To do so, we first examined the gene expression of intestinal transcellular Ca^{2+} transporting proteins following chronic CaSR activation. In wildtype and mutant mouse models, we also examined the effects of acute pharmacological or physiological activation of the CaSR on the transcellular Ca^{2+} absorption pathway *ex vivo*, using Ussing chambers. Thereafter, using a simpler transport model: *Xenopus oocytes*, we examined the effect of CaSR activation on TRPV6 channel function. Together, it is the goal of this thesis to delineate the role of the intestinal CaSR on overall Ca^{2+} homeostasis by examining its effect on Ca^{2+} absorption.

1.6 Hypothesis

I hypothesize that the basolateral intestinal CaSR directly regulates intestinal Ca^{2+} absorption. Specifically, intestinal CaSR activation (*i.e.* via high plasma Ca^{2+}) decreases intestinal Ca^{2+} absorption via acute inhibition of the transcellular Ca^{2+} transport protein TRPV6 and chronically by decreasing TRPV6 expression.

Chapter 2: Materials and Methods

2.1 Ethical approval and animals

All animal experiments were approved by the Animal Care and Use Committee for Health Science of the University of Alberta (protocol #213) following the Guide for the Care and Use of Laboratory Animals (NIH Guide). Wildtype FVB/N mice (Jackson laboratories), named after its sensitivity to Friend leukemia virus B, and $Trpv6^{D541A/D541}$ knock in mice (Weissgerber et al., 2011) were housed in virus-free conditions and maintained on a 12-h light-dark schedule. The $TRPV6^{D541A/D541}$ mice were backcrossed to FVB/N for > 5 generations. These mice express a functionally inactive Trpv6 channel under its endogenous promoter; however, no significant Ca^{2+} phenotype are observed aside from sterile homozygote males (Weissgerber et al., 2011). Standard pelleted chow (PicoLab Rodent Diet 5053: 21% wt/wt protein, 5.0% wt/wt fat, 0.81% wt/wt Ca^{2+}, and 2.2 IU/g vitamin D_3) and drinking water were available ad libitum. Chronic altered-Ca^{2+} containing diets, or experiments where $1,25[OH]_2D_3$, or cinacalcet were administered were performed in a previous study by Dr. Henrik Dimke (Dimke et al., 2013). In short, wildtype FVB/N mice were (1) fed a low (0.01%, TD.95027)-, normal (0.6%, TD.97191)-, or high (2%, TD.00374)-Ca^{2+} diet for 21 days (n = 8 each); (2) intraperitoneally injected 500 pg/g body weight of vehicle or $1,25(OH)_2D_3$ for 5-days (n = 8 each); and (3) administered 1 mg/g body weight of cinacalcet hydrochloride for 6-days by diet (n = 6 each/control and cinacalcet diet).

2.1.1 Intestinal segment isolation

Intestinal segments were dissected as previously described (Rievaj et al., 2013). In short, the first 4.6 cm of the small intestine from the pyloric sphincter represented the duodenum. The anatomical pouch at the ileocecal junction was identified as the cecum. The first 2.4 cm distal to the ileocecal junction was considered proximal colon.

2.1.2 Pig colon samples

5-week old landrace/large white cross Duroc pig colon samples were generously provided by Dr. Justine Turner (Department of Pediatrics, University of Alberta) (Levesque et al., 2017).

2.2 Real-time quantitative PCR

Total RNA from dissected intestinal segments was isolated using TRIzol Reagent and reverse transcribed into cDNA using random primers and SuperScript II reverse transcriptase (all from Invitrogen, Carlsbad, CA). Primers and probes (Integrated DNA Technologies, San Diego, CA) designed for TRPV6 (*Trpv6*), CABP9K (*S100g*), NCX1 (*Slc8a1*), PMCA1b (*Atp2b1*), and CaSR (*CaSR*) were used to quantify the expression levels with the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) (Pan et al., 2012).

2.3 Immunoblotting

Total protein was isolated from mouse parathyroid gland, brush-border or basolateral membrane of the proximal colon as described before (Cheeseman & O'Neill, 2006; Pan et al., 2012). Immunoblotting was carried out as previously described (Pan et al., 2012). In short, total protein was extracted using RIPA buffer (50 mM Tris, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 1% lgepal CA-630) containing a protease inhibitor cocktail (Calbiochem, Gibbstown, NJ) and subjected to 10% SDS-PAGE and electroeluted to 0.2 µm PVDF membrane (Millipore, Burlington, MA). Mouse primary anti-CaSR monoclonal antibody (1:2000, Gentex, Zeeland, MI), mouse primary anti-NHE3 monoclonal antibody [1:25, obtained from Amemiya et al. (Amemiya et al., 1995)], and rabbit primary anti-GLUT2 monoclonal antibody (1:1000,

Millipore, Burlington, MA) were applied overnight at 4°C, followed by incubation with a secondary horseradish peroxidase-coupled secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected with Immobilon Crescendo Western HRP substrate (Millipore, Burlington, MA) and visualized using a ChemiDoc Imaging System (Bio-Rad, Hercules, CA).

2.4 Ussing chamber experiments

$^{45}Ca^{2+}$ flux across the duodenum, cecum, and proximal colon of FVB/N, $Trpv6^{WT/WT}$, and $Trpv6^{D541A/D541}$ mice was performed essentially as previously (Rievaj et al., 2013). Following euthanasia, intestinal sections were dissected, linearized, and transversely cut into 3 mm segments. These segments were mounted in an Ussing Chamber (EM-CYS-4 system with P2400 chambers and P2407B sliders, Physiologic Instruments, San Diego, CA) and incubated with 4 mL Ringer's solution consisting of 115 mM NaCl, 2.5 mM K_2HPO_4, 40 nM KH_2PO_4, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$, bubbled with 5% vol/vol CO_2, 95% vol/vol O_2 at 37°C on both sides. 10 mM glucose was only added to the basolateral chamber to maintain electroneutrality under voltage clamp conditions; 10 mM mannitol was added to apical chamber to maintain osmotic equilibrium. 2 μM indomethacin was bilaterally added to reduce inflammation (Sigma-Aldrich, St. Louis, MO). The basolateral solution also contained 0.1 μM tetrodotoxin to eliminate any remaining nerve function (Alomone Labs, Jerusalem, Israel). The transepithelial potential difference was clamped to 0 mV by a VCC MC6 Multichannel Voltage/Current Clamp (Physiologic Instruments, San Diego, CA) and the resulting short-circuit current recorded with Acquire & Analyze software (Physiologic Instruments, San Diego, CA) through Ag-AgCl electrodes and 3 M KCL agarose

bridges. The transepithelial resistance (TER) was calculated using Ohm's law, following the measurement of the current generated in response to 2 mV pulses lasting 2.5 s, applied every 100s.

Unidirectional Ca^{2+} fluxes (*i.e.* apical-to-basolateral and basolateral-to-apical from adjacent samples of the intestinal segment from the same mouse) were measured using the protocol shown in **Figure 2**. At time 0, either the apical or basolateral solution was exchanged for a fresh solution of the same composition spiked with 5 µCi/mL $^{45}Ca^{2+}$. Three samples (50 µL each) were taken from both chambers at 15 minute intervals throughout each experimental condition (Condition A: sample taken at 20, 35 and 50 min; Condition B: samples taken at 75, 90, and 105 min); N.B. each condition is subjected to 20 minutes of incubation time prior to obtainment of samples (**Figure 4**).After the third sample was collected under condition A, the buffers were immediately changed and/or treatments applied [10 µM cinacalcet hydrochloride (cinacalcet) in ethanol, 10 µM U73122 in DMSO or both] and the tissue incubated for another 20 min before sampling for condition B occurred. Radioactivity was measured with a LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter, Brea, CA) and unidirectional flux ($J_{H\rightarrow C}$, in $nmol \cdot h^{-1} \cdot cm^{-2}$) from the hot chamber (H) to the cold chamber (C) was calculated by (34):

(i) $J_{H\rightarrow C} = R_{H\rightarrow C}/(S_H \times A)$ and (ii) $S_H = C_{^{45}Ca} / C_T$,

where $R_{H\rightarrow C}$ is the rate of $^{45}Ca^{2+}$ appearance in the cold chamber (in cpm/h); S_H is the average of specific activity in the hot chamber (in cpm/nmol); A is the surface area of the tissue (in cm^2); $C_{^{45}Ca}$ is the mean radioactivity in the hot chamber (in cpm); and C_T is the total concentration of Ca^{2+} in the hot chamber (in nmol). Unidirectional Ca^{2+} fluxes in opposite directions from the same animal and segment (apical-to-basolateral vs. basolateral-to-apical) were paired to calculate net Ca^{2+} flux (net apical-to-basolateral flux). All Ussing chamber fluxes were normalized to surface

area (cm^2) prior to analysis. A total of four pairs were made per animal but only pairs with less than 25% difference in TER were considered.

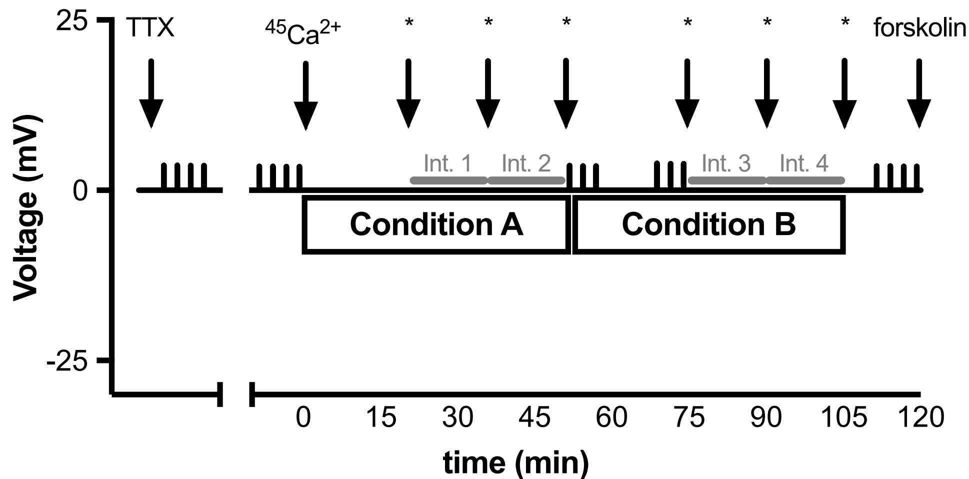

Figure 2.1. **Protocol used to measure unidirectional Ca^{2+} fluxes across intestinal preparations.**

The transepithelial voltage across tissue preparations (y-axis) was clamped to 0 mV for the duration of the experiment (x-axis). The voltage spikes along the x-axis correspond to 2 mV pulses applied and used to determine the transepithelial resistance. 0.1 µM tetrodotoxin (TTX) was added basolaterally first and the resulting short-circuit current allowed to stabilize. At time 0, the solutions were exchanged for fresh ones with one side spiked with $^{45}Ca^{2+}$. Asterisks indicate the time points when samples were taken for radioactivity measurements. Two gray horizontal lines represent 15-min time intervals, where unidirectional $^{45}Ca^{2+}$ flux was calculated for each conditions. The tissue was again pulsed and10 µM forskolin was added at the end of the experiment to confirm tissue viability.

2.5 Xenopus oocyte expression and two-electrode voltage clamp

The preparation of Xenopus oocytes and the two-electrode voltage clamp experiments were performed as previously (Zheng et al., 2018). Capped RNA of human TRPV6 [accession #: NM_018646; generated using *in vitro* transcription with mMESSAGE mMACHINE kit (Ambion, Austin, TX)] and human CaSR cDNA (Origene, Rockville, MD, Cat #RC211229) were injected into Xenopus oocytes. Two-days post injection, whole-cell currents of oocytes were recorded at

room temperature in a standard extracellular solution containing 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 10 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES; pH 7.5) with or without 5 mM Ca^{2+}. The two electrodes (capillary pipettes; Warner Instruments, Hamden, CT) impaling an oocyte were filled with 3 M KCl to form a tip resistance of 0.3–2 MΩ. A Geneclamp 500B amplifier and Digidata 1322A AD/DA converter (Molecular Devices, Union City, CA) were used to obtain the currents. pClamp 9 software (Axon Instruments, Union City, CA) was used for data acquisition and analysis. Currents and voltages were digitally recorded at 200 ms/sample and filtered at 2 kHz through a Bessel filter. Sigma Plot 14 (Systat Software, San Jose, CA) was used for plotting data.

Oocytes surface protein expression was determined with a biotinylation assay as previously (Zheng et al., 2018). In short, the oocytes were incubated with 0.5 mg/mL sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) for 30 min at room temperature and non-reacted biotin was quenched with 1 M NH_4Cl. After a wash, oocytes were harvested in ice-cold CellLytic M lysis buffer (Sigma-Aldrich, St. Louis, MO) with a 1X proteinase inhibitor mixture (Thermo Scientific, Waltham, MA). The surface proteins were absorbed by 100 µL streptavidin (Pierce, Rockford, IL) at 4°C overnight and subjected to SDS page as above.

2.6 Statistics

Data are presented as means ± standard error of the mean (SEM), and all data reported are based on measurements made on at least 6 different animals. One-way ANOVA and students' t-tests (GraphPad, La Jolla, CA) were carried out to determine statistical significance as appropriate, and values < 0.05 were considered statistically significant.

Chapter 3: Results

Modified from published work (J. J. Lee et al., 2019).
The author performed all experiments and data analysis except: Dr. Henrik Dimke performed altered Ca^{2+} homeostasis studies; Debbie O'Neill performed protein isolation for **Figure 8B** and **C**; Xiong Liu performed experiments and data analysis for **Figure 12**.

3.1 Alteration in Ca^{2+} homeostasis affects the mRNA expression of transcellular Ca^{2+} absorption mediators

3.1.1 Altered Ca^{2+} containing diets alter the mRNA expression of transcellular Ca^{2+} absorption mediators

The expression of transcellular Ca^{2+} absorption genes was measured on intestinal tissue from FVB/N mice fed a low (0.01%), normal (0.6%), or high (2%) Ca^{2+} diet for 21 days (n = 7 for each group). Consistent with the known increase in 1,25[OH]$_2$D$_3$ induced by a low Ca^{2+} diet (Dimke et al., 2013), *Trpv6* mRNA expression was increased in mice on a low Ca^{2+} diet, with the greatest, >30-fold increase, observed in the proximal colon (**Figure 3.1A**). The high Ca^{2+} diet suppressed *Trpv6* expression in the duodenum, perhaps due to a lack of 1,25[OH]$_2$D$_3$, although a direct inhibitory effect of plasma Ca^{2+} cannot be excluded. This same trend of an inverse relationship between dietary Ca^{2+} content and expression was observed for *S100g*, which encodes the intracellular Ca^{2+} buffering and shuttling protein CABP9K. The mRNA expression of the basolateral Ca^{2+} efflux transporters NCX1 (*Slc8a1*) and PMCA1b (*Atp2b1*) was unaltered in all tissues under the different dietary conditions (**Figure 3.1A-C**).

3.1.2 Changes observed in mice on the low Ca^{2+} diet are likely mediated by changes in 1,25[OH]$_2$D$_3$ levels

The ionized plasma Ca^{2+} levels of mice on altered-Ca^{2+} diets were not different (Dimke et al., 2013). This was likely the result of enhanced 1,25[OH]$_2$D$_3$ production on the low Ca^{2+} diet (Benn et al., 2008; Cui et al., 2012; G. S. Lee et al., 2009). To assess the effect of 1,25[OH]$_2$D$_3$ on intestinal expression of transcellular Ca^{2+} mediators, the hormone was directly administered (via intraperitoneal injection) to mice daily (500 pg/g body weight for 5 days) and the studies repeated (Dimke et al., 2013). It has been widely observed that low plasma Ca^{2+} stimulates PTH secretion, which indirectly enhances transcellular Ca^{2+} absorption from the intestine through

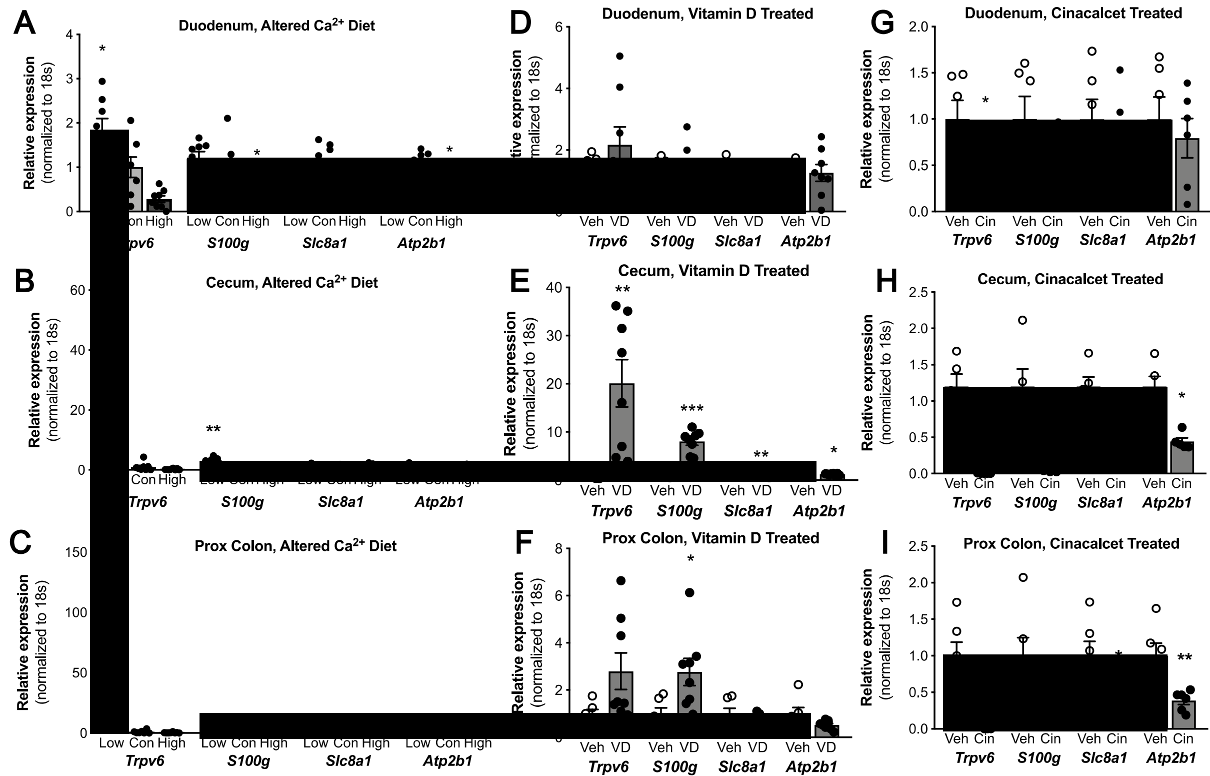

Figure 3.1. **Relative intestinal mRNA expression of transcellular Ca^{2+} transport mediators under altered extracellular Ca^{2+} conditions.**

(A-C) Relative mRNA expression of transcellular Ca^{2+} transport mediators, TRPV6 (*Trpv6*), Calbindin$_{D9K}$ (*S100g*), Na^{+}- Ca^{2+} exchanger (*Slc8a1*) or plasma membrane calcium dependent ATPase 1b (*Atp2b1*), normalized to 18S rRNA expression in mice on high-, normal- (norm), or low-Ca^{2+} diet for 21 days (n = 7 for each). (D-F) Relative mRNA expression in animals treated with 1,25[OH]$_2$D$_3$ (VD) or vehicle (Veh) (n = 8 for each). (G-I) Relative mRNA expression in animals treated with cinacalcet (Cin) or control (Con) diet (n = 8 for each). All data are presented as the mean ± SEM, normalized to the normal/control diet mice. Asterisks indicate a statistically significant difference from the normal/control mice (by one-way ANOVA or student's unpaired t-tests; *P < 0.05, **P < 0.01, ***P < 0.001). The animal experiments were designed and performed by Dr. Henrik Dimke; the author isolated mRNA and performed qPCR from collected tissue.

1,25[OH]$_2$D$_3$ activation (Christakos, 2012; Christakos et al., 2011; Wongdee & Charoenphandhu, 2015). Consistent with this, FVB/N mice that received daily injections of 1,25[OH]$_2$D$_3$ for 5 days showed increased expression of *Trpv6* and *S100g* compared to the vehicle group (**Figure 3.1E-F**). These data are consistent with the observation that 1,25[OH]$_2$D$_3$ enhances intestinal Ca^{2+} absorption via increased expression of TRPV6 (van Abel et al., 2003).

Table 1. *Real-time PCR primers and probes for mouse (Mus musculus)*

TRPV6 (*Trpv6*)	**Forward**: TCACCACCTTCCCACAATC
	Reverse: CTGTCTCCTCCCAGGTCTAATA
	Probe: CACAGAACTCTTCCCAGGGTGCTC
Calbindin-D9k (*S100g*)	**Forward**: TGGATAAGAATGGCGATGGAG
	Reverse: GCTAGAGCTTCAGGATTGGAG
	Probe: ACAGCACCTACTGATTGAACGCACG
PMCA1b (*Atp2b1*)	**Forward**: CGCCATCTTCTGCACCATT
	Reverse: CAGCCATTGCTCTATTGAAAGTTC
	Probe: CAGCTGAAAGGCTTCCCGCCAAA
NCX (Slc8a1)	**Forward**: TGGTCTGAAAGATTCCGTGAC
	Reverse: AGTGACATTGCCTATAGACGC
	Probe: AGCTACCCAGGACCAGTATGCAGA
GAPDH	CAT NO: Mn03302249-g1 (ABI)

See text for definitions.

3.1.3 Chronic cinacalcet treatment suppressed the expression of transcellular Ca^{2+} absorption mediators

To examine a direct effect of intestinal CaSR activation on *Trpv6*, *S100g*, *Slc8a1*, and *Atp2b1* expression, we administered the calcimimetic cinacalcet, a positive allosteric modulator at the CaSR, in the diet (1 mg/g body weight) for 6 days (Dimke et al., 2013). Lower *Trpv6* expression was observed in all 3 segments of the cinacalcet treated group (**Figure 3.1G-I**). Importantly, 1,25[OH]$_2$D$_3$ levels were not altered in these mice (Dimke et al., 2013). These findings are consistent with a direct effect on a Ca^{2+} sensing mechanism regulating the absorption of Ca^{2+} from the intestine, though we cannot exclude the possibility of other cinacalcet-mediated calciotropic effects (*e.g.* via PTH or FGF23). In addition, the cecum from cinacalcet treated mice showed a significant decrease in *S100g* and *Atp2b1* expression (**Figure 3.1H**), while the proximal colon of cinacalcet treated mice showed significant decreases in all genes involved in transcellular Ca^{2+} absorption (**Figure 5I**). Further, we detected the transcellular Ca^{2+} absorption mediators *Trpv6*, *S100g*, and *Atp2b1* in porcine colon tissue (**Figure 3.2A**), inferring that this pathway also exists in

pigs, a non-rodent model (Guilloteau et al., 2010). Together, these results are consistent with the concept that direct Ca^{2+} sensing by the intestine decreases transcellular Ca^{2+} absorption via direct inhibition of gene expression or increased mRNA stability.

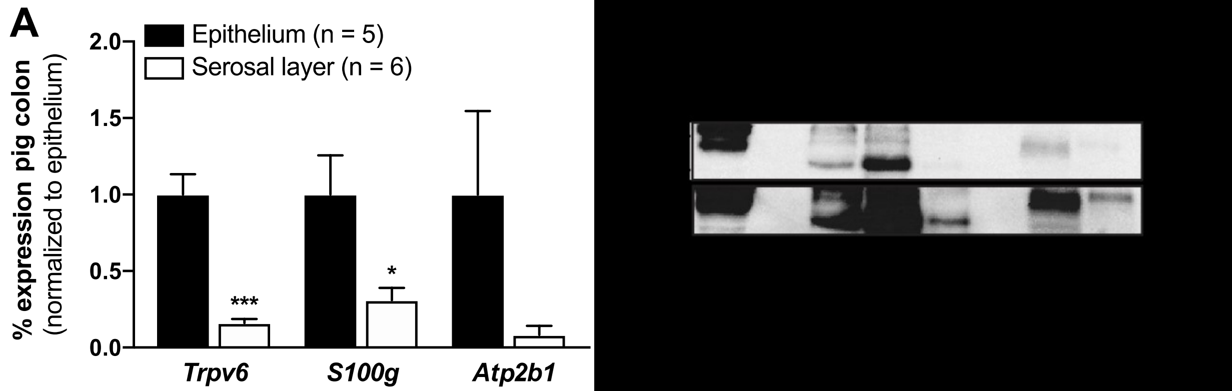

***Figure 3.2.* Relative mRNA expression of transcellular calcium absorption mediators: TRPV6 (Trpv6), CalbindinD9K (Calb9k), Pmca1b (Pmca1b) normalized to GAPDH, extracted from pig colon (A) and protein expression of CaSR in mouse and pig colon (B).**

(A) All data are presented as the mean ± SE. Asterisks indicate a statistical difference from the control mice (student's unpaired t-tests; $*P < 0.05$, $***P < 0.001$). (B) Immunoblot for the CaSR on opossum kidney cells overexpressing human CaSR (OK^+), empty vector (OK^-), mouse parathyroid gland (PT), proximal colonocyte brush-border vesicles (BBV), basolateral vesicles (BLV), pig proximal colonocyte BBV or BLV. Pig colonic tissue isolation was performed by laboratory members of Dr. Justine Turner. Debbie O'Neill isolated BBV and BLV.

Table 2. *Real-time PCR primers and probes for pig (Sus scrofa)*

TRPV6 (*Trpv6*)	**Forward**: GTAAGAGCCTGGACGTCATTC
	Reverse: TCTGCCTGTGGAGAAAGTTG
	Probe: TCTGAGCCCATGACTCCTGTCTC
Calbindin-D9k (*S100g*)	**Forward**: AGGCTGAATTCCCCAGTTTAC
	Reverse: CTCCATCTCCATTCTTGTCCAG
	Probe: TCCGAGAACCCTAGATGACCTCTTTCA
PMCA1b (*Atp2b1*)	**Forward**: AGAAGGTGAAGGTGAAACTGG
	Reverse: CTCCTGCTCAATTCGACTCTG
	Probe: TGGTGTGCGTGGTCTTGGTCA
GAPDH	**Forward**: CACTCTTCCACTTTTGATGCTG
	Reverse: CCTGTTGCTGTAGCCAAATTC
	Probe: ACCACTTCGTCAAGCTCATTTCCTGT

See text for definitions.

3.2 Extracellular Ca^{2+} inhibits transcellular Ca^{2+} absorption in the proximal colon

Given the changes observed along the intestine in response to chronic systemic cinacalcet exposure, we sought to examine the presence of a direct Ca^{2+} sensing mechanism in the intestine without the influence of calciotropic hormones (*e.g.* PTH, $1,25[OH]_2D_3$). We hypothesized the presence of an acute extracellular Ca^{2+} sensing mechanism that decreases transcellular Ca^{2+} absorption, independent of calciotropic hormones. To test our hypothesis, we measured net flux of Ca^{2+} across the proximal colon *ex vivo* in Ussing chambers. We employed Ussing chambers and intestinal preparations *ex vivo* to avoid the confounding effects of calciotropic hormones. Full thickness preparations of proximal colon from wild-type mice were mounted in Ussing chambers with equal concentrations of Ca^{2+} (1.25 mM) in the Ringer's Buffer on both apical and basolateral sides of the tissue, with the transepithelial voltage clamped to 0 mV. This eliminated a net driving force for paracellular Ca^{2+} movement, enabling us to attribute net flux to movement through the transcellular Ca^{2+} transport pathway. **Figure 2.1** illustrates the protocol used for Ca^{2+} flux experiments. The net Ca^{2+} flux obtained under Condition A (control) was compared with the one obtained under Condition B (for example bilateral application of 10 μM cinacalcet, activating the CaSR). N.B. tissues were subjected to 20 minutes of incubation time prior to obtaining samples. **Figure 3.3A** displays a typical short-circuit current trace recorded for a single channel. We found that the net Ca^{2+} absorption under the control condition (14.1 ± 5.4 $nmol \cdot h^{-1} \cdot cm^{-2}$) was significantly reduced following cinacalcet administration (3.9 ± 6.2 $nmol \cdot h^{-1} \cdot cm^{-2}$) (**Figure 3.3B**). This strongly infers that the proximal colon can sense extracellular Ca^{2+} and attenuate absorption in response to elevated Ca^{2+} levels.

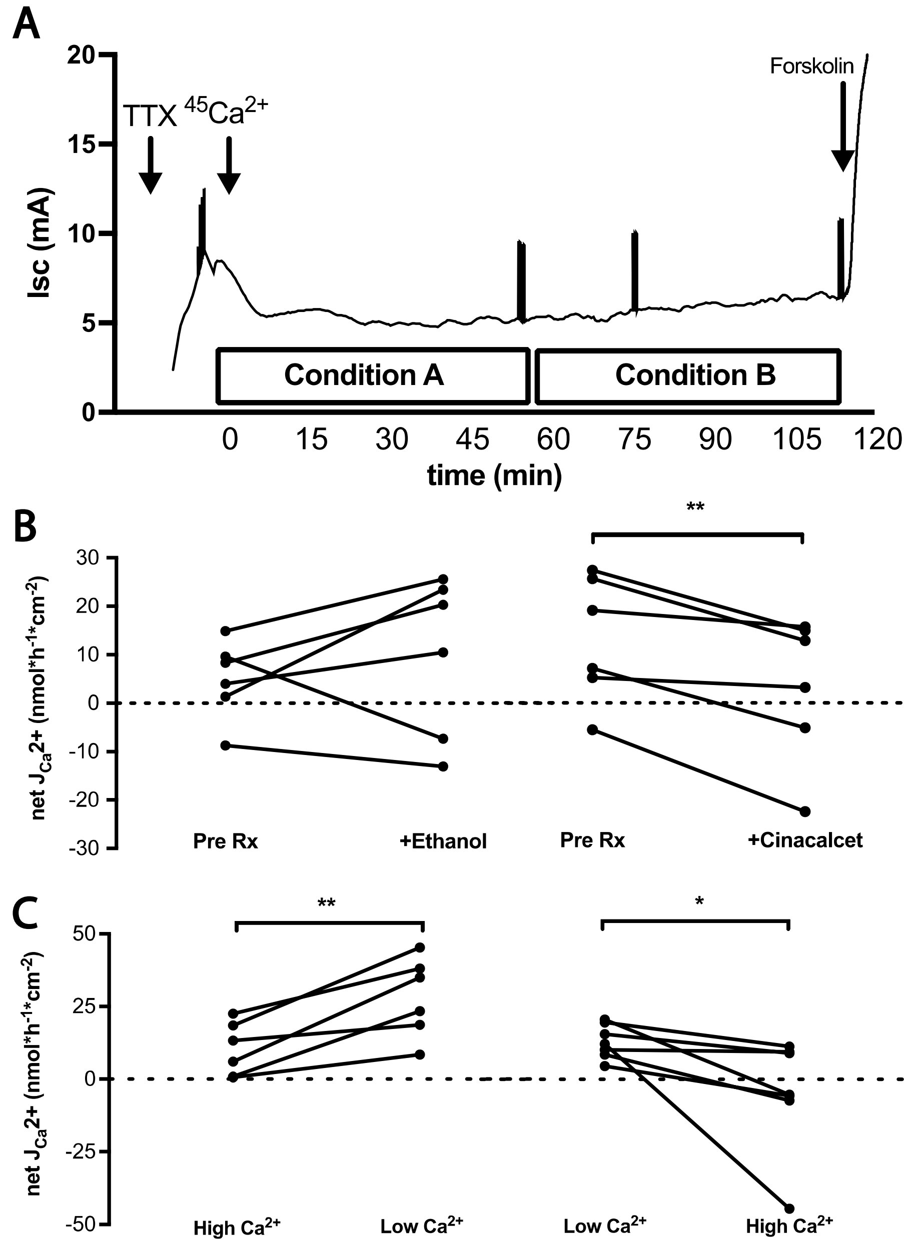

Figure 3.3. **The effect of altering extracellular Ca^{2+} on Ca^{2+} fluxes across mouse proximal colon.**

(A) An example of the short-circuit current (Isc) recorded throughout Protocol 1. Tetrodotoxin (TTX) was added and Isc allowed to stabilize. The. Isc spikes occur in response to 2 mV pulses. At the second arrow, solutions were exchanged with one side only containing $^{45}Ca^{2+}$. The tissue was deemed viable if the Isc increased > 3X with forskolin administration at the end of the experiment. (B) Changes in the net Ca^{2+} flux (net $J_{Ca}{}^{2+}$) between Condition A: pre-treatment (Pre Rx) and Condition B: vehicle (ethanol) or 10 µM cinacalcet. (C) The change in net $J_{Ca}{}^{2+}$ between Condition A: high-Ca^{2+} (2.5 mM) and Condition B: low-Ca^{2+} (0.5 mM) or the converse. Raw values are presented; transepithelial resistance (TER) are shown in Table 3; asterisks indicate a statistical difference between the conditions (student's paired t-tests; *$P < 0.05$; **$P < 0.01$).

To confirm a physiological role for extracellular Ca^{2+} in the regulation of transcellular Ca^{2+} absorption, we next examined Ca^{2+} fluxes across proximal colon in Ussing chambers *ex vivo* under high or low extracellular Ca^{2+} concentrations. To this end, net Ca^{2+} flux across proximal colon was measured before and after changing both chambers from a high-Ca^{2+} (2.5 mM) to a low-Ca^{2+} (0.5 mM) containing buffer as well as the opposite (i.e. from low-to-high Ca^{2+} containing buffers). When the extracellular Ca^{2+} concentration was decreased by changing from a high-Ca^{2+} buffer to a low-Ca^{2+} buffer, net Ca^{2+} flux significantly increased from 10.3 ± 3.8 $nmol \cdot h^{-1} \cdot cm^{-2}$ to 28.1 ± 5.6 $nmol \cdot h^{-1} \cdot cm^{-2}$. Conversely, when the extracellular Ca^{2+} concentration was increased, by changing from a low-Ca^{2+} buffer to a high-Ca^{2+} buffer, the net Ca^{2+} flux significantly decreased from 13.7 ± 2.2 $nmol \cdot h^{-1} \cdot cm^{-2}$ to -4.2 ± 7.4 $nmol \cdot h^{-1} \cdot cm^{-2}$ (**Figure 3.3C**). These results are consistent with the proximal colon directly sensing extracellular Ca^{2+} and acutely altering transcellular Ca^{2+} absorption to maintain plasma Ca^{2+} within physiological limits.

3.3 Delineating the mechanism by which intestinal CaSR inhibits Ca^{2+} absorption

3.3.1 Increased basolateral extracellular Ca^{2+} attenuates transcellular Ca^{2+} absorption

The CaSR has been found throughout the intestine, including in both the apical and basolateral membranes of proximal colonocytes (Chattopadhyay et al., 1998; S. Cheng et al., 2002; Gama et al., 1997). We confirmed the expression of the CaSR in the proximal colon by measuring the mRNA levels via quantitative real-time PCR (**Figure 3.4A**). Thereafter, we immunoblotted for the CaSR on samples isolated from proximal colon brush border vesicles, basolateral vesicles or wild type mouse parathyroid. We were able to detect CaSR in isolates of both apical and basolateral membranes (**Figure 3.4B**). The specificity of the brush border and basolateral vesicle isolation technique was confirmed by immunoblotting against NHE3 (an apical specific transporter)

and GLUT2 (a basolateral specific transporter). We also observed CaSR expression in the proximal colon of pigs (**Figure 3.4B**).

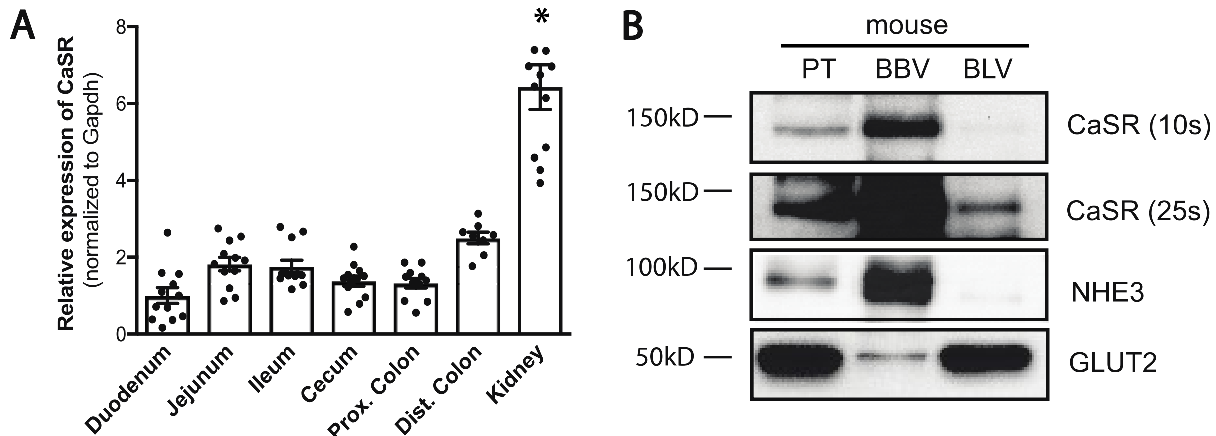

Figure 3.4. **Expression of CaSR in the mouse intestine.**

(A) Relative mRNA expression of CaSR throughout mouse intestine (n = 12), normalized to duodenum. Asterisks indicate a statistically significant difference from the duodenum (by one-way ANOVA; $^*P < 0.05$) (B) Immunoblot for the CaSR on mouse parathyroid gland (PT), proximal colonocyte brush-border vesicles (BBV) or basolateral vesicles (BLV). Na^+/H^+ Exchanger (NHE3) and glucose transporter 2 (GLUT2) were used as controls for BBV and BLV respectively. BBV and BLV isolations were performed by Debbie O'Neill.

Next, we performed net Ca^{2+} flux studies, in Ussing chambers *ex vivo*, as described above, but with 10 μM cinacalcet applied to either the basolateral or the apical hemi-chamber to determine whether apical and/or basolateral Ca^{2+} sensing mediates the decrease in transcellular Ca^{2+} absorption across the proximal colon. The apically treated hemi-chambers did not display a significant change in net Ca^{2+} flux between control and apical cinacalcet treatment (3.94 ± 6.6 $nmol \cdot h^{-1} \cdot cm^{-2}$ *vs.* 9.01 ± 9.3 $nmol \cdot h^{-1} \cdot cm^{-2}$) (**Figure 3.5A**). In contrast, the basolaterally-treated hemi-chambers showed a significant decrease in net Ca^{2+} flux between control and cinacalcet treated conditions (24.7 ± 4.7 $nmol \cdot h^{-1} \cdot cm^{-2}$ *vs* 15.1 ± 4.1 $nmol \cdot h^{-1} \cdot cm^{-2}$) (**Figure 3.5B**).

Interestingly, basolateral application of cinacalcet attenuated net Ca^{2+} flux across the duodenum as well as the cecum (**Figure 3.6**). These data support a basolateral CaSR mediating the decrease in transcellular Ca^{2+} absorption across the intestine.

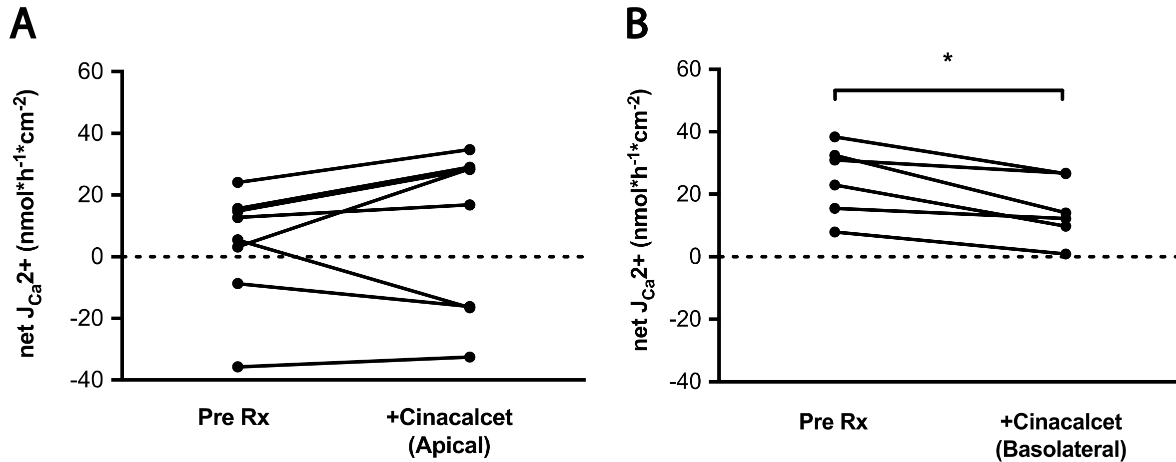

Figure 3.5. **Effect of (A) apical or (B) basolateral CaSR activation on Ca^{2+} fluxes across mouse proximal colon.**

Changes in the net Ca^{2+} flux (net $J_{Ca}{}^{2+}$) in the proximal colon of wild-type mice between Condition A: pre-treatment (Pre Rx) and Condition B: apical or basolateral 10 μM cinacalcet application (respectively). Raw values are presented; transepithelial resistance (TER) are shown in Table 3; asterisks indicate a statistical difference between the conditions (student's paired t-tests; *$P < 0.05$).

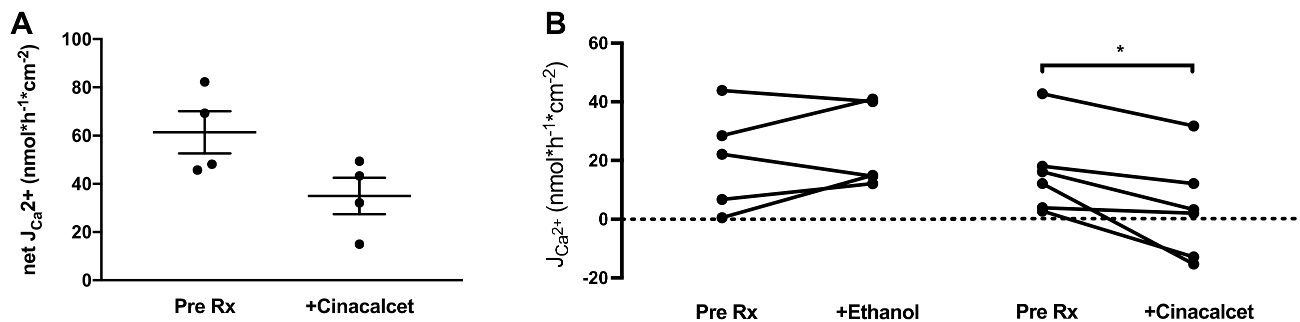

Figure 3.6. **The effect of basolateral CaSR activation on Ca^{2+} fluxes across mouse duodenum (A) and cecum (B).**

(A) The net Ca^{2+} flux (net J_{Ca}^{2+}) observed before and after ethanol treated duodenum or cinacalcet treated. N.B. duodenum could not be kept intact for long enough to perform paired experiments. (B) Changes in the net Ca^{2+} flux (net J_{Ca}^{2+}) between Condition A: pre-treatment (Pre Rx) and Condition B: vehicle (ethanol) or basolateral 10 μM cinacalcet. Raw values are presented; transepithelial resistance (TER) are shown in Table 3; asterisks indicate a statistical difference between the conditions (student's unpaired t-test for (A) and paired t-tests for (B); *P < 0.05).

3.3.2 TRPV6 is necessary for altered transcellular Ca^{2+} absorption across the proximal large bowel

To delineate the apical influx pathway mediating altered transcellular Ca^{2+} flux in response to increased basolateral extracellular Ca^{2+}, we repeated the Ca^{2+} flux studies on wild-type ($TRPV6^{WT/WT}$) and $TRPV6^{D541A/D541A}$ knock-in mice. These animals express TRPV6 with a D541A mutation in the pore loop, rendering TRPV6 non-functional (Weissgerber et al., 2011). First, we compared net Ca^{2+} fluxes of wild-type vs mutant mice. $TRPV6^{WT/WT}$ mice had significantly greater net Ca^{2+} flux across the proximal colon (11.9 ± 1.5 $nmol \cdot h^{-1} \cdot cm^{-2}$) under control condition (Condition A), compared to the $TRPV6^{D541A/D541}$ mice (-1.0 ± 3.6 $nmol \cdot h^{-1} \cdot cm^{-2}$) (**Figure 3.7A**). However, $TRPV6^{WT/WT}$ mice had significantly reduced net Ca^{2+} flux after basolateral cinacalcet treatment (0.8 ± 2.7 $nmol \cdot h^{-1} \cdot cm^{-2}$), while net Ca^{2+} flux was unchanged in $TRPV6^{D541A/D541}$ mice (9.5 ± 8.7 $nmol \cdot h^{-1} \cdot cm^{-2}$) (**Figure 3.7A**).

As $TRPV6^{D541/D541}$ mice do not display net Ca^{2+} flux at baseline, there is little baseline net Ca^{2+} flux to be inhibited by extracellular Ca^{2+}. We therefore sought to stimulate net Ca^{2+} flux in the $TRPV6^{D541/D541}$ mice. To do so, we exposed the tissue to high extracellular Ca^{2+} buffer (Condition A) and then changed to a low extracellular Ca^{2+} buffer (Condition B). Again, the $TRPV6^{WT/WT}$ mice had significantly greater net Ca^{2+} flux (10.3 ± 3.8 $nmol \cdot h^{-1} \cdot cm^{-2}$) in the presence of the initial high extracellular Ca^{2+} buffer, compared to the $TRPV6^{D541A/D541}$ mice (-6.83 ± 7.1 $nmol \cdot h^{-1} \cdot cm^{-2}$) (**Figure 3.7B**). When proximal colon from $TRPV6^{WT/WT}$ mice was exposed to the

low-Ca^{2+} buffer, net Ca^{2+} flux increased to 28.1 ± 5.6 nmol·h^{-1}·cm^{-2}, in contrast to no change in net Ca^{2+} flux across proximal colon of TRPV6$^{D541A/D541}$ mice (-0.5 ± 7.9 nmol·h^{-1}·cm^{-2}). These results implicate TRPV6 in mediating transcellular Ca^{2+} absorption across the proximal colon which acutely alters apical Ca^{2+} entry in response to changes in basolateral extracellular Ca^{2+}.

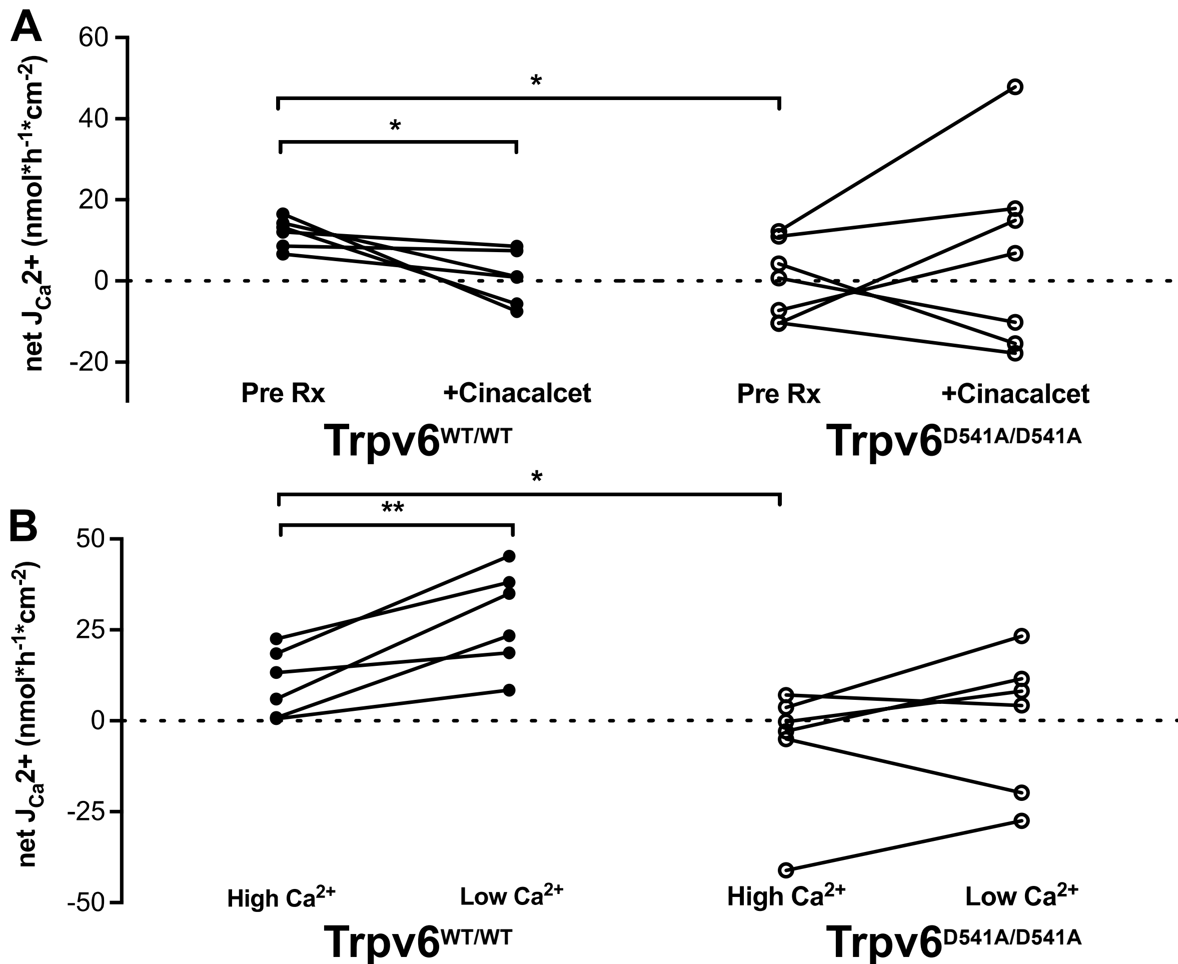

Figure 3.7. **Effect of extracellular Ca^{2+} on Ca^{2+} fluxes across proximal colon from TRPV6$^{WT/WT}$ or TRPV6$^{D541A/D541}$ mice.**

(A) Change in net Ca^{2+} flux (net $J_{Ca}{}^{2+}$) between Condition A: pre-treatment (Pre Rx) and Condition B: basolateral 10 μM cinacalcet application. (B) Change in net $J_{Ca}{}^{2+}$ between Condition A: high-Ca^{2+} (2.5 mM) and Condition B: low-Ca^{2+} (0.5 mM). Raw values are presented; transepithelial resistance (TER) are shown in Table 3; asterisks indicate a statistical difference between conditions (student's paired t-test, for within genotype comparisons or unpaired t-tests for between genotype comparison; *P < 0.05; **P < 0.01).

3.3.3 CaSR expression is sufficient for TRPV6 to respond to extracellular Ca^{2+} *in Xenopus oocytes*

To understand how the CaSR may confer acute inhibition of Ca^{2+} flux through TRPV6, we sought to reconstitute the system *in vitro*. To this end, we expressed human TRPV6 and the CaSR in *Xenopus* oocytes and measured Ca^{2+} currents (I_{Ca}). Oocytes expressing TRPV6 alone failed to decrease I_{Ca} after a 30 min incubation with 10 μM cinacalcet (**Figure 3.8A**) (Peng et al., 1999). In contrast, oocytes co-expressing the CaSR and TRPV6 displayed a significant reduction in I_{Ca} after cinacalcet treatment compared to vehicle treatment (**Figure 3.8B**). These results are consistent with our *ex vivo* observation that CaSR activation inhibits Ca^{2+} flux through TRPV6 acutely.

CaSR-mediated inhibition of TRPV6 activity could be due to activation of one of the small G- proteins including G_i, $G_{q/11}$, and $G_{12/13}$, and their respective downstream signaling (Conigrave & Ward, 2013). Previous studies identified a role for phospholipase C (PLC) in the regulation of Trpv6 *in vitro* (Nilius et al., 2008; Thyagarajan et al., 2009; Thyagarajan et al., 2008; Vachel et al., 2015; Zakharian et al., 2011). We therefore measured normalized I_{Ca} in TRPV6 and CaSR expressing oocytes in the presence of U73122 (5 μM), a PLC inhibitor, or in the presence of U73122 and cinacalcet. Consistent with a role for PLC in inactivating TRPV6, the PLC inhibitor increased I_{Ca} even in the absence of the CaSR (**Figure 3.8A**). Further, PLC inhibition increased I_{ca} in the absence and presence of cinacalcet, implicating PLC inhibition in the CaSR mediated decrease in TRPV6 activity (**Figure 3.8A** and **B**). We next examined the effects of cinacalcet and U73122 on total and surface expression of TRPV6 and the CaSR in *Xenopus* oocytes and found that their membrane expression was not altered by either drug (**Figure 3.8C** and **D**). Together, these data strongly support that the PLC pathway mediates the inhibition of TRPV6 channel activity by the CaSR.

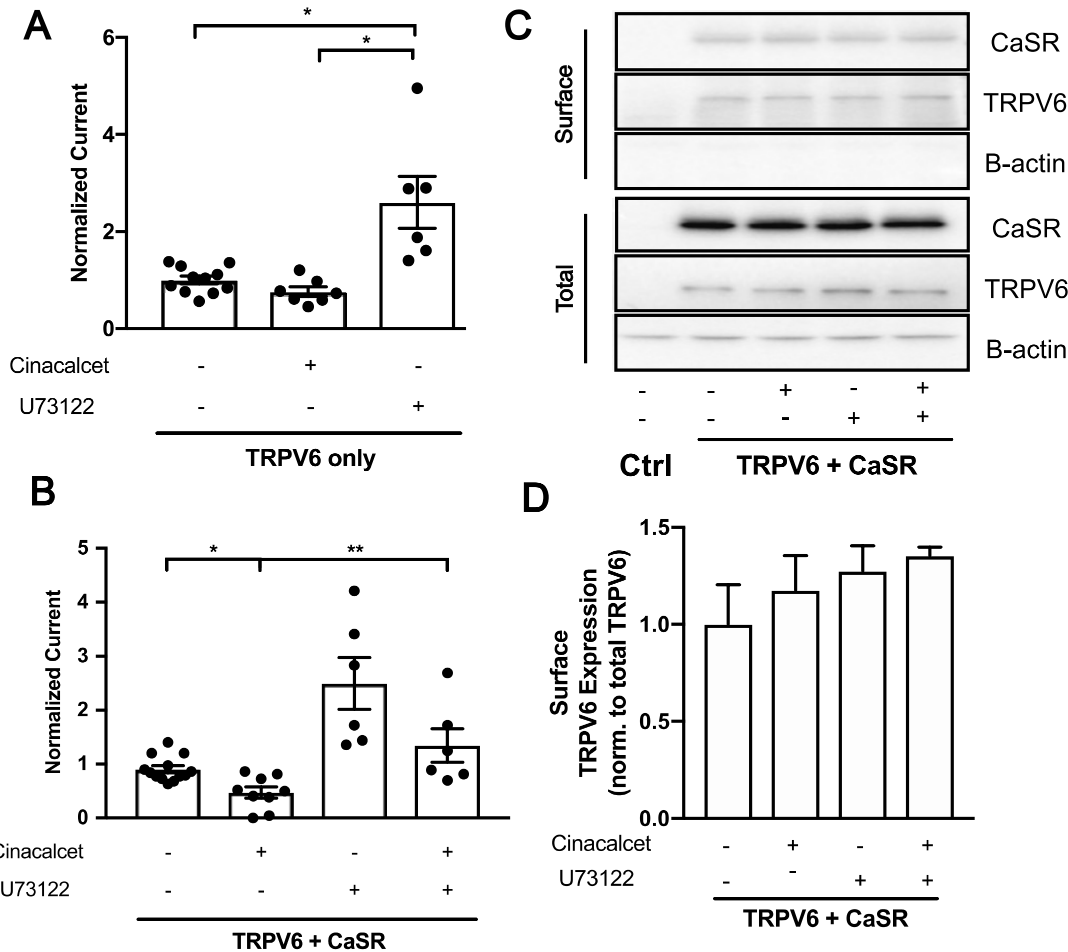

Figure 3.8. **Characterization of CaSR activation on TRPV6 function in Xenopus oocytes expressing TRPV6 and CaSR.**

(A) Ca^{2+}-induced currents (I_{Ca}) in TRPV6 only expressing oocytes in the presence or absence of cinacalcet and/or U73122 measured at – 50 mV with 30 min incubation time. (B) Effect of CaSR activation on I_{Ca} in TRPV6 expressing oocytes in the presence and absence of cinacalcet and/or U73122, a PLC inhibitor. Mean I_{Ca} values obtained from TRPV6 and CaSR expressing oocytes were normalized to vehicle I_{Ca} values from TRPV6 only expressing oocytes ± SEM (A); asterisks indicate a statistically significant difference between the conditions (multiple comparisons non-parametric test; *$P < 0.05$; **$P < 0.01$). (C) Effect of cinacalcet and/or U73122 on the plasma membrane expression of TRPV6 and CaSR in oocytes determined by immunoblot. β-actin was blotted (bottom) as a loading control. (D) Quantification of surface TRPV6 expression, normalized to total TRPV6 (n = 3 each). Experiments were performed by Xiong Liu from Dr. Xing Zhen Chen laboratory.

3.3.4 CaSR activation inhibits transcellular Ca^{2+} *absorption via phospholipase C activation*

Finally, the involvement of PLC in CaSR-mediated regulation of TRPV6 was investigated in our murine model *ex vivo*. To do so, we again employed the PLC inhibitor U73122 in combination with cinacalcet in Ussing chambers mounted with wild-type mouse proximal colon. In our previous experimental approach, net Ca^{2+} flux was compared between Condition A (control) and Condition B (in the presence of cinacalcet in the basolateral hemi-chamber). For these experiments, we had a similar condition A, but for condition B, we administered either cinacalcet (10 μM) + vehicle (DMSO) or cinacalcet (10 μM) + U73122 (10 μM). In the cinacalcet/vehicle treatment group, we observed a significant decrease in net Ca^{2+} flux (**Figure 3.9**), consistent with our previous experiments. However, co-incubation with the PLC inhibitor prevented the inhibitory effect of cinacalcet (**Figure 3.9**). These data are thus in agreement with our *in vitro* data (**Figure 3.8**), and together infer that a basolateral Ca^{2+} sensing mechanism mediates a decrease in transcellular Ca^{2+} transport through TRPV6 via PLC in the proximal colon.

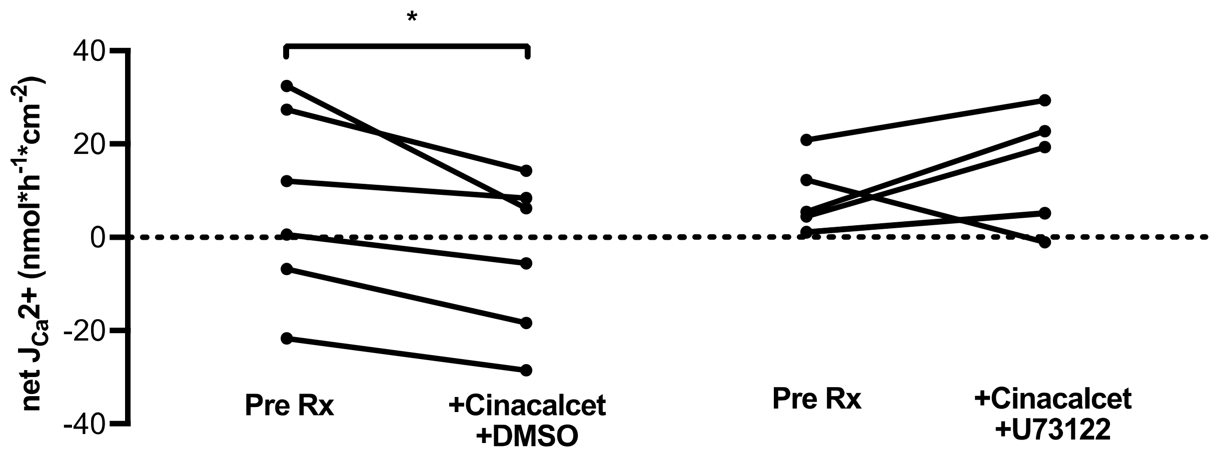

Figure 3.9. **Effect of phospholipase C (PLC) inhibition on CaSR-mediated inhibition of Ca^{2+} absorption from mouse proximal colon.**

Raw net Ca^{2+} flux (net $J_{Ca}{}^{2+}$) values are plotted for Condition A: pre-treatment (Pre Rx) and Condition B: basolateral 10 µM cinacalcet and vehicle (DMSO) or PLC inhibitor U73122 application (10 µM final concentration). Raw values are presented; transepithelial resistance (TER) are shown in Table 3; asterisks indicate a statistical difference between the conditions (student's paired t-tests; $*P < 0.05$).

Table 3. Transepithelial Resistance (Ω*cm^2) of Ussing Chamber Experiments (Figures. 3.3, 3.5 - 3.7, 3.9)

	Before (Condition A)	After (Condition B)
Figure 3.3B. Vehicle	68.7 ±3.9	45.1 ±1.0
Figure 3.3B. Cinacalcet	52.4 ±2.3	40.5 ±3.2
Figure 3.3C. High to Low	56.8 ±1.6	37.2 ±0.9
Figure 3.3C. Low to High	64.7 ±2.5	43.6 ±1.9
Figure 3.5A. Cinacalcet (AP)	65.1 ±3.7	44.2 ±2.5
Figure 3.5B. Cinacalcet (BL)	60.9 ±3.4	37.0 ±2.0
Figure 3.6A. Duodenum	(Vehicle)	(Cinacalcet)
Figure 3.6B. Cecum Vehicle		
Figure 3.6B. Cecum Cinacalcet		
Figure 3.7A. $TRPV6^{WT/WT}$	60.4 ±1.2	38.3 ±2.0
Figure 3.7A. $TRPV6^{D541A/D541A}$	64.2 ±2.4	38.8 ±2.3
Figure 3.7B. $TRPV6^{WT/WT}$	56.8 ±1.6	37.2 ±0.9
Figure 3.7B. $TRPV6^{D541A/D541A}$	59.2 ±2.4	41.1 ±2.4
Figure 3.9. Vehicle	66.3 ±3.3	39.6 ±2.2
Figure 3.9. U73122	66.9 ±2.0	47.2 ±3.1

Chapter 4: Discussion

The CaSR is expressed throughout the intestine, however, a direct role for the intestinal CaSR in maintaining Ca^{2+} homeostasis has not been described (E. M. Brown, 2013; Tang et al., 2016). In general, adjustment of intestinal Ca^{2+} absorption has been thought to occur by reducing circulating $1,25[OH]_2D_3$, secondary to a decrease in PTH secretion induced by lower blood Ca^{2+} levels. However, such a mechanism would be slow to respond to acute elevations in plasma Ca^{2+}. We therefore assessed whether the intestine can directly adjust Ca^{2+} absorption in response to extracellular Ca^{2+}. Herein, we report that the intestine has an extracellular Ca^{2+} sensing mechanism, which alters transcellular Ca^{2+} absorption through TRPV6. This is predominantly based on three observations. 1) Both increased extracellular Ca^{2+} and a calcimimetic decreased transcellular Ca^{2+} absorption in Ussing chambers *ex vivo*. 2) This alteration in transcellular Ca^{2+} absorption is driven by TRPV6 since $TRPV6^{WT/WT}$, but not $TRPV6^{D541A/D541A}$ mice, alter transcellular Ca^{2+} flux in response to changes in extracellular Ca^{2+}; and 3) extracellular Ca^{2+} in the presence of the CaSR, but not in its absence, inhibits Ca^{2+} mediated TRPV6 currents in oocytes, a process involving PLC *in vitro* and *ex vivo*. Taken together, these results reveal a mechanism in the bowel whereby alterations in plasma Ca^{2+} are detected by a basolateral CaSR, which regulates Ca^{2+} absorption via a TRPV6 pathway so as to maintain Ca^{2+} homeostasis (**Figure 4.1**).

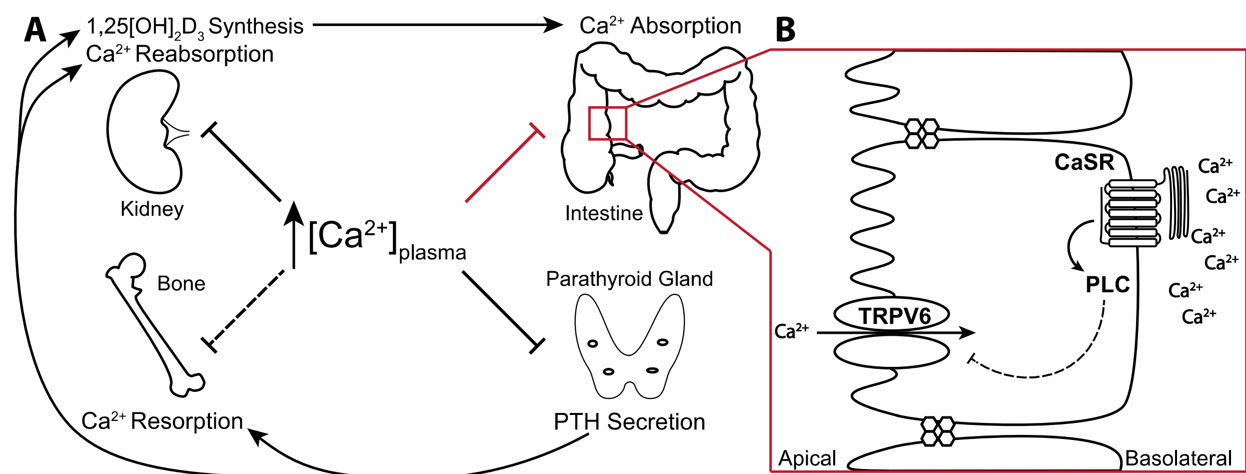

Figure 4.1. **Proposed Model of CaSR-mediated Inhibition of Ca^{2+} Absorption.**

A) In addition to the intestinal CaSR, high plasma Ca^{2+} is detected by the CaSR expressed in kidneys, bone and parathyroid glands and respectively decreases Ca^{2+} reabsorption, resorption, and parathyroid hormone (PTH) secretion. In concert, these actions mediated by the CaSR to reduce plasma Ca^{2+}. This is compounded by reduced PTH actions, which increases plasma Ca^{2+} by increasing Ca^{2+} resorption, reabsorption, and indirectly absorption by activating active vitamin D_3 ($1,25[OH]_2D_3$) in the kidneys, ultimately normalizing plasma Ca^{2+}. B) High plasma Ca^{2+} is detected by the intestinal CaSR, which inhibits TRPV6-mediated transcellular Ca^{2+} transport via phospholipase C (PLC).

4.1 Proposed model for the CaSR-mediated reduced intestinal Ca^{2+} absorption by TRPV6

PTH increases production of $1,25[OH]_2D_3$, which acts on the intestine to increase Ca^{2+} absorption (Alexander et al., 2009; Christakos et al., 2011; Pan et al., 2012). Consistent with this, our data demonstrate that low Ca^{2+} diet fed mice had increased plasma $1,25[OH]_2D_3$, but maintained normal plasma Ca^{2+} (Dimke et al., 2013), and increased expression of transcellular Ca^{2+} absorption mediators (**Figure 3.1**). In addition, direct administration of $1,25[OH]_2D_3$ increased expression of intestinal transcellular Ca^{2+} absorption mediators. However, the degree of increased expression observed was less in the $1,25[OH]_2D_3$ injected group than the mice on a low Ca^{2+} diet. Interestingly, the mice administered $1,25[OH]_2D_3$ also had increased plasma Ca^{2+}, which could have attenuated gene expression via a direct effect on the intestinal CaSR (Dimke et al., 2013). Conversely, a high Ca^{2+} diet decreased expression of these mediators of transcellular Ca^{2+} absorption. This may be due to decreased secretion of PTH and therefore decreased activation of $1,25[OH]_2D_3$ (Dimke et al., 2013). However, it might also be a result of chronic activation of the basolateral intestinal CaSR directly altering expression of transcellular Ca^{2+} absorption mediators. Consistent with this, administration of the calcimimetic, cinacalcet, suppressed plasma PTH levels and *Trpv6* and *S100g* expression, without altering plasma $1,25[OH]_2D_3$ (Dimke et al., 2013). Reduced circulating PTH could decrease $1,25[OH]_2D_3$ levels and consequently reduce the expression of transcellular Ca^{2+} absorption mediators. However, cinacalcet treated mice did not

have reduced circulating $1,25[OH]_2D_3$ (Dimke et al., 2013). Thus, decreased *Trpv6* and *S100g* expression are not a result of PTH-dependent reduction in $1,25[OH]_2D_3$, but instead are due to a potential direct activation of an intestinal CaSR. It is noteworthy that we and others observed CaSR expression along the intestine (Chattopadhyay et al., 1998; S. Cheng et al., 2002). Together, the data is consistent with the bowel altering transcellular Ca^{2+} absorption via transcriptional down regulation directly in response to increased extracellular Ca^{2+}, independent of $1,25[OH]_2D_3$.

The current model of transcellular Ca^{2+} absorption suggests a significant role for TRPV6 (Benn et al., 2008; Cui et al., 2012; Woudenberg-Vrenken et al., 2012). TRPV6 is transcriptionally regulated by $1,25[OH]_2D_3$ and estrogen (Cui et al., 2009; J. C. Fleet et al., 2002; G. S. Lee et al., 2009; Song et al., 2003; Walters et al., 2006). Here, we report alterations in *Trpv6* expression in response to extracellular Ca^{2+}, in the absence of altered $1,25[OH]_2D_3$, adding intestinal CaSR activation to the list of transcriptional regulators. It should be noted that since CABP9K expression is regulated by cytosolic Ca^{2+}, the corresponding changes in CABP9k expression observed likely reflect decreased Ca^{2+} absorption, and therefore, decreased cytosolic Ca^{2+}, rather than a direct transcriptional response to CaSR activation (Cui et al., 2012; G. S. Lee et al., 2009).

Not only have we observed a chronic transcriptional effect of extracellular Ca^{2+} on TRPV6 expression, we also identified an acute direct regulatory role of extracellular Ca^{2+} on TRPV6 activity *ex vivo*. Decreased net Ca^{2+} flux was observed across proximal colon of $TRPV6^{WT/WT}$ mice, but not $TRPV6^{D541A/D541A}$ mutant mice, following basolateral CaSR activation. Similarly, the increased net intestinal Ca^{2+} absorption observed in $TRPV6^{WT/WT}$ mice in response to lower extracellular Ca^{2+} was not observed in $TRPV6^{D541A/D541A}$ mice. These observations directly implicate TRPV6 in mediating altered transcellular Ca^{2+} absorption in response to CaSR activation. This was confirmed *in vitro* with *Xenopus* oocytes. CaSR activation in oocytes expressing TRPV6

and the CaSR decreased TRPV6-mediated Ca^{2+} currents. Previous work found evidence of CaSR-mediated alterations in paracellular Ca^{2+} permeability in colonic and renal epithelium (Dimke et al., 2013; Geibel et al., 2006; Plain et al., 2016). However, our experimental setup allowed us to eliminate the driving force for passive paracellular Ca^{2+} transport (i.e. a transepithelial electrochemical gradient). Thus our results reflect changes in the net Ca^{2+} flux via an active transcellular pathway. Together, these data strongly support the presence of an acute regulatory effect of the CaSR in modifying cellular Ca^{2+} uptake, and thus transcellular Ca^{2+} absorption, via TRPV6.

Acute regulation of epithelial membrane channels can be accomplished by alterations in channel function or membrane expression. Membrane expression of TRPV5, a close family member of TRPV6, is altered in the DCT/CNT thereby regulating channel activity (Hoenderop et al., 2001a; Topala et al., 2009). Therefore, we assessed whether CaSR-mediated TRPV6 regulation was the result of alterations in membrane expression. This was not the case. In *Xenopus* oocytes expressing TRPV6 and the CaSR, cinacalcet had no effect on membrane expression of TRPV6. Unlike the changes in intestinal expression of *Trpv6* mediated by chronic cinacalcet administration, acute changes in TRPV6-mediated Ca^{2+} flux are likely due to a CaSR mediated regulation of TRPV6 activity, rather than expression.

Activation of the CaSR stimulates a network of cell-signalling pathways. In colonocytes, CaSR activation alters fluid absorption via PLC (S. Cheng et al., 2002; Geibel et al., 2006). Consistent with this, PLC inhibition prevented decreased Ca^{2+} flux through TRPV6 in response to activation of the CaSR both *in vitro* and *ex vivo*. PLC is a membrane bound phospholipase that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol and inositol triphosphate (IP3), and IP3 increases intracellular Ca^{2+} (Conigrave & Ward, 2013), a

signalling pathway utilized by the CaSR in parathyroid (Geibel et al., 2006). TRPV6 activity is up-regulated by PIP_2 and down-regulated by intracellular Ca^{2+} (Bodding & Flockerzi, 2004). Extracellular Ca^{2+} inhibits TRPV6 via PIP_2 hydrolysis in whole-cell patch clamp experiments and everted duodenal gut sac $^{45}Ca^{2+}$ transport assays (Thyagarajan et al., 2009; Thyagarajan et al., 2008). Furthermore, increased intracellular Ca^{2+}, another consequence of PLC activation, directly inhibits TRPV6, providing another molecular explanation for how CaSR activation could inhibit TRPV6 (Bodding & Flockerzi, 2004; Hoenderop et al., 2001b). Regardless of the exact downstream mechanism, our data provide evidence of intestinal CaSR-mediated PLC regulation of TRPV6 function.

4.2 Contribution to our Understanding of Intestinal Ca^{2+} Absorption and Regulation

The currently accepted model of intestinal Ca^{2+} absorption is that the duodenum, cecum, and proximal colon are capable of both transcellular and paracellular Ca^{2+} absorption while the jejunum and ileum only contribute paracellular Ca^{2+} absorption and/or secretion (Bronner et al., 1986; Favus, 1985; Hoenderop et al., 2005). There has been greater emphasis on the duodenum as a site of Ca^{2+} absorption and regulation recently (Bronner & Pansu, 1999), however, a significant role for the proximal large bowel in mediating intestinal Ca^{2+} absorption in humans and rodents has been appreciated for decades (Hylander et al., 2009, 2010; Karbach & Feldmeier, 1993; Petith & Schedl, 1976). In addition, multiple studies support the presence of $1,25[OH]_2D_3$-mediated regulation of transcellular Ca^{2+} absorption from the proximal large bowel (Favus et al., 1981; Favus et al., 1980; Harrison & Harrison, 1969; Karbach & Feldmeier, 1993; Petith & Schedl, 1976). Thus, the contribution of this segment to overall Ca^{2+} homeostasis should be considered. Our work provides further evidence that the proximal colon plays a regulatory role in Ca^{2+} homeostasis. We

have identified a novel regulatory mechanism present in the proximal large bowel, which includes a Ca^{2+} sensing mechanism that detects altered extracellular Ca^{2+} and amends Ca^{2+} absorption to restore plasma Ca^{2+}. We hypothesize that luminal Ca^{2+} not absorbed from the duodenum and distal small bowel is likely subjected to fine regulation by the proximal large bowel, which senses the body's extracellular Ca^{2+} and fine-tunes Ca^{2+} absorption and consequently fecal excretion to maintain plasma Ca^{2+} within the physiological range. Interestingly, a similar Ca^{2+} handling mechanism is observed in renal tubules. After significant paracellular reabsorption from the proximal tubule and the TAL, urinary Ca^{2+} excretion is fine-tuned in the more distal DCT/CNT segments, by a transcellular pathway analogous to the one observed in the proximal large bowel (**Figure 4.2**) (Huang et al., 2007; Topala et al., 2009). Our results reveal that these pathways share a similar regulatory mechanism, a direct Ca^{2+} sensing mechanism that affects Ca^{2+} transport across the sensing segment. Further, the DCT/CNT and the large bowel have both been estimated to contribute 10% of Ca^{2+} reabsorption in their respective organ (Bronner & Pansu, 1999; Moor & Bonny, 2016). Together, our findings highlight a significant Ca^{2+} regulatory role in the proximal large bowel and challenge the prevailing contention that this segment is not important for Ca^{2+} homeostasis.

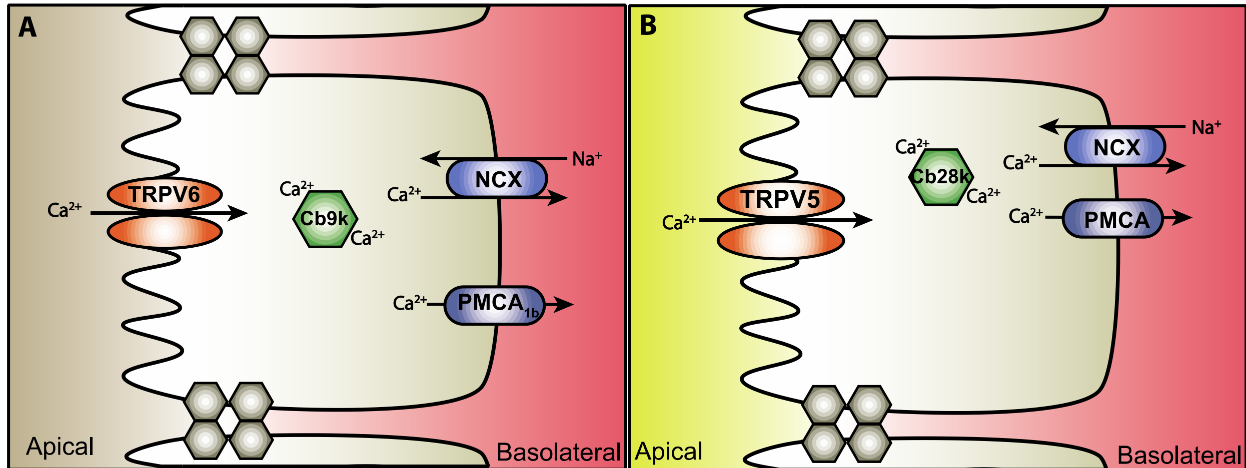

Figure 4.2. **Distal Convoluted Tubule has an Analogous Transcellular Ca^{2+} Transport Pathway Observed in the Intestine.**

A) Active transcellular Ca^{2+} transport pathway in the intestine mediated by transient receptor potential channel, subfamily V, member 6 (TRPV6), Calbindin-D_{9k} (CABP9K; Cb9k), plasma membrane Ca^{2+} ATPase (PMCA1b) and Na^{+}-Ca^{2+} exchanger 1 (NCX1). B) Active transcellular Ca^{2+} transport pathway in the distal convoluted tubule mediated by transient receptor potential channel, subfamily V, member 5 (TRPV5), Calbindin-D_{28k} (CABP28K; Cb28k), plasma membrane Ca^{2+} ATPase (PMCA) and Na^{+}-Ca^{2+} exchanger (NCX).

4.3 Therapeutic considerations

Dietary Ca^{2+} intake is carefully monitored in patients with kidney failure (Block et al., 2004; Brunelli et al., 2015; Floege et al., 2018; "Office of the Surgeon General (US). 2004. Bone Health and Osteoporosis: A Report of the Surgeon General. Rockville (MD): Office of the Surgeon General (US)."). This is due to the common comorbidities seen in chronic kidney disease caused by Ca^{2+} imbalance. Hypercalcaemia in renal failure is associated with vascular calcifications, cardiovascular disease and mortality. Conversely, hypocalcaemia as a consequence to renal failure is also not infrequently observed, resulting in tetany. Both are detrimental for the patient and thus a neutral Ca^{2+} balance is sought. Patients on dialysis, who are at risk of hypercalcaemia, are sometimes treated with cinacalcet; unfortunately, this often causes hypocalcaemia (Block et al., 2004; Brunelli et al., 2015; Floege et al., 2018). This has been attributed to hungry bone syndrome, where the body rapidly lowers plasma PTH, consequently limiting Ca^{2+} import into the blood and increasing export at various epithelial sites of Ca^{2+} transport, including intestine, kidneys, and bone. Our work provides an additional explanation for this observation (Block et al., 2004; Brunelli et al., 2015; Floege et al., 2018). Cinacalcet administration would not only attenuate release of PTH from the parathyroid, but also inhibit Ca^{2+} absorption from the intestine, thereby lowering plasma Ca^{2+} levels in these patients. Thus, one should consider this effect on bowel when prescribing cinacalcet, especially to children who are still mineralizing their bone.

4.4 Conclusion

In conclusion, we demonstrate a Ca^{2+}-sensing mechanism present in proximal large bowel that regulates Ca^{2+} absorption through a transcellular pathway, both acutely and chronically. The transcellular pathway mediating this effect relies on apical Ca^{2+} influx through TRPV6, as this

effect was absent in $TRPV6^{D541A/D541A}$ mutant mice. The CaSR appears to be the sensor of extracellular Ca^{2+} as the pathway can be reconstituted *in vitro* by co-expressing the CaSR and TRPV6 in *Xenopus* oocytes. The cellular mechanism contributing to acute CaSR modulation of TRPV6 function depends on PLC activation, which ultimately results in TRPV6 inactivation. This might be via a decrease in PIP_2 levels and/or an increase in intracellular Ca^{2+} levels. These studies contribute to our understanding of Ca^{2+} homeostasis, providing evidence that the proximal large bowel can sense extracellular Ca^{2+} and adjust intestinal Ca^{2+} absorption to maintain plasma Ca^{2+} levels.

4.5 Future Directions

Ex vivo radioactive Ca^{2+} flux in Ussing chambers have provided a powerful technique to isolate the intestinal transcellular Ca^{2+} transport pathway and implicate CaSR-mediated modulation of TRPV6 activity. A future direction will be to determine the cellular signalling mediators between CaSR activation and TRPV6. Our results described above indicate a possibility of $G\alpha_{q/11}$-protein mediated signalling pathway, where PIP_2 cleavage results in the inactivation of TRPV6. To examine this observation further, we can use intestinal cell lines that endogenously express the CaSR and TRPV6 (if not expressing, we can transfect exogenous CaSR and TRPV6), then measure the effects of $G\alpha_{q/11}$ signalling inhibition or activation on TRPV6 channel function. TRPV6 channel function can be measured using the whole-cell patch clamp technique, where the Ca^{2+} current can be isolated and measured. In conjunction, we can introduce pharmacological agents altering the $G\alpha_{q/11}$ pathway and its downstream mediators, including U73122 (a phospholipase C inhibitor), intracellular Ca^{2+} chelators, and/or PIP_2 analogs to confirm the role of $G\alpha_{q/11}$-protein signalling in CaSR-mediated TRPV6 regulation. Additionally, genetic mutants of

$G\alpha_{11}$ which are activators or suppressors of CaSR signalling (e.g. R181G and I200del) that have been implicated in familial hypocalciuric hypercalcaemia type 2 and autosomal dominant hypocalcaemia type 2 can be introduced into the cell model, thereby providing additional insight into the abnormal Ca^{2+} homeostasis associated with these mutations (Nesbit et al., 2013). Ultimately, this study can provide an in-depth mechanistic understanding of the abovementioned local minute-to-minute regulation of Ca^{2+} absorption by the intestinal CaSR.

There is evidence of acute regulation in paracellular ionic transport mediated by claudins (Weber et al., 2015). Thus, it begs the question whether the intestinal CaSR can also acutely modulate Ca^{2+} absorption through the paracellular pathway. Preliminary data based on basolateral-to-apical unidirectional flux from this thesis, which is theoretically mediated by the paracellular pathway only, reveals increased flux with basolateral cinacalcet application (N.B. transcellular Ca^{2+} transport is unidirectional as the active transporters extrudes cytosolic Ca^{2+} across the basolateral membrane). This suggests that basolateral application of cinacalcet increases paracellular Ca^{2+} permeability, potentially as a means to increase net Ca^{2+} secretion across the colon. These observations were also apparent when bilateral Ringer solutions were exchanged from low-to-high Ca^{2+} containing. Importantly, consistent with an alteration in the paracellular pathway, we did not observe a difference between wildtype *and* TRPV6 mutant mice. Again the physiological explanation could be that high plasma Ca^{2+} increases the permeability of the paracellular Ca^{2+} transport pathway to increase secretion of Ca^{2+} down its electrochemical gradient ultimately leading to the excretion of Ca^{2+}. The permeability of Ca^{2+} across the epithelium can be measured using Ussing chambers and bi-ionic dilution potential experiments. This experimental setup uses a potential difference with known concentration of sodium and Ca^{2+} across the epithelium and measures the arising changes in the current using Ohm's law. Following, the

paracellular permeability of Ca^{2+} can be calculated using the Goldman-Hodgkins-Katz equation (Plain et al., 2016). This methodology can be applied to TRPV6 mutant mice (ensuring limited transcellular Ca^{2+} transport), in the presence and absence of cinacalcet, in order to eliminate potential confounding effects of altered transcellular transport. If altered paracellular permeability is confirmed, intestinal CaSR activation may acutely increase the paracellular permeability of Ca^{2+} in concert with decreased transcellular Ca^{2+} absorption, ultimately increasing Ca^{2+} excretion and limiting absorption.

The CaSR is ubiquitously expressed throughout the body, it would therefore be noteworthy to examine the effect of intestine-specific CaSR knockout on Ca^{2+} homeostasis. Currently, an epithelial-specific CaSR knockout mice have been described (S. X. Cheng et al., 2014); however, its Ca^{2+} phenotype has not been examined. It is likely that these mice would not have altered Ca^{2+} homeostasis, unless exposed to hypercalcaemia, as our work found an acute regulatory role of intestinal CaSR in Ca^{2+} homeostasis in response to elevated plasma Ca^{2+}. We can impose a chronically altered Ca^{2+} environment either by diet or pharmacological agents, and then measure urinary and fecal Ca^{2+} excretion over time. In conjunction, the temporal changes in blood Ca^{2+} levels of the animals can also be measured. When the wildtype mice become hypercalcemic with high Ca^{2+} diet or cinacalcet administration (Dimke et al., 2013), thus activating CaSR, we expect an acute response by the intestinal CaSR, which will decrease Ca^{2+} absorption and increase Ca^{2+} excretion via feces. If the intestinal CaSR knockout mice become more hypercalcemic by these perturbations, it would highlight the physiological importance of the intestinal CaSR in the regulation of Ca^{2+} absorption in relation to overall Ca^{2+} homeostasis. Together, these experiments would provide foundational insight to scientists and clinicians that can be used to manipulate Ca^{2+} absorption and treat diseases of Ca^{2+} imbalance.

BIBLIOGRAPHY

Alexander, R. T., Woudenberg-Vrenken, T. E., Buurman, J., et al. (2009). Klotho prevents renal calcium loss. *J Am Soc Nephrol, 20*(11), 2371-2379. doi:10.1681/asn.2008121273

Amasheh, S., Meiri, N., Gitter, A. H., et al. (2002). Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J Cell Sci, 115*(Pt 24), 4969-4976.

Amemiya, M., Loffing, J., Lotscher, M., et al. (1995). Expression of NHE-3 in the apical membrane of rat renal proximal tubule and thick ascending limb. *Kidney Int, 48*(4), 1206-1215.

Barley, N. F., Howard, A., O'Callaghan, D., Legon, S., & Walters, J. R. (2001). Epithelial calcium transporter expression in human duodenum. *Am J Physiol Gastrointest Liver Physiol, 280*(2), G285-290. doi:10.1152/ajpgi.2001.280.2.G285

Benn, B. S., Ajibade, D., Porta, A., et al. (2008). Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D9k. *Endocrinology, 149*(6), 3196-3205. doi:10.1210/en.2007-1655

Bergeim, O., Stewart, F. T., & Hawk, P. B. (1914). Calcium Metabolism After Thyro-parathyroidectomy. *J Exp Med, 20*(3), 225-236.

Bianco, S. D., Peng, J. B., Takanaga, H., et al. (2007). Marked disturbance of calcium homeostasis in mice with targeted disruption of the Trpv6 calcium channel gene. *J Bone Miner Res, 22*(2), 274-285. doi:10.1359/jbmr.061110

Blau, J. E., & Collins, M. T. (2015). The PTH-Vitamin D-FGF23 axis. *Rev Endocr Metab Disord, 16*(2), 165 -174. doi:10.1007/s11154-015-9318-z

Block, G. A., Martin, K. J., de Francisco, A. L., et al. (2004). Cinacalcet for secondary hyperparathyroidism in patients receiving hemodialysis. *N Engl J Med, 350*(15), 1516-1525. doi:10.1056/NEJMoa031633

Bodding, M., & Flockerzi, V. (2004). Ca^{2+} dependence of the Ca^{2+}-selective TRPV6 channel. *J Biol Chem, 279*(35), 36546-36552. doi:10.1074/jbc.M404679200

Bonny, O., & Edwards, A. (2013). Calcium reabsorption in the distal tubule: regulation by sodium, pH, and flow. *Am J Physiol Renal Physiol, 304*(5), F585-600. doi:10.1152/ajprenal.00493.2012

Bronner, F., & Pansu, D. (1999). Nutritional aspects of calcium absorption. *J Nutr, 129*(1), 9-12. doi:10.1093/jn/129.1.9

Bronner, F., Pansu, D., & Stein, W. D. (1986). An analysis of intestinal calcium transport across the rat intestine. *Am J Physiol, 250*(5 Pt 1), G561-569. doi:10.1152/ajpgi.1986.250.5.G561

Brown, E., Enyedi, P., LeBoff, M., et al. (1987). High extracellular Ca^{2+} and Mg^{2+} stimulate accumulation of inositol phosphates in bovine parathyroid cells. *FEBS Lett, 218*(1), 113-118.

Brown, E. M. (2013). Role of the calcium-sensing receptor in extracellular calcium homeostasis. *Best Pract Res Clin Endocrinol Metab, 27*(3), 333-343. doi:10.1016/j.beem.2013.02.006

Brown, E. M., Brennan, M. F., Hurwitz, S., et al. (1978a). Dispersed cells prepared from human parathyroid glands: distinct calcium sensitivity of adenomas vs. primary hyperplasia. *J Clin Endocrinol Metab, 46*(2), 267-275. doi:10.1210/jcem-46-2-267

Brown, E. M., Fuleihan, G. e.-H., Chen, C. J., & Kifor, O. (1990). A comparison of the effects of divalent and trivalent cations on parathyroid hormone release, 3',5'-cyclic-adenosine monophosphate accumulation, and the levels of inositol phosphates in bovine parathyroid cells. *Endocrinology, 127*(3), 1064-1071. doi:10.1210/endo-127-3-1064

Brown, E. M., Gamba, G., Riccardi, D., et al. (1993). Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. *Nature, 366*(6455), 575-580. doi:10.1038/366575a0

Brown, E. M., Gardner, D. G., Windeck, R. A., & Aurbach, G. D. (1978b). Relationship of intracellular 3',5'-adenosine monophosphate accumulation to parathyroid hormone release from dispersed bovine parathyroid cells. *Endocrinology, 103*(6), 2323-2333. doi:10.1210/endo-103-6-2323

Brown, E. M., Gardner, D. G., Windeck, R. A., et al. (1979). Beta-adrenergically stimulated adenosine 3',5'-monophosphate accumulation in and parathyroid hormone release from dispersed human parathyroid cells. *J Clin Endocrinol Metab, 48*(4), 618-626. doi:10.1210/jcem-48-4-618

Brown, E. M., Hurwitz, S., & Aurbach, G. D. (1976). Preparation of viable isolated bovine parathyroid cells. *Endocrinology, 99*(6), 1582-1588. doi:10.1210/endo-99-6-1582

Brown, E. M., Hurwitz, S., & Aurbach, G. D. (1977). Beta-adrenergic stimulation of cyclic AMP content and parathyroid hormone release from isolated bovine parathyroid cells. *Endocrinology, 100*(6), 1696-1702. doi:10.1210/endo-100-6-1696

Brown, E. M., Leombruno, R., Thatcher, J., & Burrowes, M. (1985). The acute secretory response to alterations in extracellular calcium concentration and dopamine in perifused bovine parathyroid cells. *Endocrinology, 116*(3), 1123-1132. doi:10.1210/endo-116-3-1123

Brown, W. H. (1929). Calcium and Inorganic Phosphorus in the Blood of Rabbts: IV. Influence of Light Environment on Normal Rabbits. *J Exp Med, 49*(1), 103-126.

Brunelli, S. M., Dluzniewski, P. J., Cooper, K., et al. (2015). Management of serum calcium reductions among patients on hemodialysis following cinacalcet initiation. *Pharmacoepidemiol Drug Saf, 24*(10), 1058-1067. doi:10.1002/pds.3845

Centeno, V. A., Diaz de Barboza, G. E., Marchionatti, A. M., et al. (2004). Dietary calcium deficiency increases Ca^{2+} uptake and Ca^{2+} extrusion mechanisms in chick enterocytes. *Comp Biochem Physiol A Mol Integr Physiol, 139*(2), 133-141. doi:10.1016/j.cbpb.2004.08.002

Chambers, T. J., & Magnus, C. J. (1982). Calcitonin alters behaviour of isolated osteoclasts. *J Pathol, 136*(1), 27-39. doi:10.1002/path.1711360104

Chang, W., Tu, C., Chen, T. H., Bikle, D., & Shoback, D. (2008). The extracellular calcium-sensing receptor (CaSR) is a critical modulator of skeletal development. *Sci Signal, 1*(35), ra1. doi:10.1126/scisignal.1159945

Chattopadhyay, N., Cheng, I., Rogers, K., et al. (1998). Identification and localization of extracellular Ca^{2+}-sensing receptor in rat intestine. *American Journal of Physiology, 274*(1), G122-G130.

Cheeseman, C. I., & O'Neill, D. (2006). Isolation of intestinal brush-border membranes. *Curr Protoc Cell Biol, Chapter 3*, Unit 3.21. doi:10.1002/0471143030.cb0321s30

Cheng, S., Okuda, M., Hall, A., Geibel, J., & Hebert, S. (2002). Expression of calcium-sensing receptor in rat colonic epithelium: evidence for modulation of fluid secretion. *Am J Physiol Gastrointest Liver Physiol, 283*(1), 50. doi:10.1152/ajpgi.00500.2001

Cheng, S. X., Lightfoot, Y. L., Yang, T., et al. (2014). Epithelial CaSR deficiency alters intestinal integrity and promotes proinflammatory immune responses. *FEBS Lett, 588*(22), 4158-4166. doi:10.1016/j.febslet.2014.05.007

Cho, C. H., Lee, S. Y., Shin, H. S., Philipson, K. D., & Lee, C. O. (2003). Partial rescue of the Na^{+}-Ca^{2+} exchanger (NCX1) knock-out mouse by transgenic expression of NCX1. *Exp Mol Med, 35*(2), 125-135. doi:10.1038/emm.2003.18

Choi, K. J., Cho, D. S., Kim, J. Y., et al. (2011). Ca^{2+}-induced Ca^{2+} Release from Internal Stores in INS-1 Rat Insulinoma Cells. *Korean J Physiol Pharmacol, 15*(1), 53-59. doi:10.4196/kjpp.2011.15.1.53

Christakos, S. (2012). Mechanism of action of 1,25-dihydroxyvitamin D_3 on intestinal calcium absorption. *Rev Endocr Metab Disord, 13*(1), 39-44. doi:10.1007/s11154-011-9197-x

Christakos, S., Dhawan, P., Porta, A., Mady, L. J., & Seth, T. (2011). Vitamin D and intestinal calcium absorption. *Mol Cell Endocrinol, 347*(1-2), 25-29. doi:10.1016/j.mce.2011.05.038

Cipriani, C., Pepe, J., Colangelo, L., & Minisola, S. (2018). Vitamin D and Secondary Hyperparathyroid States. *Front Horm Res, 50*, 138-148. doi:10.1159/000486077

Collip, J. B. (1925). Clinical Use of the Parathyroid Hormone. *Can Med Assoc J, 15*(11), 1158.

Conigrave, A. D., Mun, H. C., Delbridge, L., et al. (2004). L-amino acids regulate parathyroid hormone secretion. *J Biol Chem, 279*(37), 38151-38159. doi:10.1074/jbc.M406373200

Conigrave, A. D., & Ward, D. T. (2013). Calcium-sensing receptor (CaSR): pharmacological properties and signaling pathways. *Best Pract Res Clin Endocrinol Metab, 27*(3), 315-331. doi:10.1016/j.beem.2013.05.010

Copp, D. H., & Cameron, E. C. (1961). Demonstration of a hypocalcemic factor (calcitonin) in commercial parathyroid extract. *Science, 134*(3495), 2038.

Copp, D. H., Cameron, E. C., Cheney, B. A., Davidson, A. G., & Henze, K. G. (1962). Evidence for calcitonin-a new hormone from the parathyroid that lowers blood calcium. *Endocrinology, 70*, 638-649. doi:10.1210/endo-70-5-638

Cui, M., Li, Q., Johnson, R., & Fleet, J. C. (2012). Villin promoter-mediated transgenic expression of transient receptor potential cation channel, subfamily V, member 6 (TRPV6) increases intestinal calcium absorption in wild-type and vitamin D receptor knockout mice. *J Bone Miner Res, 27*(10), 2097-2107. doi:10.1002/jbmr.1662

Cui, M., Zhao, Y., Hance, K. W., et al. (2009). Effects of MAPK signaling on 1,25-dihydroxyvitamin D-mediated CYP24 gene expression in the enterocyte-like cell line, Caco-2. *J Cell Physiol, 219*(1), 132-142. doi:10.1002/jcp.21657

Dacquin, R., Davey, R. A., Laplace, C., et al. (2004). Amylin inhibits bone resorption while the calcitonin receptor controls bone formation *in vivo*. *J Cell Biol, 164*(4), 509-514. doi:10.1083/jcb.200312135

Datta, N. S., & Abou-Samra, A. B. (2009). PTH and PTHrP signaling in osteoblasts. *Cell Signal, 21*(8), 1245-1254. doi:10.1016/j.cellsig.2009.02.012

Davey, R. A., Turner, A. G., McManus, J. F., et al. (2008). Calcitonin receptor plays a physiological role to protect against hypercalcemia in mice. *J Bone Miner Res, 23*(8), 1182-1193. doi:10.1359/jbmr.080310

Di Leva, F., Domi, T., Fedrizzi, L., Lim, D., & Carafoli, E. (2008). The plasma membrane Ca^{2+} ATPase of animal cells: structure, function and regulation. *Arch Biochem Biophys, 476*(1), 65-74. doi:10.1016/j.abb.2008.02.026

Dimke, H., Desai, P., Borovac, J., et al. (2013). Activation of the Ca^{2+}-sensing receptor increases renal claudin-14 expression and urinary Ca^{2+} excretion. *Am J Physiol Renal Physiol, 304*(6), F761-769. doi:10.1152/ajprenal.00263.2012

Dvorak, M. M., Siddiqua, A., Ward, D. T., et al. (2004). Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. *Proc Natl Acad Sci U S A, 101*(14), 5140-5145. doi:10.1073/pnas.0306141101

E. White, K., E. Evans, W., L.H. O'Riordan, J., et al. (2000). *Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23* (Vol. 26).

Edwards, B. R., Sutton, R. A., & Dirks, J. H. (1974). Effect of calcium infusion on renal tubular reabsorption in the dog. *Am J Physiol, 227*(1), 13-18. doi:10.1152/ajplegacy.1974.227.1.13

Favus, M. J. (1985). Factors that influence absorption and secretion of calcium in the small intestine and colon. *Am J Physiol, 248*(2 Pt 1), G147-157. doi:10.1152/ajpgi.1985.248.2.G147

Favus, M. J., Kathpalia, S. C., & Coe, F. L. (1981). Kinetic characteristics of calcium absorption and secretion by rat colon. *Am J Physiol, 240*(5), G350-354. doi:10.1152/ajpgi.1981.240.5.G350

Favus, M. J., Kathpalia, S. C., Coe, F. L., & Mond, A. E. (1980). Effects of diet calcium and 1,25-dihydroxyvitamin D_3 on colon calcium active transport. *Am J Physiol, 238*(2), G75-78. doi:10.1152/ajpgi.1980.238.2.G75

Fleet, J. C. (2017). The role of vitamin D in the endocrinology controlling calcium homeostasis. *Molecular and Cellular Endocrinology*, 1 -10. doi:10.1016/j.mce.2017.04.008

Fleet, J. C., Eksir, F., Hance, K. W., & Wood, R. J. (2002). Vitamin D-inducible calcium transport and gene expression in three Caco-2 cell lines. *Am J Physiol Gastrointest Liver Physiol, 283*(3), G618-625. doi:10.1152/ajpgi.00269.2001

Floege, J., Tsirtsonis, K., Iles, J., et al. (2018). Incidence, predictors and therapeutic consequences of hypocalcemia in patients treated with cinacalcet in the EVOLVE trial. *Kidney Int, 93*(6), 1475-1482. doi:10.1016/j.kint.2017.12.014

Freeman, T. C., Howard, A., Bentsen, B. S., Legon, S., & Walters, J. R. (1995). Cellular and regional expression of transcripts of the plasma membrane calcium pump PMCA1 in rabbit intestine. *Am J Physiol, 269*(1 Pt 1), G126-131. doi:10.1152/ajpgi.1995.269.1.G126

Fujita, H., Chiba, H., Yokozaki, H., et al. (2006). Differential expression and subcellular localization of claudin-7, -8, -12, -13, and -15 along the mouse intestine. *J Histochem Cytochem, 54*(8), 933-944. doi:10.1369/jhc.6A6944.2006

Fujita, H., Sugimoto, K., Inatomi, S., et al. (2008). Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca^{2+} absorption between enterocytes. *Mol Biol Cell, 19*(5), 1912-1921. doi:10.1091/mbc.E07-09-0973

Gama, L., Baxendale-Cox, L. M., & Breitwieser, G. E. (1997). Ca^{2+}-sensing receptors in intestinal epithelium. *American Journal of Physiology-Cell Physiology, 273*(4), C1168-C1175. doi:10.1152/ajpcell.1997.273.4.C1168

Garabedian, M., Pavlovitch, H., Fellot, C., & Balsan, S. (1974). Metabolism of 25-hydroxyvitamin D_3 in anephric rats: a new active metabolite. *Proc Natl Acad Sci U S A, 71*(2), 554-557.

Garrett, J. E., Capuano, I. V., Hammerland, L. G., et al. (1995). Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. *J Biol Chem, 270*(21), 12919-12925.

Geibel, J., Sritharan, K., Geibel, R., et al. (2006). Calcium-sensing receptor abrogates secretagogue-induced increases in intestinal net fluid secretion by enhancing cyclic nucleotide destruction. *Proc Natl Acad Sci U S A, 103*(25), 9390-9397. doi:10.1073/pnas.0602996103

Glassman, V. P., Safirstein, R., & DiScala, V. A. (1974). Effects of metabolic acidosis on proximal tubule ion reabsorption in dog kidney. *Am J Physiol, 227*(4), 759-765. doi:10.1152/ajplegacy.1974.227.4.759

Goltzman, D., & Hendy, G. N. (2015). The calcium-sensing receptor in bone-mechanistic and therapeutic insights. *Nat Rev Endocrinol, 11*(5), 298-307. doi:10.1038/nrendo.2015.30

Guilloteau, P., Zabielski, R., Hammon, H. M., & Metges, C. C. (2010). Nutritional programming of gastrointestinal tract development. Is the pig a good model for man? *Nutr Res Rev, 23*(1), 4-22. doi:10.1017/s0954422410000077

Harrison, H. C., & Harrison, H. E. (1969). Calcium transport by rat colon in vitro. *Am J Physiol, 217*(1), 121-125. doi:10.1152/ajplegacy.1969.217.1.121

Havard, R. E., & Hoyle, J. C. (1928). Vitamin D in adults: Its effect on the calcium and inorganic phosphate of the blood. *Biochem J, 22*(3), 713-716.

Hess, A. F., Unger, L. J., & Pappenheimer, A. M. (1922). Experimental Rickets in Rats: VII. the Prevention of Rickets by Sunlight, by Rays of the Mercury Vapor Lamp, and by the Carbonic Arc Lamp. *J Exp Med, 36*(4), 427-446.

Hoenderop, J. G., Muller, D., Van Der Kemp, A. W., et al. (2001a). Calcitriol controls the epithelial calcium channel in kidney. *J Am Soc Nephrol, 12*(7), 1342-1349.

Hoenderop, J. G., Nilius, B., & Bindels, R. J. (2005). Calcium absorption across epithelia. *Physiol Rev, 85*(1), 373-422. doi:10.1152/physrev.00003.2004

Hoenderop, J. G., Vennekens, R., Muller, D., et al. (2001b). Function and expression of the epithelial Ca^{2+} channel family: comparison of mammalian ECaC1 and 2. *J Physiol, 537*(Pt 3), 747-761.

Holick, M. F., & DeLuca, H. F. (1971). A new chromatographic system for vitamin D_3 and its metabolites: resoluation of a new vitamin D_3 metabolite. *J Lipid Res, 12*(4), 460-465.

Holick, M. F., Frommer, J. E., McNeill, S. C., et al. (1977). Photometabolism of 7-dehydrocholesterol to previtamin D_3 in skin. *Biochem Biophys Res Commun, 76*(1), 107-114.

Horsting, M., & DeLuca, H. F. (1969). *In vitro* production of 25-hydroxycholecalciferol. *Biochem Biophys Res Commun, 36*(2), 251-256.

Huang, C., Sindic, A., Hill, C. E., et al. (2007). Interaction of the Ca^{2+}-sensing receptor with the inwardly rectifying potassium channels Kir4.1 and Kir4.2 results in inhibition of channel function. *American Journal of Physiology-Renal Physiology, 292*(3), F1073-F1081. doi:10.1152/ajprenal.00269.2006

Hylander, E., Ladefoged, K., & Jarnum, S. (2009). Calcium Absorption after Intestinal Resection: The Importance of a Preserved Colon. *Scandinavian Journal of Gastroenterology, 25*(7), 705-710. doi:10.3109/00365529008997596

Hylander, E., Ladefoged, K., & Jarnum, S. (2010). The Importance of the Colon in Calcium Absorption Following Small-Intestinal Resection. *Scandinavian Journal of Gastroenterology, 15*(1), 55-60. doi:10.3109/00365528009181432

Kanatani, M., Sugimoto, T., Kanzawa, M., Yano, S., & Chihara, K. (1999). High extracellular calcium inhibits osteoclast-like cell formation by directly acting on the calcium-sensing receptor existing in osteoclast precursor cells. *Biochem Biophys Res Commun, 261*(1), 144-148. doi:10.1006/bbrc.1999.0932

Kantham, L., Quinn, S. J., Egbuna, O. I., et al. (2009). The calcium-sensing receptor (CaSR) defends against hypercalcemia independently of its regulation of parathyroid hormone secretion. *Am J Physiol Endocrinol Metab, 297*(4), E915-923. doi:10.1152/ajpendo.00315.2009

Karbach, U. (1989). Mechanism of intestinal calcium transport and clinical aspects of disturbed calcium absorption. *Dig Dis, 7*(1), 1-18. doi:10.1159/000171202

Karbach, U. (1992). Paracellular calcium transport across the small intestine. *J Nutr, 122*(3 Suppl), 672-677. doi:10.1093/jn/122.suppl_3.672

Karbach, U., & Feldmeier, H. (1993). The cecum is the site with the highest calcium absorption in rat intestine. *Dig Dis Sci, 38*(10), 1815-1824.

Kawashima, H., Torikai, S., & Kurokawa, K. (1981). Localization of 25-hydroxyvitamin-D_3 1-alpha-hydroxylase and 24-hydroxylase along the rat nephron. *Proc Natl Acad Sci U S A, 78*(2), 1199-1203.

Khundmiri, S. J., Murray, R. D., & Lederer, E. (2016). PTH and Vitamin D. *Comprehensive Physiology, 6*(April), 561- 601. doi:10.1002/cphy.c140071

Kim, J. A., Yang, H., Hwang, I., et al. (2011). Expression patterns and potential action of the calcium transport genes Trpv5, Trpv6, Ncx1 and Pmca1b in the canine duodenum, kidney and uterus. *In Vivo, 25*(5), 773-780.

Ko, S. H., Choi, K. C., Oh, G. T., & Jeung, E. B. (2009a). Effect of dietary calcium and $1,25(OH)_2D_3$ on the expression of calcium transport genes in calbindin-D9k and -D28k double knockout mice. *Biochem Biophys Res Commun, 379*(2), 227-232. doi:10.1016/j.bbrc.2008.12.029

Ko, S. H., Lee, G. S., Vo, T. T., et al. (2009b). Dietary calcium and 1,25-dihydroxyvitamin D_3 regulate transcription of calcium transporter genes in calbindin-D9k knockout mice. *J Reprod Dev, 55*(2), 137-142.

Koushik, S. V., Wang, J., Rogers, R., et al. (2001). Targeted inactivation of the sodium-calcium exchanger (NCX1) results in the lack of a heartbeat and abnormal myofibrillar organization. *Faseb j, 15*(7), 1209-1211.

Krause, G., Winkler, L., Piehl, C., et al. (2009). Structure and function of extracellular claudin domains. *Ann N Y Acad Sci, 1165*, 34-43. doi:10.1111/j.1749-6632.2009.04057.x

Kremer, R., & Goltzman, D. (1982). Parathyroid hormone stimulates mammalian renal 25-hydroxyvitamin-D_3 1-alpha-hydroxylase *in vitro*. *Endocrinology, 110*(1), 294-296. doi:10.1210/endo-110-1-294

Lee, G. S., Jung, E. M., Choi, K. C., Oh, G. T., & Jeung, E. B. (2009). Compensatory induction of the TRPV6 channel in a calbindin$_{D9k}$ knockout mouse: Its regulation by 1,25-hydroxyvitamin D_3. *J Cell Biochem, 108*(5), 1175-1183. doi:10.1002/jcb.22347

Lee, G. S., Lee, K. Y., Choi, K. C., et al. (2007). Phenotype of a calbindin-D9k gene knockout is compensated for by the induction of other calcium transporter genes in a mouse model. *J Bone Miner Res, 22*(12), 1968-1978. doi:10.1359/jbmr.070801

Lee, J. J., Liu, X., O'Neil, D., et al. (2019). Activation of the calcium sensing receptor attenuates TRPV6-dependent intestinal calcium absorption. *JCI Insight, 5*. doi:10.1172/jci.insight.128013

Lee, J. J., Plain, A., Beggs, M. R., Dimke, H., & Alexander, R. T. (2017). Effects of phospho- and calciotropic hormones on electrolyte transport in the proximal tubule. *F1000Res, 6*, 1797. doi:10.12688/f1000research.12097.1

Levesque, C. L., Turner, J., Li, J., et al. (2017). In a Neonatal Piglet Model of Intestinal Failure, Administration of Antibiotics and Lack of Enteral Nutrition Have a Greater Impact on Intestinal Microflora Than Surgical Resection Alone. *JPEN J Parenter Enteral Nutr, 41*(6), 938-945. doi:10.1177/0148607115626903

Lieben, L., Benn, B. S., Ajibade, D., et al. (2010). Trpv6 mediates intestinal calcium absorption during calcium restriction and contributes to bone homeostasis. *Bone, 47*(2), 301-308. doi:10.1016/j.bone.2010.04.595

Liu, C., Weng, H., Chen, L., et al. (2013). Impaired intestinal calcium absorption in protein 4.1R-deficient mice due to altered expression of plasma membrane calcium ATPase 1b (PMCA1b). *J Biol Chem, 288*(16), 11407-11415. doi:10.1074/jbc.M112.436659

Loretz, C. A., Pollina, C., Hyodo, S., et al. (2004). cDNA cloning and functional expression of a Ca^{2+}-sensing receptor with truncated C-terminal tail from the Mozambique tilapia (Oreochromis mossambicus). *J Biol Chem, 279*(51), 53288-53297. doi:10.1074/jbc.M410098200

Loupy, A., Ramakrishnan, S. K., Wootla, B., et al. (2012). PTH-independent regulation of blood calcium concentration by the calcium-sensing receptor. *J Clin Invest, 122*(9), 3355-3367. doi:10.1172/JCI57407

Maccallum, W. G., & Voegtlin, C. (1909). On The Relation Of Tetany To The Parathyroid Glands And To Calcium Metabolism. *J Exp Med, 11*(1), 118-151.

Mancilla, E. E., De Luca, F., & Baron, J. (1998). Activating mutations of the Ca^{2+}-sensing receptor. *Mol Genet Metab, 64*(3), 198-204. doi:10.1006/mgme.1998.2716

Mannstadt, M., Juppner, H., & Gardella, T. J. (1999). Receptors for PTH and PTHrP: their biological importance and functional properties. *Am J Physiol, 277*(5), F665-675. doi:10.1152/ajprenal.1999.277.5.F665

Marie, P. J. (2010). The calcium-sensing receptor in bone cells: A potential therapeutic target in osteoporosis. *Bone, 46*(3), 571 - 576. doi:https://doi.org/10.1016/j.bone.2009.07.082

Martin, A., David, V., & Quarles, L. D. (2012). Regulation and function of the FGF23/klotho endocrine pathways. *Physiol Rev, 92*(1), 131-155. doi:10.1152/physrev.00002.2011

Mohammad, G., Di Scala, V., & Stein, R. M. (1974). Effects of chronic sodium depletion on tubular sodium and water reabsorption in the dog. *Am J Physiol, 227*(2), 469-476. doi:10.1152/ajplegacy.1974.227.2.469

Moor, M. B., & Bonny, O. (2016). Ways of calcium reabsorption in the kidney. *Am J Physiol Renal Physiol, 310*(11), F1337-1350. doi:10.1152/ajprenal.00273.2015

Morrissey, J. J., & Cohn, D. V. (1978). The effects of calcium and magnesium on the secretion of parathormone and parathyroid secretory protein by isolated porcine parathyroid cells. *Endocrinology, 103*(6), 2081-2090. doi:10.1210/endo-103-6-2081

Nemeth, E. F., & Scarpa, A. (1986). Cytosolic Ca^{2+} and the regulation of secretion in parathyroid cells. *FEBS Lett, 203*(1), 15-19.

Nesbit, M. A., Hannan, F. M., Howles, S. A., et al. (2013). Mutations affecting G-protein subunit alpha11 in hypercalcemia and hypocalcemia. *N Engl J Med, 368*(26), 2476-2486. doi:10.1056/NEJMoa1300253

Nilius, B., Owsianik, G., & Voets, T. (2008). Transient receptor potential channels meet phosphoinositides. *EMBO Journal, 27*(21), 2809-2816. doi:10.1038/emboj.2008.217

. Office of the Surgeon General (US). 2004. Bone Health and Osteoporosis: A Report of the Surgeon General. Rockville (MD): Office of the Surgeon General (US). In *Diseases of Bone*.

Okano, T., Yasumura, M., Mizuno, K., & Kobayashi, T. (1978). In vivo and in vitro conversion of 7-dehydrocholesterol into vitamin D_3 in rat skin by ultraviolet ray's irradiation. *J Nutr Sci Vitaminol (Tokyo), 24*(1), 47-56.

Ottolia, M., & Philipson, K. D. (2013). NCX1: mechanism of transport. *Adv Exp Med Biol, 961*, 49-54. doi:10.1007/978-1-4614-4756-6_5

Pan, W., Borovac, J., Spicer, Z., et al. (2012). The epithelial sodium/proton exchanger, NHE3, is necessary for renal and intestinal calcium (re)absorption. *Am J Physiol Renal Physiol, 302*(8), F943-956. doi:10.1152/ajprenal.00504.2010

Peng, J. B., Brown, E. M., & Hediger, M. A. (2001). Structural conservation of the genes encoding CaT1, CaT2, and related cation channels. *Genomics, 76*(1-3), 99-109. doi:10.1006/geno.2001.6606

Peng, J. B., Chen, X. Z., Berger, U. V., et al. (1999). Molecular cloning and characterization of a channel-like transporter mediating intestinal calcium absorption. *J Biol Chem, 274*(32), 22739-22746.

Perwad, F., Zhang, M. Y., Tenenhouse, H. S., & Portale, A. A. (2007). Fibroblast growth factor 23 impairs phosphorus and vitamin D metabolism *in vivo* and suppresses 25-

hydroxyvitamin D-1alpha-hydroxylase expression *in vitro*. *Am J Physiol Renal Physiol, 293*(5), F1577-1583. doi:10.1152/ajprenal.00463.2006

Petith, M. M., & Schedl, H. P. (1976). Intestinal Adaptation to Dietary Calcium Restriction: In Vivo Cecal and Colonic Calcium Transport in The Rat. *Gastroenterology, 71*(6), 1039-1042. doi:10.1016/S0016-5085(76)80055-8

Pi, M., Hinson, T. K., & Quarles, L. (1999). Failure to detect the extracellular calcium-sensing receptor (CasR) in human osteoblast cell lines. *J Bone Miner Res, 14*(8), 1310-1319. doi:10.1359/jbmr.1999.14.8.1310

Pidasheva, S., Canaff, L., Simonds, W. F., Marx, S. J., & Hendy, G. N. (2005). Impaired cotranslational processing of the calcium-sensing receptor due to signal peptide missense mutations in familial hypocalciuric hypercalcemia. *Hum Mol Genet, 14*(12), 1679-1690. doi:10.1093/hmg/ddi176

Plain, A., Wulfmeyer, V. C., Milatz, S., et al. (2016). Corticomedullary difference in the effects of dietary Ca^{2+} on tight junction properties in thick ascending limbs of Henle's loop. *Pflugers Arch, 468*(2), 293-303. doi:10.1007/s00424-015-1748-7

Poole, K. E., & Reeve, J. (2005). Parathyroid hormone - a bone anabolic and catabolic agent. *Curr Opin Pharmacol, 5*(6), 612-617. doi:10.1016/j.coph.2005.07.004

Potts, J. T., Jr., Murray, T. M., Peacock, M., et al. (1971). Parathyroid hormone: sequence, synthesis, immunoassay studies. *Am J Med, 50*(5), 639-649.

Prasad, V., Okunade, G. W., Miller, M. L., & Shull, G. E. (2004). Phenotypes of SERCA and PMCA knockout mice. *Biochem Biophys Res Commun, 322*(4), 1192-1203. doi:10.1016/j.bbrc.2004.07.156

Raggatt, L. J., & Partridge, N. C. (2010). Cellular and molecular mechanisms of bone remodeling. *J Biol Chem, 285*(33), 25103-25108. doi:10.1074/jbc.R109.041087

Reilly, R. F., & Shugrue, C. A. (1992). cDNA cloning of a renal Na^{+}-Ca^{2+} exchanger. *Am J Physiol, 262*(6 Pt 2), F1105-1109. doi:10.1152/ajprenal.1992.262.6.F1105

Riccardi, D., Hall, A. E., Chattopadhyay, N., et al. (1998). Localization of the extracellular Ca^{2+}/polyvalent cation-sensing protein in rat kidney. *Am J Physiol, 274*(3 Pt 2), F611-622.

Riccardi, D., Park, J., Lee, W. S., et al. (1995). Cloning and functional expression of a rat kidney extracellular calcium/polyvalent cation-sensing receptor. *Proc Natl Acad Sci U S A, 92*(1), 131-135.

Rievaj, J., Pan, W., Cordat, E., & Alexander, R. T. (2013). The Na^+/H^+ exchanger isoform 3 is required for active paracellular and transcellular Ca^{2+} transport across murine cecum. *Am J Physiol Gastrointest Liver Physiol, 305*(4), G303-313. doi:10.1152/ajpgi.00490.2012

Rodriguez, M. E., Almaden, Y., Canadillas, S., et al. (2007). The calcimimetic R-568 increases vitamin D receptor expression in rat parathyroid glands. *Am J Physiol Renal Physiol, 292*(5), F1390-1395. doi:10.1152/ajprenal.00262.2006

Rodriguez-Ortiz, M. E., & Rodriguez, M. (2015). FGF23 as a calciotropic hormone. *F1000Res, 4*. doi:10.12688/f1000research.7189.1

Rosenthal, R., Gunzel, D., Theune, D., et al. (2017). Water channels and barriers formed by claudins. *Ann N Y Acad Sci, 1397*(1), 100-109. doi:10.1111/nyas.13383

Roth, S. I., Feinblatt, J. D., & Raisz, L. G. (1974). Effect of calcium on embryonic rat thyroid C cells in vitro. *Am J Pathol, 75*(1), 27-44.

Shimura, F., & Wasserman, R. H. (1984). Membrane-associated vitamin D-induced calcium-binding protein (CaBP): quantification by a radioimmunoassay and evidence for a specific CaBP in purified intestinal brush borders. *Endocrinology, 115*(5), 1964-1972. doi:10.1210/endo-115-5-1964

Siegel, N., Wongsurawat, N., & Armbrecht, H. J. (1986). Parathyroid hormone stimulates dephosphorylation of the renoredoxin component of the 25-hydroxyvitamin-D_3 1-alpha-hydroxylase from rat renal cortex. *J Biol Chem, 261*(36), 16998-17003.

Silver, J., & Naveh-Many, T. (2010). FGF23 and the parathyroid glands. *Pediatr Nephrol, 25*(11), 2241-2245. doi:10.1007/s00467-010-1565-3

Song, Y., Peng, X., Porta, A., et al. (2003). Calcium transporter 1 and epithelial calcium channel messenger ribonucleic acid are differentially regulated by 1,25 dihydroxyvitamin D_3 in the intestine and kidney of mice. *Endocrinology, 144*(9), 3885-3894. doi:10.1210/en.2003-0314

Sugimoto, T., Kanatani, M., Kano, J., et al. (1993). Effects of high calcium concentration on the functions and interactions of osteoblastic cells and monocytes and on the formation of osteoclast-like cells. *J Bone Miner Res, 8*(12), 1445-1452. doi:10.1002/jbmr.5650081206

Sugimoto, T., Kanatani, M., Kano, J., et al. (1994). IGF-I mediates the stimulatory effect of high calcium concentration on osteoblastic cell proliferation. *American Journal of Physiology-Endocrinology and Metabolism, 266*(5), E709-E716. doi:10.1152/ajpendo.1994.266.5.E709

Takeshita, A., Kawakami, K., Furushima, K., Miyajima, M., & Sakaguchi, K. (2018). Central role of the proximal tubular alpha-Klotho/FGF receptor complex in FGF23-regulated

phosphate and vitamin D metabolism. *Sci Rep, 8*(1), 6917. doi:10.1038/s41598-018-25087-3

Talmage, R. V., Grubb, S. A., Norimatsu, H., & Vanderwiel, C. J. (1980). Evidence for an important physiological role for calcitonin. *Proc Natl Acad Sci U S A, 77*(1), 609-613.

Tang, L., Cheng, C. Y., Sun, X., et al. (2016). The Extracellular Calcium-Sensing Receptor in the Intestine: Evidence for Regulation of Colonic Absorption, Secretion, Motility, and Immunity. *Front Physiol, 7*. doi:10.3389/fphys.2016.00245

Thyagarajan, B., Benn, B. S., Christakos, S., & Rohcs, T. (2009). Phospholipase C-mediated regulation of transient receptor potential vanilloid 6 channels: implications in active intestinal Ca^{2+} transport. *Molecular pharmacology, 75*(3), 608-616. doi:10.1124/mol.108.052449

Thyagarajan, B., Lukacs, V., & Rohacs, T. (2008). Hydrolysis of phosphatidylinositol 4,5-bisphosphate mediates calcium-induced inactivation of TRPV6 channels. *Journal of Biological Chemistry, 283*(22), 14980-14987. doi:10.1074/jbc.M704224200

Toka, H. R., Al-Romaih, K., Koshy, J. M., et al. (2012). Deficiency of the calcium-sensing receptor in the kidney causes parathyroid hormone-independent hypocalciuria. *J Am Soc Nephrol, 23*(11), 1879-1890. doi:10.1681/asn.2012030323

Toka, H. R., Pollak, M. R., & Houillier, P. (2015). Calcium Sensing in the Renal Tubule. *Physiology (Bethesda), 30*(4), 317-326. doi:10.1152/physiol.00042.2014

Topala, C. N., Schoeber, J. P., Searchfield, L. E., et al. (2009). Activation of the Ca^{2+}-sensing receptor stimulates the activity of the epithelial Ca^{2+} channel TRPV5. *Cell Calcium, 45*(4), 331-339. doi:10.1016/j.ceca.2008.12.003

Tucker, G., 3rd, Gagnon, R. E., & Haussler, M. R. (1973). Vitamin D_3-25-hydroxylase: tissue occurrence and apparent lack of regulation. *Arch Biochem Biophys, 155*(1), 47-57.

Vachel, L., Norez, C., Jayle, C., Becq, F., & Vandebrouck, C. (2015). The low PLC-delta1 expression in cystic fibrosis bronchial epithelial cells induces upregulation of TRPV6 channel activity. *Cell Calcium, 57*(1), 38-48. doi:10.1016/j.ceca.2014.11.005

van Abel, M., Hoenderop, J. G., van der Kemp, A. W., van Leeuwen, J. P., & Bindels, R. J. (2003). Regulation of the epithelial Ca^{2+} channels in small intestine as studied by quantitative mRNA detection. *Am J Physiol Gastrointest Liver Physiol, 285*(1), G78-85. doi:10.1152/ajpgi.00036.2003

Van Cromphaut, S. J., Dewerchin, M., Hoenderop, J. G., et al. (2001). Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci USA, 98*(23), 13324-13329. doi:10.1073/pnas.231474698

Vassilev, P. M., Peng, J. B., Hediger, M. A., & Brown, E. M. (2001). Single-channel activities of the human epithelial Ca^{2+} transport proteins CaT1 and CaT2. *J Membr Biol, 184*(2), 113-120.

Wada, M., Tamura, A., Takahashi, N., & Tsukita, S. (2013). Loss of claudins 2 and 15 from mice causes defects in paracellular Na^{+} flow and nutrient transport in gut and leads to death from malnutrition. *Gastroenterology, 144*(2), 369-380. doi:10.1053/j.gastro.2012.10.035

Walters, J. R., Balesaria, S., Chavele, K. M., et al. (2006). Calcium channel TRPV6 expression in human duodenum: different relationships to the vitamin D system and aging in men and women. *J Bone Miner Res, 21*(11), 1770-1777. doi:10.1359/jbmr.060721

Wasserman, R. H. (2004). Vitamin D and the dual processes of intestinal calcium absorption. *J Nutr, 134*(11), 3137-3139. doi:10.1093/jn/134.11.3137

Wasserman, R. H., Corradino, R. A., & Taylor, A. N. (1969). Binding proteins from animals with possible transport function. *J Gen Physiol, 54*(1), 114-137.

Wasserman, R. H., & Fullmer, C. S. (1995). Vitamin D and intestinal calcium transport: facts, speculations and hypotheses. *J Nutr, 125*(7 Suppl), 1971s-1979s. doi:10.1093/jn/125.suppl_7.1971S

Wasserman, R. H., & Taylor, A. N. (1966). Vitamin D_3-induced calcium-binding protein in chick intestinal mucosa. *Science, 152*(3723), 791-793. doi:10.1126/science.152.3723.791

Weber, C. R., Liang, G. H., Wang, Y., et al. (2015). Claudin-2-dependent paracellular channels are dynamically gated. *Elife, 4*, e09906. doi:10.7554/eLife.09906

Weissgerber, P., Kriebs, U., Tsvilovskyy, V., et al. (2011). Male fertility depends on Ca^{2+} absorption by TRPV6 in epididymal epithelia. *Sci Signal, 4*(171), 27. doi:10.1126/scisignal.2001791

Welsh, J., Weaver, V., & Simboli-Campbell, M. (1991). Regulation of renal 25(OH)D_3 1-alpha-hydroxylase: signal transduction pathways. *Biochem Cell Biol, 69*(12), 768-770.

Wongdee, K., & Charoenphandhu, N. (2015). Vitamin D-enhanced duodenal calcium transport. *Vitam Horm, 98*, 407-440. doi:10.1016/bs.vh.2014.12.010

Woudenberg-Vrenken, T. E., Lameris, A. L., Weissgerber, P., et al. (2012). Functional TRPV6 channels are crucial for transepithelial Ca^{2+} absorption. *Am J Physiol Gastrointest Liver Physiol, 303*(7), G879-885. doi:10.1152/ajpgi.00089.2012

Xue, Y., & Fleet, J. C. (2009). Intestinal vitamin D receptor is required for normal calcium and bone metabolism in mice. *Gastroenterology, 136*(4), 1317-1327, e1311-1312. doi:10.1053/j.gastro.2008.12.051

Yamaguchi, T., Chattopadhyay, N., Kifor, O., et al. (1998). Mouse osteoblastic cell line (MC3T3-E1) expresses extracellular calcium ($Ca^{2+}{}_o$)-sensing receptor and its agonists stimulate chemotaxis and proliferation of MC3T3-E1 cells. *J Bone Miner Res, 13*(10), 1530-1538. doi:10.1359/jbmr.1998.13.10.1530

Yamaguchi, T., & Sugimoto, T. (2007). [Impaired bone mineralization in calcium-sensing receptor (CaSR) knockout mice : the physiological action of CaSR in bone microenvironments]. *Clin Calcium, 17*(10), 1567-1573. doi:CliCa071015671573

Yu, A. S., Cheng, M. H., Angelow, S., et al. (2009). Molecular basis for cation selectivity in claudin-2-based paracellular pores: identification of an electrostatic interaction site. *J Gen Physiol, 133*(1), 111-127. doi:10.1085/jgp.200810154

Yu, A. S., Cheng, M. H., & Coalson, R. D. (2010). Calcium inhibits paracellular sodium conductance through claudin-2 by competitive binding. *J Biol Chem, 285*(47), 37060-37069. doi:10.1074/jbc.M110.146621

Zakharian, E., Cao, C., & Rohacs, T. (2011). Intracellular ATP supports TRPV6 activity via lipid kinases and the generation of PtdIns(4,5)P_2. *The FASEB Journal, 25*(11), 3915--3928. doi:10.1096/fj.11-184630

Zheng, W., Yang, X., Hu, R., et al. (2018). Hydrophobic pore gates regulate ion permeation in polycystic kidney disease 2 and 2L1 channels. *Nat Commun, 9*. doi:10.1038/s41467-018-04586-x